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On the Effect of Ca^{2+} and La^{3+} on the Colloidal Stability of Liposomes

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This work deals with the effect of Ca^{2+} and La^{3+} on the colloidal stability of phosphatidylcholine (PC) liposomes in aqueous media. As physical techniques, nephelometry, photon correlation spectroscopy, electrophoretic mobility, and surface tension were used. The theoretical predictions of the colloidal stability of liposomes were followed using the Derjaguin−Landau−Verwey−Overbeek theory. Changes in the size of liposomes and high polydispersity values were observed as La^{3+} concentration increases, suggesting that this cation induces the aggregation of liposomes. However, changes in polydispersity were not observed with Ca^{2+}, suggesting a coalescence mechanism or fusion of liposomes. The stability factor (W), calculated from the nephelometry measurements indicated that aggregation/fusion occurs at a critical concentration (c.c.) of 0.3 and 0.7 M for La^{3+} and Ca^{2+}, respectively. To gain a better insight into the interaction mechanism between the liposomes and the studied ions, the interaction between PC monolayers and Ca^{2+} and La^{3+} was studied. Changes in the surface area per lipid molecule (A_0) in the monolayer at the c.c. values were found for both ions, with a more pronounced effect in the case of Ca^{2+}. This corresponds with a larger reduction of the steric repulsive interaction between the headgroups at the phospholipid membrane (π_{head}). The experimental result validates the hypothesis made on the liposome fusion in the presence of Ca^{2+} and liposome aggregation in the presence of La^{3+}. These aggregation mechanisms have also been confirmed by transmission electron microscopy.

Introduction

Liposomes are spherical, self-closed structures consisting of one or more concentric lipid bilayers encapsulating aqueous volume, their size ranging from 20 nm to several micrometers, whereas the thickness of the membranes is around 4 nm.[1] The high biocompatibility of liposomes makes it possible to use them as models for the study of biological membranes, but in the past decades, liposomes are finding a lot of different biotechnology applications as drug delivery systems.[2−4] The properties of the liposomes depend on the composition and concentration of constituents of phospholipids and the ionic strength of aqueous medium, as well as the method of preparation.[5]

The L-α-phosphatidylcholines (PC) are the most common phospholipid components of biological membranes. They are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a zwiterionic hydrophilic headgroup. Their low cost, neutral charge, and chemical inertness make them ideal for the "in vitro" preparation of liposomes as biomembranes models.[6] Biological membranes are surrounded by an aqueous buffer containing Na^+ K^+, Ca^{2+}, Mg^{2+}, or Cl^- ions with different concentrations inside and outside of the cells. Electrostatic interactions between these ionic buffers and the lipid molecules are crucial for membrane fusion, phase transitions, or transport across the membrane. Divalent cations interact very strongly with charged lipids, but also moderately with zwitterionic lipids.[7] Lanthanum (La^{+3}) plays an important role in the structure and function of biomembranes or phospholipid membranes. La^{+3} can modulate the gating properties of a voltage-gated sodium channel. Also, the lanthanides exert a marked effect on the structure and stability of phospholipid membranes. The interaction of calcium and lanthanum with neutral zwitterionic membranes is the subject of this study.

In general, conventional PC liposomes exhibit moderate colloidal stability due to the aggregation/fusion processes that can prevent drug-loaded liposomes from reaching a targeted site in biomedical applications.[8] It has also been reported[9] that large phospholipid structures (such as liposome aggregates) are cleaned from the bloodstream more rapidly than small structures. Therefore, the studies of the aggregation and fusion processes of the PC liposomes are necessary if efficient liposomes as drug delivery system are to be designed. This tendency of aggregation is attributable to attractive van der Waals forces. The existence of other interactions between particles determines the stability, rheology, and many other properties

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of these colloidal dispersions. The colloidal stability warrants detailed attention since the development of these liposomes to biophysics, pharmacy, agriculture, and medicine are dependent on a large extent on a better understanding of the colloidal interactions.

Liposomes can be characterized as a type of association colloid governed by interparticle forces. Such forces have usually been described by the Derjaguin−Landau−Verwey−Overbeek (DLVO) theory, which, in the past 50 years has been a standard model for describing interparticle forces.[10,11] This theory assumes that two types of forces control colloidal interactions: electrostatic forces, which are typically repulsive for like-charged particles, and van der Waals forces, which are typically attractive.

The DLVO theory can be a very powerful assay in the prediction of the colloidal stability of liposomes at different ion concentrations, temperatures, etc. The aim of this work is to test the validity of the DLVO theory in the case of dynamic processes such as aggregation or fusion. We have experimentally estimated the stability of the liposomes at different concentrations of $La(NO_3)_3$ and $Ca(NO_3)_2$ using two different optical techniques, nephelometry and photon correlation spectroscopy (PCS), and compared the results with the stability predicted by the DLVO theory. In addition, the ion adsorption onto liposome surfaces using measurements of electrophoretic mobilities has been characterized. To consider the mechanism/explanation of the fusion/aggregation of PC liposomes induced by Ca^{2+} and La^{3+}, the effect of Ca^{2+} and La^{3+} on the PC monolayers was investigated. We have analyzed the dynamic surface tension of PC by adding Ca^{2+} and La^{3+}. The active research of Langmuir−Blodgett monolayers and multilayers of fatty acids and phospholipids[12,13] suggested significant influence of divalent cations on the packing order and structure of the films.

Experimental Section

Materials. PC from egg yolk (No. P 3556) was from Sigma and used as received. Organic solvents methanol and chloroform were from Aldrich and Merck, respectively. All used salts ($La(NO_3)_3$ and $Ca(NO_3)_2$ were of analytical grade with purity higher than 99%. Polycarbonate membrane filters were purchased from Millipore.

Vesicle Preparation. Large unilamellar vesicles (LUVs) were prepared by the thin-film hydration method.[1] A solution of PC in chloroform/methanol (4:1) was evaporated in a rotary evaporator to dryness under a stream of nitrogen, and the resultant lipid film hydrated with double distilled, degassed, and deionized water. This mixture was extruded five times through polycarbonate filters of 800 nm pore size and five times through polycarbonate filters of 200 nm to form LUVs.[14−16] The extrusion through pores larger than 100 nm generally yielded liposomes smaller than the pore size.[17] In our case, liposomes with a (140 ± 2) nm diameter were obtained.

