Physical Stability of Sonicated Arsonoliposomes: Effect of Calcium Ions

D.G. FATOUROS,[1] S. PIPEROUDI,[1] O. GORTZI,[1] P.V. IOANNOU,[2] P. FREDERIK,[3] S.G. ANTIMISIARIS[1]

[1]Laboratory of Pharmaceutical Technology, Department of Pharmacy, School of Health Sciences, University of Patras, 26500 Patras, Greece

[2]Department of Chemistry, University of Patras, Rio 26500, Patras, Greece

[3]EM-Unit/Department of Pathology, Maastricht University, P.O. Box 616, Maastricht, The Netherlands

Received 23 June 2004; revised 26 August 2004; accepted 27 August 2004

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20221

ABSTRACT: The physical stability of sonicated arsonoliposomes in the absence and presence of Ca^{2+} ions is evaluated. Cholesterol-containing arsonoliposomes composed of arsonolipids [having different acyl chains (C_{12}–C_{18})], or mixtures of arsonolipids with phospholipids (phosphatidylcholine or distearoyl-phosphatidylcholine) were prepared, and physical stability was evaluated in the absence and presence of $CaCl_2$, by vesicle dispersions turbidity measurements and cryo-electron microscopy morphological assessment. In some cases, vesicle ζ-potential was measured, under identical conditions. Results demonstrate that self-aggregation of the vesicles studied is low and influenced by the acyl chain length of the arsonolipid used, whereas calcium-induced aggregation is higher, correlating well with the decreased values of vesicle ζ-potential in the presence of Ca^{2+} ions (weaker electrostatic repulsion). Acyl chain length of arsonolipids used has a significant quantitative effect on Ca^{2+}-induced vesicle aggregation mainly for arsonoliposomes that contain phospholipids (mixed), compared with the vesicles that consist of plain arsonolipids (significant effect only for initial aggregation at time 0). Another difference between plain and mixed arsonoliposomes is that for mixed arsonoliposomes Ca^{2+}-induced increases in turbidity are irreversible by ethylenediaminotetraacetic acid, suggesting that vesicle fusion is taking place. This was confirmed by cryo-electron microscopy observations. Finally, when phosphatidylcholine is replaced by distearoyl-phosphatidylcholine, arsonoliposomes are more stable in terms of self-aggregation, but in the presence of calcium, the turbidity and morphology results are similar.
 J Pharm Sci 94:46–55, 2005
Keywords: liposomes; lipids; morphology; physical stability; particle size; light scattering (dynamic); microscopy

INTRODUCTION

We recently prepared and characterized vesicles containing arsonolipids,[1] attempting to combine the demonstrated antileukemic activity of arsenic-containing compounds[2] with the ability of liposomes to deliver cytotoxic drugs in an activity-enhancing and toxicity-reducing manor.[3,4] In view of the promising results obtained with some of the arsonoliposomes prepared, for which a differential toxicity toward cancer and normal cells was demonstrated[5,6] as well as *in vitro* antiparasitic activity,[7] further characterization of these vesicles is required, in respect to their physical stability.

It is well known that liposomes have a tendency to aggregate and subsequently fuse on storage.[8–10]

D.G. Fatouros's present address is Department of Pharmaceutics, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, 2100, Copenhagen, Denmark.

Correspondence to: S.G. Antimisiaris (Telephone: 30-2610-969332; Fax: 30-2610-996 302;
E-mail: s.antimisiaris@upatras.gr)

Journal of Pharmaceutical Sciences, Vol. 94, 46–55 (2005)

The presence of metal ions, especially divalent cations such as calcium,[9–12] are known to induce liposome aggregation and ultimately fusion. Aggregation by Ca^{2+} ions has been extensively studied in membranes containing negatively charged lipids. In liposomes made from phosphatidylserine, the addition of Ca^{2+} ions leads to aggregation, followed by vesicle fusion and leakage of aqueous content.[13] In contrast, calcium-induced aggregation of vesicles consisting of other lipids, such as digalactosyldiacylglycerol[14] or galactosylceramide and cerebroside sulfate[15] is completely reversible by addition of ethylenediaminetetraacetic acid (EDTA).