Transmission Electron Microscopy. The morphological examination of the liposomes was performed by transmission electron microscopy (TEM) (CM-12 Philips). The samples were stained with 2% (w/v) phosphotungstic acid[18] and placed on copper grids with Formvart films for viewing by TEM.

Electrophoretic Mobility. Electrophoretic mobility of the PC liposome particles was measured using a Malvern Instruments Zetamaster 5002 by taking the average of five measurements at stationary level. The cell used was a 5 mm × 2 mm rectangular quartz capillary. The temperature of the experiments was 25 °C, controlled by a Haake temperature controller. The zeta potential was calculated from the electrophoretic mobilities, μ_E, by means of the Henry correction of Smoluchowski's equation[19]

$$\zeta = \frac{3\mu_E\eta}{2\epsilon_0\epsilon_r}\frac{1}{f(\kappa a)} \tag{1}$$

where ϵ_0 is the permittivity of the vacuum, ϵ_r is the relative permittivity dielectric constant, a is the particle radius, κ is the Debye length, and η is the viscosity of water. The function $f(\kappa a)$ depends on the particle shape and, for our system, was determined by

$$f(\kappa a) = \frac{3}{2} - \frac{9}{2\kappa a} + \frac{75}{2\kappa^2 a^2} \tag{2}$$

which is valid for $\kappa a > 1$.

Photon Correlation Spectroscopy (PCS). Measurements of the size and polydispersities of the liposome systems were performed by PCS using a Spectrometer Autosizer 4800 from Malvern Instruments equipped with a Uniphase 75 mW Ar laser operating at 488 nm with vertically polarized light at a scattering angle of 90° at 298.15 ± 0.01 K. Time correlation was analyzed by a digital autocorrelator PCS7132 from Malvern Instruments and using the CONTIN algorithm.

Nephelometry. The aggregation/fusion rates of the liposomes were measured by nephelometry measuring at a low angle to the static light scattering. Scattered light intensity (Malvern 4700 System, U.K.) was followed at 10° during 120 s in a rectangular flow cell with 2 mm path length. The cell was thoroughly cleaned with chromic acid and rinsed with distilled water. Equals volumes (1 mL) of salt and liposome solutions were mixed and introduced into the cell by an automatic mixing device. The dead time was quite short.[20]

The scattered light intensity at low angles increases linearly with time, and then an absolute aggregation/fusion rate (k) can be obtained from the slope of intensity versus time. When the repulsive potential between liposomes disappears, all the collisions are efficient and cause the aggregation or fusion of the liposomes. This is, usually, denominated as the *rapid regime*.[21]

The stability ratio (W) is a criterion of stability of the colloidal system:

$$W = \frac{k_r}{k_s} \tag{3}$$

in which k_r describes the rate constant in the *rapid regime* and k_s is the rate constant for the *slow regime*. Thus, the inverse of the stability ratio provides a measure of the effectiveness of collisions leading to aggregation or fusion.

Surface Tension. The experiments were performed with a constant surface pressure penetration Langmuir balance based on axisymmetric drop shape analysis (ADSA) which is described in detail in refs 22 and 23. The whole setup (image capturing, microinjector, ADSA algorithm, and the fuzzy pressure control) is managed by a Windows-integrated program (DINATEN).

A solution droplet is formed at the tip of the coaxial double capillary connected to a double microinjector. The program fits experimental drop profiles, extracted from digital drop micrographs, to the Young−Laplace equation of capillarity by using

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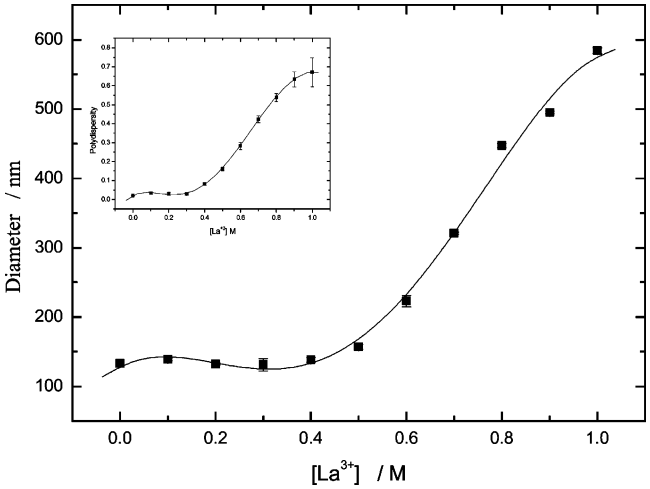

Figure 1. Values of diameter and polydispersity index of the PC liposomes for different La^{3+} concentrations measured by PCS.

ADSA, and provides as outputs the drop volume, V, the interfacial tension, γ, and the surface area, A. Pressure and area control use a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control). Changing the drop volume in a controlled manner and simultaneously measuring surface tension and surface area generate the isotherms.

The surface pressure values are obtained from the relationship $\pi = \gamma_0 - \gamma$, where π is the surface pressure, γ_0 is the surface tension of pure liquid, and γ is the surface tension of the liquid covered by the monolayer.

Phospholipid Monolayers. PC was dissolved in a methanol/chloroform mixture (1:4) to obtain solutions of $(7 \times 10^{-5}$ to $4 \times 10^{-5})$ M concentration range. Then, an aliquot of 1 μL was spread on the subphase using a microsyringe. Four minutes were allowed for solvent evaporation before starting the expansion until a volume of 25 μL was reached at a rate of 0.2 μL/s. When expansion was finished, the program maintains the drop area constant for 118 s to reach the monolayer equilibrium and then the compression started at the same rate of expansion.

The cation concentrations in the subphase were 0.2, 0.4, 0.6, 0.7, 0.8, and 1 M for Ca^{2+} and 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 M for La^{3+}.