Arsonolipids are negatively charged lipids and thus arsonoliposomes have a negative surface charge that can be easily modulated by changing the vesicle lipid composition [by using mixtures of arsonolipids with phospholipids (mixed arsonoliposomes), or plain arsonolipids (plain arsonoliposomes)] or by using arsonolipids with different acyl chains for vesicle preparation (C_{12}–C_{18} arsonolipids have been synthesized). Thereby, before selecting arsonoliposomes for further *in vivo* studies, it is important to gain knowledge about the effect of these parameters on the physical stability or tendency of such vesicles for self-aggregation as well as on divalent cation-induced aggregation. The main purpose for conducting this study was to gain knowledge about the effect of divalent cations on the *in vitro* and *in vivo* behavior of arsonoliposomes, and mainly on the mechanism of any aggregation observed. Clarification of the mechanism of interaction between different types of arsonoliposomes (aggregation or fusion) may help clarify the mechanism of interaction between arsonoliposomes and cells.

In this study, we examined the effect of calcium ions on the size, surface charge, and morphology of arsonolipid-containing liposomes. Vesicle self-aggregation was also evaluated. For this, vesicle dispersion turbidity was measured (as an estimate of vesicle size) and vesicle ζ-potential. In addition, the morphology of the vesicles was observed by cryo-electron microscopy. Arsonolipids with different acyl chains [lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}), and stearic acid (C_{18})]—which were previously demonstrated[1] to form vesicles with different properties and morphology—were used, in order to investigate whether arsonolipid acyl chain length influences arsonoliposome behavior in the presence of Ca^{2+} ions. Another factor investigated was the arsonolipid content of liposomes. Plain arsonolipids or mixtures of arsonolipids with phosphatidylcholine (PC) were used for liposome preparation. In all cases, cholesterol was added in the liposomes prepared in a 2:1 molar ratio (lipid/Chol). Furthermore, the effect of replacing PC in mixed arsonoliposomes, with the saturated synthetic phospholipid distearoyl-phosphatidylcholine (DSPC), known to produce more rigid liposomes, was evaluated.

MATERIALS AND METHODS

Materials

Egg L-α-phosphatidylcholine (PC) and distearoylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids. The 99% purity of this lipid was verified by thin-layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4 v/v/v) as the solvent system and iodine staining.[16] Cholesterol (Chol) (pure), and all buffer salts were obtained from Sigma. All other reagents used were of analytical grade and were purchased from Sigma (Poole, UK). The water was deionized and then distilled.

Arsenic-containing analogs of phosphonolipids, the *rac*-2,3-diacyloxypropylarsonic acids [arsonolipids (C_{12}, C_{14}, C_{16}, and C_{18})], were synthesized as described previously.[17,18]

Liposome Composition

Using the arsonolipids (Ars) C_{12}–C_{18}, Chol, egg PC, and DSPC, we prepared liposomes with lipid compositions of Ars/Chol (2:1 mol/mol) and Ars/PC/Chol (or Ars/DSPC/Chol) 8:12:10 mol/mol/mol (40 mol % of total lipid is Ars).

Preparation of Liposomes

For the preparation of all types of liposomes studied, we used the "one step method" as previously reported.[1] In brief, the lipid or lipids (as powders) were mixed with water (or phosphate buffer, pH 7.40) and magnetically stirred vigorously on a hot plate for 6–12 h at 70–90°C, depending on the transition temperature of the lipid used in each case. The liposome suspensions, produced initially, were decreased in size by sonication for at least two 10-min cycles. A probe type Vibra-cell sonicator (Sonics and Materials, UK) equipped with a tapered micro tip was used at a power setting of 600 W. In all cases, the initially turbid liposomal suspension was clarified after

sonication. After sonication, the liposome suspensions were allowed to anneal for 2 h at a temperature higher than the transition temperature of the lipid used in each case, in order to correct any structural defects. Any titanium fragments (from the probe), as well as multilamellar vesicles or liposomal aggregates present in the samples, were removed by centrifugation at 10.000*g* for 15 min. The lipid content of the samples was routinely determined using a colorimetric technique that is widely applied for phospholipids, the Stewart assay,[19] in which the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution is utilized. This assay was found to also detect arsonolipids (at the high concentrations of the initial dispersions). In brief, liposome samples (20 μL) are vortexed with 2 mL of a solution of ammonium ferrothiocyanate (0.1 M) and 2 mL of chloroform. The OD-485 nm of the chloroform phase is measured and the lipid concentration of samples is calculated by comparison with an appropriate standard curve.

Vesicle ζ-Potential

Liposome dispersions were diluted with TBS pH 7.40, or H_2O, or $CaCl_2$ (depending on the experiment), and their electrophoretic mobility was measured at 25°C by photon correlation spectroscopy (Zetasizer 5000; Malvern Instruments, UK). ζ-Potential values of the dispersions were calculated (by the instrument), according to the Helmholtz-Smoulukowski equation.

Calcium-Induced Aggregation and Self-Aggregation of Liposomes

Calcium-induced vesicle aggregation, as well as self-aggregation were studied by measuring the turbidity of the vesicle dispersions.

Arsonoliposomes, which were prepared in water, were diluted with water (for self-aggregation studies), or with solutions of calcium chloride (final calcium concentration ranged from 0.43 to 1.8 mM) for calcium-induced aggregation studies, to give a final lipid concentration of 0.065 mM.

Higher calcium concentrations (calcium concentration in plasma is somewhat higher than the highest concentration used in this study: >2 mM compared with 1.8 mM, respectively), were not used, because our experiments were conducted in water and not in the presence of serum proteins, and thus all Ca^{2+} ions were available for interaction with the vesicles, which is not the case in serum, where some of the Ca^{2+} ions may be bound to proteins.

Aggregation was evaluated by measuring the turbidity of liposome dispersions at a wavelength of 500 nm, using a Shimatzu RF-1501 Spectrofluorophotometer (emission and excitation both set at 500 nm, slit 10–10) equipped with a thermostated sample holder and a magnetic stirrer.

Initial turbidity of the vesicle dispersions in H_2O at the beginning of each experiment (time 0) was taken as starting point. Turbidity was measured for mixed arsonoliposomes composed of Ars/PC/Chol or Ars/DSPC/Chol (8:12:10 mol/mol/mol) as well as plain arsonoliposomes composed of Ars/Chol (20:10 mol/mol). Three different arsonolipids were used, the C_{12}, C_{16}, and C_{18}, and their turbidity was measured immediately after mixing with $CaCl_2$—or plain H_2O, for self aggregation—as well as after 2 h, 4 h, and 24 h.

In some cases, the effect of EDTA on the calcium-induced turbidity change of the vesicle dispersions was evaluated by remeasuring the turbidity after adding a tenfold amount of EDTA (compared with the final calcium concentration of the samples), and correcting the turbidity value measured by the dilution factor. The decrease in turbidity due to sample dilution was accounted for by performing blank experiments (diluting some samples with H_2O). This control experiment would indicate if the vesicles are fusing or perhaps changing morphology, or if only loose aggregates—that can be easily disassembled upon Ca^{2+} removal—are formed.

Cryo-Electron Microscopy Study

The morphology of some arsonoliposome samples was studied by cryogenic transmission electron microscopy, before and after incubation in the presence of 1 mM $CaCl_2$ (for 6 or 24 h). For this, small aliquots (3 μL) were taken from the arsonolipid-containing liposome suspensions. Aliquots were applied to Quantifoil grids (R2/2 Quantifoil Jena) within the environmental chamber (relative humidity 100%, 24°C) of the Vitrobot (UM patent licensed to FEI). Excess liquid was blotted away with filter paper using an automatic blotting device within the environmental chamber of the Vitrobot. The grid was subsequently shot through a shutter into melting ethane placed just outside the environmental chamber.[20] The vitrified specimens were stored under liquid nitrogen and observed at −170°C (Gatan 626 cryo holder) in a

Philips CM12 microscope. Micrographs were taken at 120 kV using low dose conditions.

Statistical Analysis

For the statistical evaluation of differences between results, several statistical tests were used. The paired t test was used to check the significance between turbidity differences, under the same conditions (time and calcium) of different liposome types. For checking the significance of self-aggregation as well as the effect of EDTA on reversing aggregation, we used single-sample t test (relative turbidity values compared with 1). Finally, to check the significance of the effect of arsonoliposome type (arsonolipid acyl chain length and lipid composition), time of incubation, and calcium concentration on relative turbidity values, we used two-way analysis of variance, and thus evaluated the significance of independent variables alone and in combinations (Statistica for Windows, 4.3).