Results and Discussion

Experimental and Predicted DLVO Stability of the Liposomes in $La(NO_3)_3$ Solution. To compare with the DLVO theory predictions, measurements of stability of the liposomes at different concentrations of La^{3+} were made by two different methods. First, the size and polydispersity of the liposomes in La^{3+} solutions using PCS were measured. Figure 1 shows an initial liposome size of 140 nm and a small polydispersity of 0.025. However, there is a sharp increase of the size and polydispersity starting at a La^{3+} concentration of 0.3 M. At higher concentration, the system is unstable and large aggregates (>500 nm) of different sizes are originated. There is an extensive bibliography reporting that the aggregation of liposomes in the presence of La^{3+} predominates more than in the fusion process.[24,25] The micrographs obtained by TEM (Figure 2a) confirm that the aggregation processes are causing the increase in size and polydispersity of the system when the La^{3+} concentration is increasing. The particle concentration used in the TEM micrograph was 1.2×10^{19} liposome/m^3.

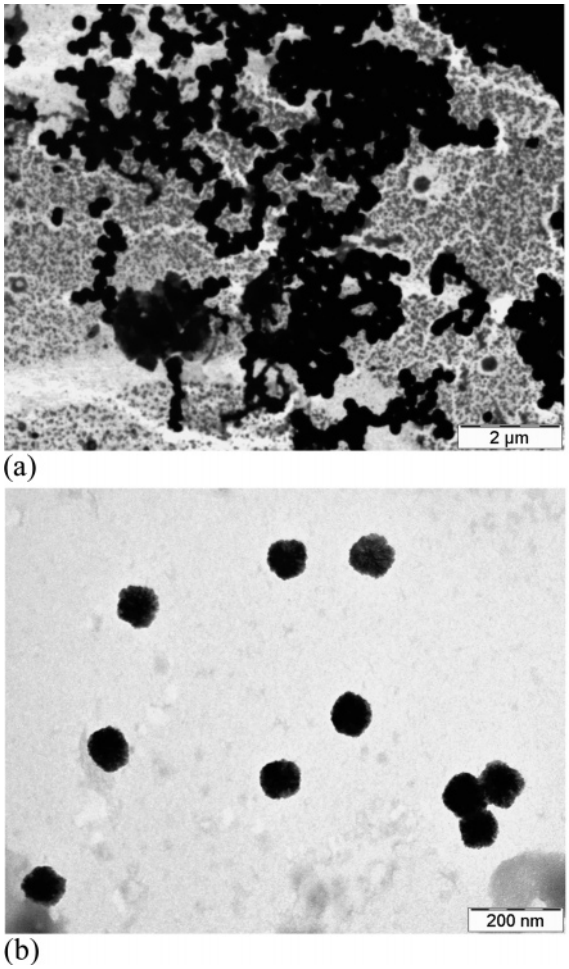

Figure 2. Transmission electron micrograph of spontaneous PC liposomes in the presence of (a) 0.7 M Ca^{2+} and (b) 0.3 M La^{3+}.

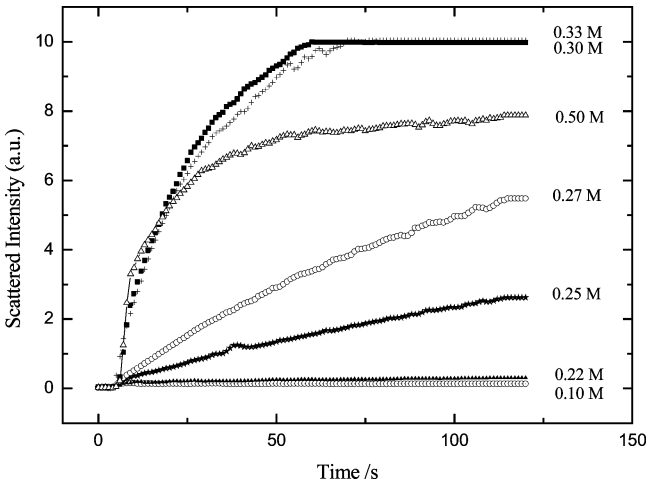

Figure 3. Scattered light intensity (arbitrary unit) vs time for a typical nephelometry experience with PC liposomes at different concentrations of La^{3+}.

To get a more complete characterization of the stability of the liposomes in La^{3+} solution, the stability constant (W) at different La^{3+} concentrations was measured using the nephelometry technique. From the slopes of the intensity versus time of Figure 3, W was calculated using eq 3. The results are shown in Figure 4. Usually the critical concentration (c.c.) is defined as the intersection of the linear fit in the slow regime and the linear fit in the rapid

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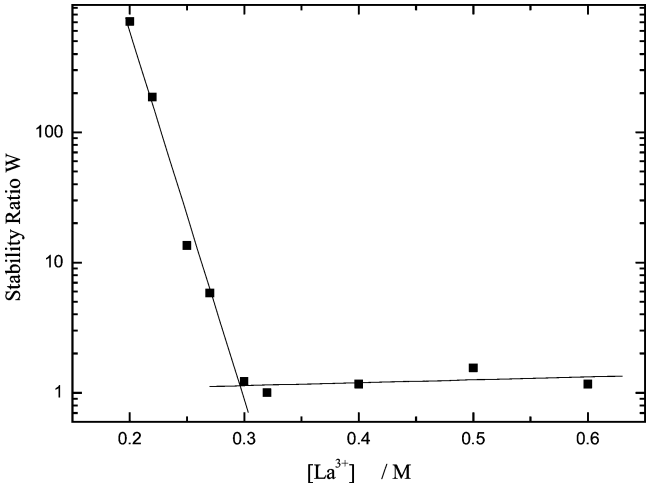

Figure 4. Variation of stability ratio (W) with La^{3+} concentration measured by nephelometry.