In all cases, a probability value of <0.05 was considered to be significant

RESULTS AND DISCUSSION

Vesicle Dispersion Turbidity

The results of the turbidity experiments, expressed as relative turbidity, are presented in Figure 1A–D. From the results presented in Figure 1,

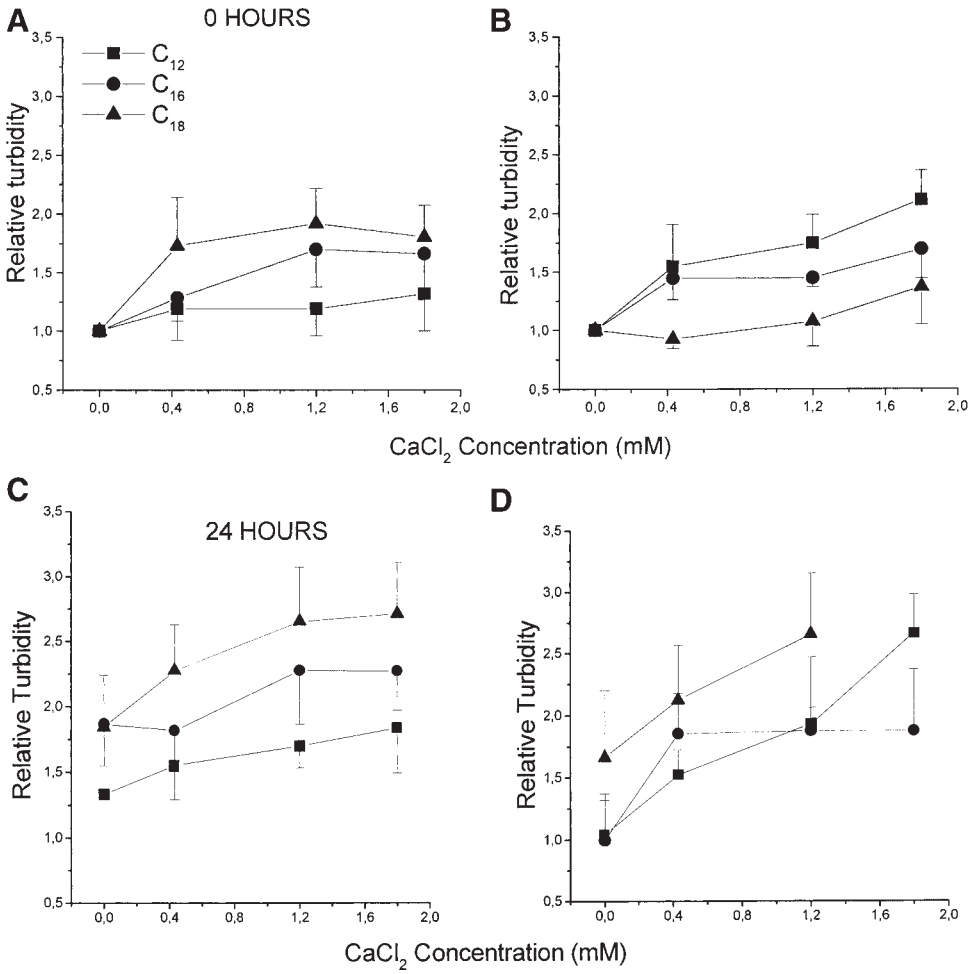


Figure 1. Calcium-induced aggregation of sonicated arsonoliposomes estimated by the increase in turbidity (relative turbidity) of the vesicle dispersions in water (caused by the presence of various concentrations of $CaCl_2$). Each value is the mean of at least four different experiments. Bars represent standard deviations. Experiments were performed as presented in detail in the Materials and Methods section. (A, C) Ars/PC/Chol arsonoliposomes; (B, D) Ars/Chol arsonoliposomes. Key: as presented in the inset in (A).

although standard deviation values of most experimental points (calculated for at least four different experiments) are high, we can extract some interesting conclusions.