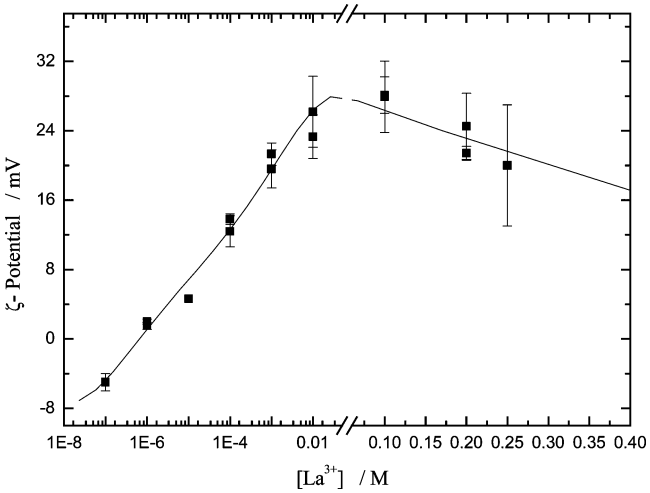

Figure 5. Zeta potential of PC liposomes as a function of La^{3+} concentrations.

regime. In that case, the c.c. is 0.3 M, which agrees with the result obtained by PCS.

Now, the interest is to compare the experimental results of stability with the DLVO theory predictions. The DLVO theory considers the interaction potential between liposomes such as the sum of an attractive London−van der Waals interaction $V_A(x)$ and a repulsive interaction $V_R(x)$ due to the electric charge on the particles:

$$V_T(x) = V_A(x) + V_R(x) \tag{4}$$

$V_A(x)$ for the case of two spherical shells of equal radius, a, and thickness, d, derived by Hamaker,[10,26] is given by[27]

$$V_A(x) = -\frac{Aa}{12}\left[\frac{1}{x+2d} - \frac{2}{x+d} + \frac{1}{d}\right] - \frac{A}{6}\ln\frac{x(x+2d)}{(x+d)^2} \tag{5}$$

where A is the attractive Hamaker constant, a the initial radius of the liposomes, and x the distance between the two spherical shells.

The Hamaker constant, A, was calculated using an algorithm based on a SIMPLEX method, which minimizes the error fit of the nephelometry data to the function:

$$W = \frac{\int_0^\infty \frac{\beta(u)}{(u+2a)^2}\exp\frac{V_T}{k_B T}\,du}{\int_0^\infty \frac{\beta(u)}{(u+2a)^2}\exp\frac{V_A}{k_B T}\,du} \tag{6}$$

where $\beta(u)$ is the hydrodynamic correlation factor, related to retardation on the particles diffusion due to the friction with the medium:

$$\beta(u) = \frac{6u^2 + 13u + 2a^2}{6u^2 + 4ua} \tag{7}$$

where $u = (x - 2a)/a$. The Hamaker constants were in the range of $(0.1 \times 10^{-20}$ to $7 \times 10^{-20})$ J with no changes in the DLVO potentials in that range of values.

The expression for the repulsive potential, $V_R(x)$, by area unit has the form[28−30]

$$V_R(x) = 2\pi\epsilon_0\epsilon_r(a+\Delta)\psi_0^2\exp[-\kappa(x-2\Delta)] \tag{8}$$

where k_B is the Boltzmann constant, T is the absolute temperature, ψ_0 is the surface potential which coincides with ζ for high values,[31] and κ is the reciprocal Debye length:

$$\kappa^2 = \frac{(z_1^2 z_2 + z_2^2 z_1)c_s N_A e^2}{\epsilon_0\epsilon_r k_B T} \tag{9}$$

where c_s is the concentration, e the elementary charge, z_i the valence of the ions, and N_A Avogadro's number. Finally, Δ is the thickness of the Stern layer, and it was taken as the hydrated radius of the adsorbed lanthanum ion, 0.49 nm.[32,33]

To know $V_r(x)$, we need to measure the zeta potential ($\zeta \approx \Psi_0$) of the liposomes for each La^{3+} concentration. The results are shown in Figure 5, where a change in the sign and a fast increase of the zeta potential until +25 mV due to the adsorption of La^{3+} onto the liposome surface can be seen. For higher concentrations than 0.05 M, there is a small decrease of the ζ-potential due to the adsorption of NO^{-3}. Using the values of ζ, we can represent the DLVO potential for each La^{3+} concentration, as is shown in Figure 6. The aggregation of the liposomes will occur when the repulsive barrier is lower than $k_B T$ at the contact surface (hydration surface $\Delta = 0.49$ nm). As can be seen in Figure 6, the repulsive barrier is below $k_B T$ for La^{3+} concentrations larger than 0.27 M. This agrees with the experimental data obtained by nephelometry and PCS, proving the good accuracy of the DLVO theory to describe the stability of liposomes in the presence of La^{3+}.

Experimental and Predicted DLVO Stability of the Liposomes in a $Ca(NO_3)_2$ Solutions. Similar experiments to the above-mentioned one have been performed with liposomes in $Ca(NO_3)_2$ solutions. Figure 7 shows the sizes and polydispersities of the liposomes at different concentrations of Ca^{2+}. It can be observed an

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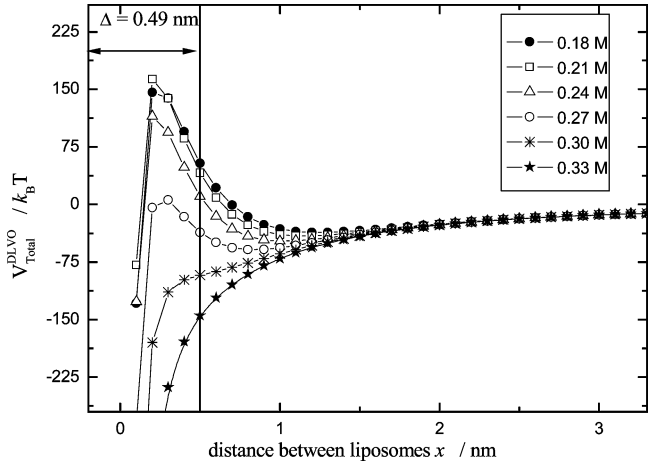

Figure 6. DLVO potentials of PC liposomes as a function of the distance between two liposomes for different La^{3+} concentrations. (●) 0.18, (□) 0.21, (△) 0.24, (○) 0.27, (*) 0.30, and (★) 0.33 M.