First, self-aggregation [evidenced by the increase in vesicle turbidity after incubation in absence (0 mM) of $CaCl_2$] for both types of arsonoliposomes studied is demonstrated only after 24 h for the C_{12}/PC/Chol, C_{16}/PC/Chol, C_{18}/PC/Chol (Fig. 1C), and the C_{18}/Chol vesicles (Fig. 1D). Statistically, the aggregation values are at significance (at $p < 0.05$) for C_{12}/PC/Chol ($p = 0.0023$) and C_{16}/PC/Chol ($p = 0.0023$), and insignificant for the other cases. This corresponds well with a previous study[1] in which the size distribution of similar types of vesicles was measured by photon correlation spectroscopy after 24 and 48 h of incubation in buffer, and minimum increases in vesicle sizes were demonstrated. In addition, mixed arsonoliposomes (composed of mixtures of Ars with PC) are physically less stable compared with plain arsonoliposomes [for which no significant self-aggregation was observed (Fig. 1B and D)].

Second, concerning calcium-induced vesicle aggregation, in general the arsonolipid acyl chain length seems to have a significant effect on the extent of aggregation (as discussed in detail below); however, in all cases, the aggregation profile is similar for similar types (lipid composition) of vesicles (as confirmed by two-factor analysis of variance). Nevertheless, the different types of arsonoliposomes studied demonstrate several differences in the mode of Ca^{2+}-induced aggregation. More analytically, at time 0 (Fig. 1A and B), it is evident that for the mixed arsonoliposomes (Fig. 1A) and plain arsonoliposomes (Fig. 1B) there is a quantitative difference concerning the effect of arsonolipid acyl chain length. The effect of Ca^{2+} on relative vesicle turbidity in the case of the mixed arsonoliposomes is highest for vesicles composed of C_{18} arsonolipids followed by C_{16} and then C_{12} (Fig. 1A). In the case of plain arsonoliposomes, vesicles composed of C_{12} aggregate the most—almost in linear dependence with the concentration of Ca^{2+} ions present. The C_{16} vesicles have an intermediate aggregation profile and, finally, vesicles composed of the C_{18} arsonolipid are almost not affected by the presence of Ca^{2+} ions, and their turbidity is only minimally increased at the highest concentration of $CaCl_2$ (Fig. 1B). Analysis of variance confirmed that the effect of acyl chain length is significant in both types of vesicles ($p = 0.0097$ for mixed and 0.0014 for plain arsonoliposomes). Another difference between the two types of arsonoliposomes in terms of initial Ca-induced aggregation is the fact that the effect of calcium ions is saturated for mixed arsonoliposomes (calcium concentrations >0.43 mM have no further effect on vesicle turbidity, $p = 0.1784$) (see Fig. 1A) but not for plain ones ($p = 0.0077$) (see Fig. 1B), suggesting a different mechanism of aggregation for the two types of arsonoliposomes, at least for the initially dominant mechanism, if aggregation is a combination of several concurring events.

After 24 h of incubation, the arsonolipid acyl chain length continues to have an effect on calcium-induced aggregation of mixed arsonoliposomes ($p = 0.0007$) (see Fig. 1C), but not of plain arsonolipid vesicles ($p = 0.2252$) (see Fig. 1D). However, at this time point, the effect of increasing calcium concentration on the turbidity of mixed arsonoliposomes is not saturated, as observed in Figure 1A. Indeed, increase in calcium concentration (in the range 0.43–1.80 mM) significantly increases vesicle aggregation ($p = 0.0110$). This implies that perhaps more than one concurrent mechanisms of aggregation with different rates or kinetic profiles are taking place.

When considering the effect of time of incubation on the calcium-induced increase in vesicle turbidity, it should be mentioned that turbidity values measured after 2 and 4 h of incubation did not have significant differences ($p > 0.05$) from those measured at time 0, and are not shown here. However, it is evident (Fig. 1C and D) that, in addition to the differences mentioned above, after 24 h of incubation, vesicle aggregation is considerably higher (at $p = 0.05$) compared with the values obtained for the same vesicles at time 0) although for most of the experimental points in this case, especially for the plain arsonoliposomes (Fig. 1D), there is higher variation.