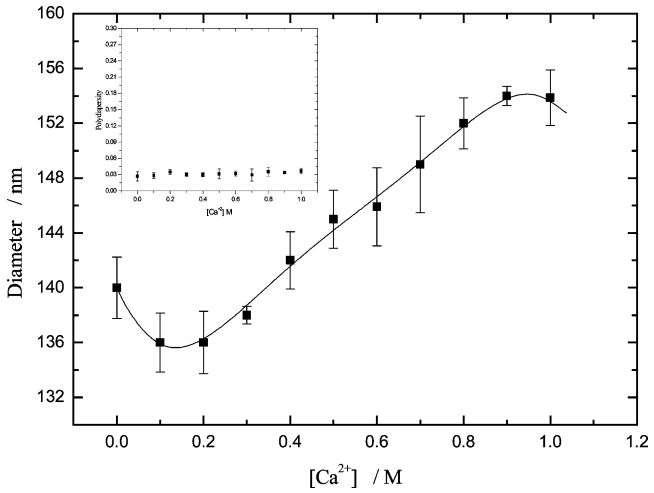

Figure 7. Values of diameters and polydispersity index of the PC liposomes for different Ca^{2+} concentrations measured by PCS.

initial decrease of their size at low Ca^{2+} concentrations due to the osmotic pressure[34−36] and a slow increase of the size until a maximum of 155 nm in the range of 0.3−0.9 M. The TEM micrographs of Figure 2b and the unchanged polydispersity (Figure 7) reflect that no aggregation process occurs. This means that the increase of the size of the liposomes in the presence of Ca^{2+} are, essentially, due to a fusion process between the liposomes particles, as other authors have reported.[37−39]

Due to the fusion being a slow process, it is not possible to define the critical concentration by PCS; therefore, the nephelometry technique was used to calculate it. The results are plotted in Figure 8, showing the c.c at 0.7 M.

To calculate the DLVO potentials, we have estimated the ζ-potential for different Ca^{2+} concentrations. The results are plotted in Figure 9. The initial negative value drastically decreases with increasing ion concentration,

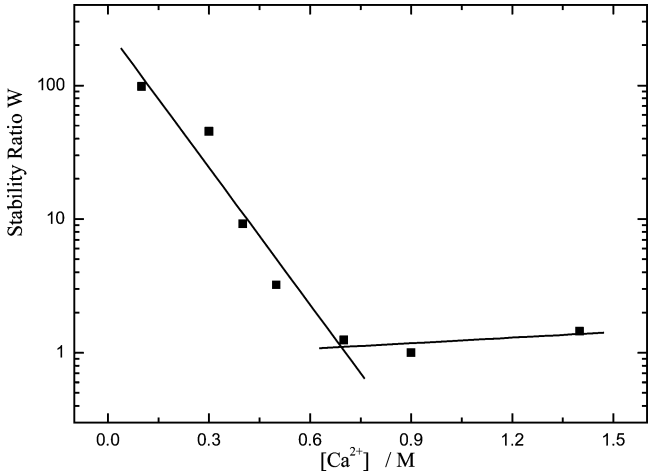

Figure 8. Variation of log W with Ca^{2+} concentration measured by nephelometry.

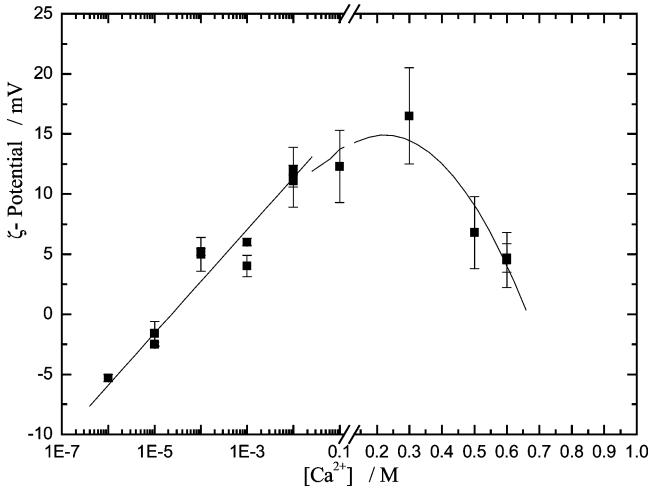

Figure 9. Zeta potential of PC liposomes as a function of Ca^{2+} concentrations.

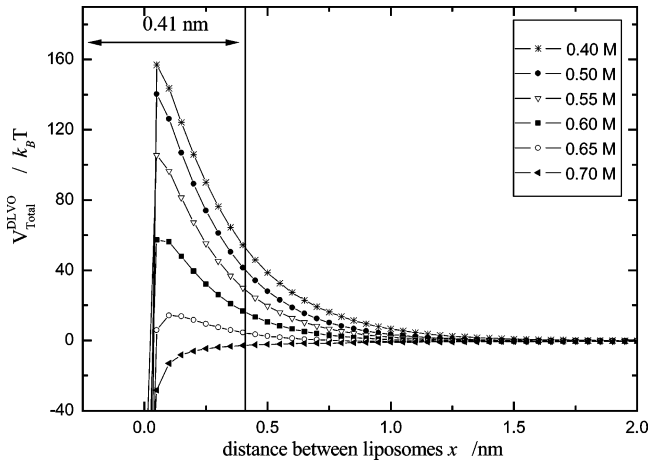

Figure 10. DLVO potentials of PC liposomes as a function of the distance between two liposomes for different Ca^{2+} concentrations. (*) 0.4, (●) 0.5, (▽) 0.55, (■) 0.6, (○) 0.65, and (▲) 0.7 M.

suggesting the binding of cations to the liposome surface until a maximum, after which a slight decrease with ion concentration is observed. Figure 10 shows DLVO potential curves calculated through the eqs 4, 5, and 7. As can be seen in Figure 10, the repulsive potential barrier at the hydrated surface (Δ = 0.41 nm)[11] disappears for larger Ca^{2+} concentrations than 0.65 M. The theoretical

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Table 1. Zeta Potential Reversal (pzr), $(d\zeta/d \log c)_\zeta = 0$, Number of Adsorption, N_1, Adsorption Constant, k_2, and Free Energy of Adsorption, ΔG_{ads} of Ca^{2+} and La^{3+} to PC Liposomes

	$(d\zeta/d\log c)_\zeta = 0$ (mV)	N_1 (m^{-2})	k_2 (m^3/mol)	ΔG_{ads} (kJ)
La^{3+}	6.05	5.12×10^{14}	6.1×10^6	−48.7
Ca^{2+}	4.1	1.41×10^{15}	1.81×10^6	−40.0

data are in reasonable agreement with the experimental data obtained by nephelometry.

Schulze−Hardy Rule. Following the Schulze−Hardy rule, the c.c. has to change with the inverse quadratic power of the valence, z, of the electrolyte counterions; i.e., c.c.c. $\propto 1/z^2$; if the ζ-potential is less than 100 mV.[11]

In our case, the Schulze−Hardy rule implies that $c.c._{Ca}/c.c._{La} = z_{La}^2/z_{Ca}^2 = 2.25$. Using the experimental measurements of the c.c. from nephelometry, we observe that $c.c._{Ca}/c.c._{La} = 2.3$. Since the Schulze−Hardy rule is derived directly from DLVO theory, the good agreement between the experimental data and the Schulze−Hardy rule is another proof of the suitability of DLVO theory to predict the colloidal stability of liposomes.

Characterization of the Ion Adsorption onto Liposome Surface. In an attempt to analyze the colloidal stability of liposomes, the ion adsorption has been studied. Figures 5 and 9 show ζ-potential values of liposomes as a function of La^{3+} and Ca^{2+} concentrations.

Considering the slope of ζ-potential versus log c at the ζ-potential reversal (pzr), the number of adsorption sites per unit area (N_1), can be calculated using the equation:[19]

$$\left(\frac{d\zeta}{d \log c}\right)_{\zeta=0} = 2.303\zeta^0\left[\frac{\epsilon\zeta^0(1+\kappa a)}{aze N_1} - 1\right] \qquad (10)$$

where ζ^0 is the ζ-potential in the absence of salt and the other symbols have their usual meaning.

The adsorption constant, k_2, can be calculated from the equation

$$\frac{1}{c_0} = k_2\left\{\frac{aze N_1}{\epsilon\zeta^0(1+\kappa a)} - 1\right\} \qquad (11)$$

where c_0 is the concentration at the pzr.

The standard free energy of adsorption, $\Delta G_{ads}{}^0$ can be obtained from the equation

$$k_2 = \exp\left(-\frac{\Delta G_{ads}{}^0}{k_B T}\right) \qquad (12)$$

Table 1 shows the number of adsorption sites, the adsorption constant, and the Gibbs free energy of the adsorption of La^{3+} and Ca^{2+} onto the PC liposome surfaces.

Although the adsorption constant k_2 is larger for La^{3+} than for Ca^{2+}, the number of adsorption sites, N_1, is smaller for La^{3+} than for Ca^{2+}. It is due to the electrostatic forces between La^{3+} ions onto the liposome surface do not allowing them to be close to each other. The larger number of calcium ions binding onto the liposome surface can be an indication of the increment of distance between the lipid molecules in the external surface of liposomes. This fact suggests that our liposome surface with Ca^{2+} is more favorable to fusion due to the decrease of the possible steric effects, which are opposite to fusion. Also, we can assume that the repulsion between lanthanum ions is more important, giving place to an aggregation process. To test this hypothesis, an experimental study on the effect of ion concentration on the interfacial behavior of PC molecules at the air−water interface was performed.

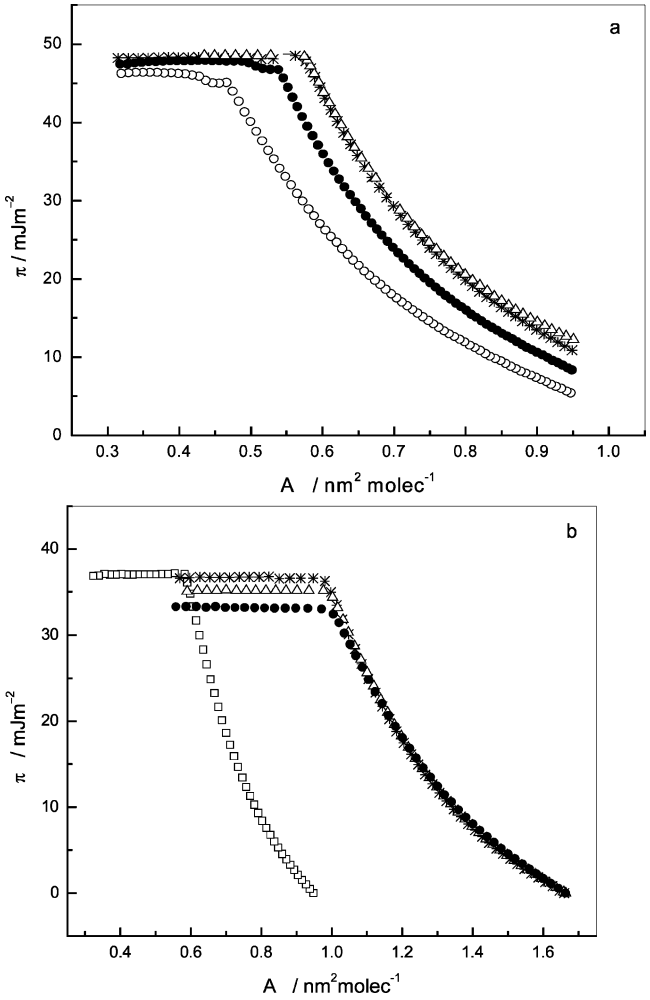

Figure 11. (a) Effect of La^{3+} on the PC monolayer. (○) 0.1, (●) 0.15, (*) 0.20, and (▲) 0.3 M La^{3+}. (b) Effect of Ca^{2+} on the PC monolayer. (□) 0.4, (*) 0.7, (△) 0.8, and (●) 1 M Ca^{2+}.

Phosholipid Monolayers. To confirm the effect of the Ca^{2+} and La^{3+} ions on the bilayer of liposomes, the behavior of PC monolayers at the air−water interface at different ionic strength values of the aqueous subphase has been studied. The interaction of La^{3+} and Ca^{2+} with PC molecules was analyzed using Langmuir monolayers. Cations were introduced into the subphase of the phospholipid monolayers, and their effects were characterized with surface pressure measurements. Figure 11a and b shows surface pressure/molecular area (π/A) isotherms for PC monolayers in the presence of different La^{3+} and Ca^{2+} concentration, respectively. Similar curves were obtained for all cation concentration values. The PC monolayer is characterized by a sharp transition between the liquid expanded (LE) and liquid condensed (LC) phases, which occurs at 25 °C. The extrapolated area, A_0, in the LC phase and the surface pressure (π) are shown in Figures 12 and 13. Figure 12 displays the variation of A_0 with the cation concentration. The form of curves suggests a pronounced effect of divalent and trivalent cations on the packing order and structure of the PC film. The surface area per lipid molecule increases significantly with the increase of cation concentration. This is due to the adsorption of hydrated cations onto the charged points of the lipid molecules. It is generally accepted that the cations are adsorbed specifically onto lipid bilayers.[40] This

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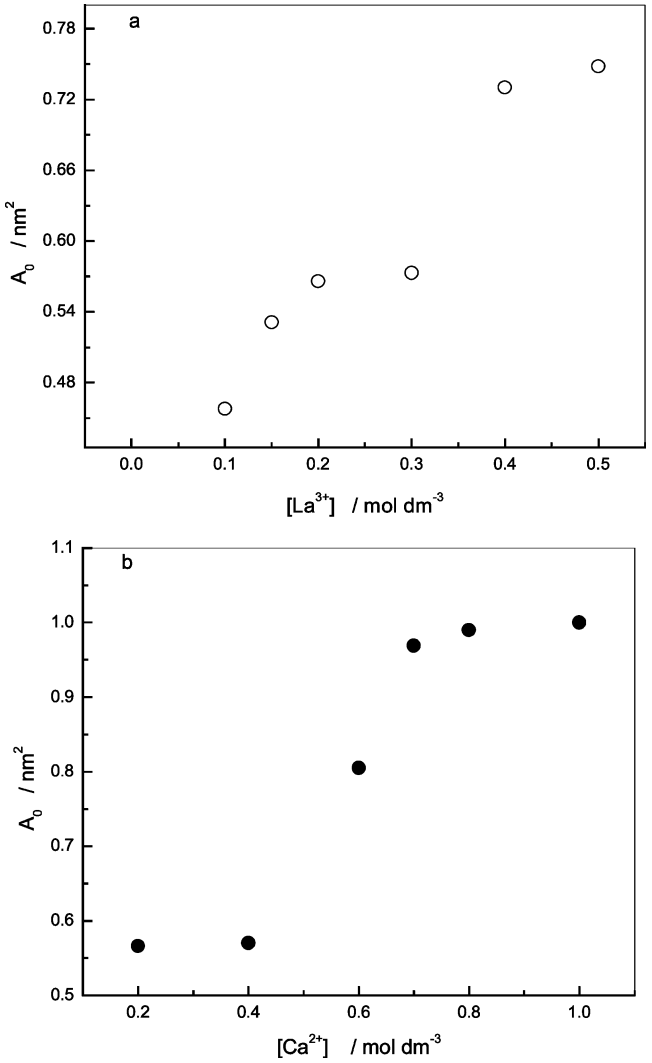

Figure 12. Variation of A_0 versus ion concentration. (a) La^{3+} and (b) Ca^{2+}.

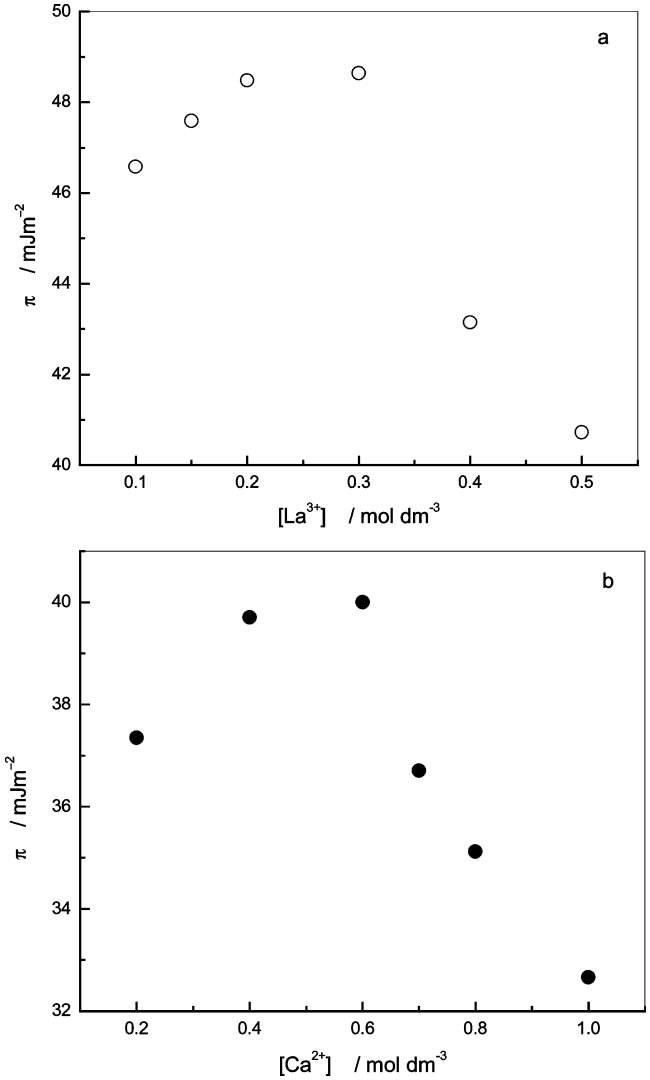

Figure 13. Variation of π versus ion concentration. (a) La^{3+} and (b) Ca^{2+}.

specific adsorption causes an inevitable change of the form and effective size of hydrated headgroups. A_0 increases simultaneously with cation concentration, but there is a concentration at which the increment in the A_0 value is greater. This concentration is the c.c. determined by nephelometry techniques. Figure 13 shows variation of π with cation concentration. The form of the curves exhibits a decrease of π from a certain salt concentration. This concentration corresponds with c.c. In equilibrium, three kinds of lateral pressures in the monolayer have to be balanced, i.e, $\pi_{head} + \pi_{chain} = \pi$, where π_{head} is the steric repulsive interaction between the headgroups in the phospholipid membrane, π_{chain} is the repulsive chain pressure, and π is the attractive interfacial pressure due to the hydrophobic interaction between the alkyl chains and water at the membrane interface.

Recent studies on the structure of phospholipid membranes clearly show that their membrane interfaces have a dynamic structure and are made of a complex and thermally disordered mixture of hydrophilic segments of headgroups, hydrophobic segments of alkyl chains, and water molecules,[11,41] The binding or interaction of substances with the membrane interface largely changes its structure, its surface area, and also intermolecular interactions such as π_{head} in the membrane interface.[42−43] Hence, in the case of the membrane interface of a phospholipid monolayer, the change in the surface free energy is not determined by the adsorption (i.e., surface excess) of substrates on the membrane according to Gibbs surface tension equation. Therefore, we have to consider the effect of the substances with the membrane interface on its structure and physical properties.

Cations can specifically adsorb onto headgroups of an electrically neutral PC membrane and induce orientation changes of the headgroup moving the N end of the P → N vector (see Figure 14) perpendicular to the water phase. In water (in absence of these ions), the headgroup orients itself almost parallel to the membrane surface. La^{3+} and Ca^{2+} may bind with the headgroup of the PC membrane near the phosphate group, which induces an electrostatic attraction between the negative charges of the phosphate groups (O^-) of neighboring phospholipids. This conformational change decreases π_{head}. The increase in the lateral compression pressure of the membrane can reasonably explain the disappearance of the undulation motion of the bilayer in the presence of cations and the fact that the transition LE → LC moves to a high A_0 value.

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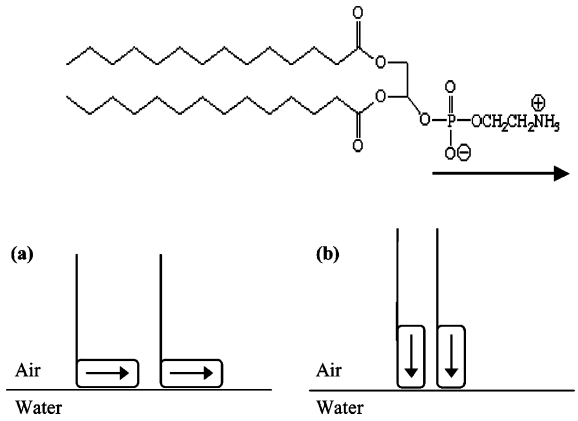

Figure 14. Phosphatidylcholine molecule (arrows indicate orientation of P → N vector in the headgroup). (a) In water; (b) in water + Ca^{2+}

If we translate these results to liposomes, a reduction of π_{head} at the liposome surface due to the presence of divalent and trivalent cations should increase electrostatic attraction between lipid molecules and favor their aggregation or fusion.

As can be seen in Figure 12, the Ca^{2+} increases the value of A_0 much more than does La^{3+}, which corresponds with a larger reduction of π_{head}. This result confirms our interpretation of liposome fusion in the presence of Ca^{2+} detected by TEM, while in the presence of La^{3+}, we can only observe liposome aggregation. In the presence of La^{3+}, the fusion among liposome particles is never observed. On the other hand, a smaller La^{3+} concentration is needed to commence the reduction of the π_{head}; this fact is related to the greater charge that the lanthanum ion possesses. Therefore, a smaller concentration of this ion is needed to generate the liposome aggregation.

Conclusions

Interactions between cations (La^{3+}, Ca^{2+}) and phospholipid liposomes have been studied by different techniques: PCS, electrophoretic mobility, and nephelometry. To corroborate the experimental results, we have used the DLVO theory to describe the colloidal stability of liposomes. Also, we have studied the effect of the two cations mentioned on the phospholipid monolayers. Light scattering shows an increase of size and polidispersity of the liposomes in the presence of lanthanum at concentration up to 0.3 M. This result can indicate an aggregation process of the liposomes. This is confirmed by micrographs obtained by TEM. The c.c. determined by nephelometry, using the stability constant (W), confirms that the aggregation processes start at a concentration of 0.3 M of La^{3+}. The liposome, upon addition of calcium, shows a different pathway in its behavior. The PCS measurements show a decrease in the size of the liposome until its concentration reaches 0.2 M, probably due the osmotic pressure. At larger concentration, the size and the polydispersity index increase, suggesting that the fusion process is progressing. In the PCS measurements, we cannot ensure when the process begins. However, by nephelometry measurements, we can determine the c.c. so the fusion process commences at a concentration of 0.7 M for Ca^{2+}. Also, the micrographs obtained by TEM confirm this fusion process. The DLVO theory, in the case of two spherical shells and the hydrated ions, illustrates that this theory is applicable to study the stability of this system. In both cases, the DLVO theory and experimental data agree. To confirm the consistency of DLVO, we applied the Schulze−Hardy rule and the results were reasonable. By means of electrophoretic measurements, we have characterized the adsorption of ions onto the liposome surface. The larger number of molecules of calcium suggests that the distance between lipid molecules in the external surface is larger than that in the lanthanum adsorption. To study the ion effect on the bilayer of liposome, we have analyzed the behavior of these ions on the phospholipid monolayer. The results indicate the specific adsorption of hydrated cations on the charged groups of the lipid molecules. The extrapolated area (A_0) increases simultaneously with cation concentration; however, there is a concentration at which the increment in the A_0 value is greater. This concentration corresponds with the c.c. determined by nephelometry. Ca^{2+} increases the value of A_0 much more than does La^{2+}, which corresponds with a higher reduction of π_{head}. This fact validates the phenomenon of liposome fusion in the presence of Ca^{2+} detected by TEM, while in the presence of La^{3+}, we can observe the liposome aggregation. In presence of La^{3+}, fusion is not reached. Also, the electrophoretic measurements predict a larger Ca^{2+} adsorption onto the liposome surface, which implies that the area, A_0, is bigger than that in the case of La^{3+}.

On the other hand, a smaller La^{3+} concentration is needed to commence the reduction of the π_{head}; this is related to the greater charge that the lanthanum ion possesses. Therefore, a smaller concentration of this ion is needed to generate the liposome aggregation

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