When replacing PC in mixed arsonoliposomes composed of the C_{16} arsonolipid with the saturated synthetic DSPC, the arsonoliposomes prepared, as seen in Figure 2, are extremely stable in terms of self-aggregation (increase of turbidity at 0 mM $CaCl_2$). Only a very small increase in turbidity is measured after 24 h of incubation; which is statistically insignificant ($p = 0.066$). Although we would expect that these arsonoliposomes would be more stable, compared with the PC-based ones, because DSPC is known to produce more rigid membranes, and this is indeed the case for stability in water; their behavior in the presence of calcium is absolutely comparable to that of

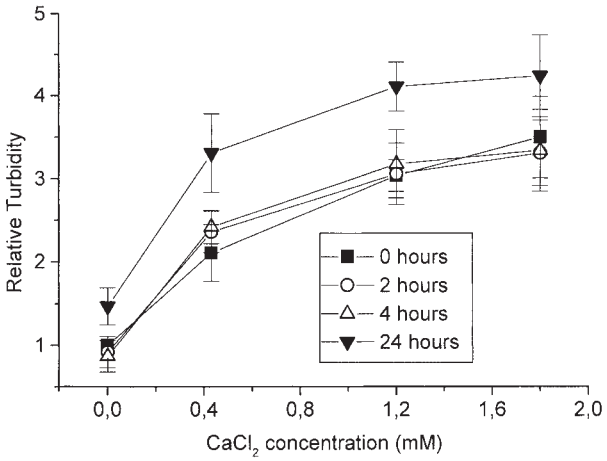
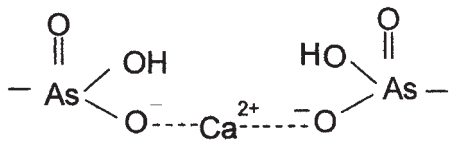


Figure 2. Calcium-induced aggregation of sonicated C_{16}/DSPC/Chol (8:12:10, mol/mol/mol) arsonoliposomes estimated by the increase in turbidity (relative turbidity) of the vesicle dispersions in water (caused by the presence of various concentrations of $CaCl_2$), at various time periods of incubation (0, 2, 4, and 24 h) Each value is the mean of at least four different experiments. Bars represent standard deviations. Experiments were performed as presented in detail in the Materials and Methods section. Key: as presented in the inset.

PC-based arsonoliposomes, with the exception that initial calcium-induced turbidity is calcium dependent ($p < 0.05$) in the full range studied (and not saturated as observed for mixed arsonoliposomes with PC).

In general, calcium is expected to induce aggregation of arsonoliposomes, after removal of water, by acting as bridge according to Scheme 1 (as suggested previously for phosphatidylserine-containing vesicles[13]).

As mentioned above, the effect of EDTA on turbidity of vesicle dispersions that have been



Scheme 1.

aggregated by Ca^{2+} ions may provide some insight on the aggregation mechanism.[21]For this, we added EDTA (in a tenfold molar excess) in the aggregated samples and remeasured their turbidity. The relative turbidity values measured after EDTA addition (after being corrected for dilution) are presented in Table 1. Regardless of the high variability of these results, the relative turbidity values are significantly higher than 1 (at $p = 0.05$) in most of the mixed arsonoliposomes studied (especially C_{16} and C_{18} arsonolipid-containing vesicles) as well as the vesicles in which PC was replaced by DSPC. It is thereby evident that, for these arsonoliposomes, the increase in turbidity measured after incubation with calcium is not reversible or, in other words, that the aggregates formed are not loose aggregates that rapidly break down when Ca^{2+} ions are removed by EDTA complexation. On the contrary, in almost all cases of plain arsonoliposomes studied, the results of relative turbidity after EDTA addition (after statistical evaluation), do not provide proof of a nonreversible process, with the exception of the C_{12}/Chol arsonoliposomes. Therefore, lipid composition of arsonoliposomes seems to have an effect on the mechanism of calcium-induced increase of vesicle dispersion turbidity, and the results of Table 1 imply that, from all the Ars/PC/Chol (and the C_{16}/DSPC/Chol) arsonoliposomes studied, new larger structures are formed in the presence of

Table 1. Relative Turbidity Values for Plain and Mixed Sonicated Arsonoliposomes, Measured after 24 h of Incubation in Water or in the Presence of $CaCl_2$, after the Addition of a Tenfold Excess Concentration of EDTA

$CaCl_2$ concentration	0 mM $CaCl_2$	0.43 mM $CaCl_2$	1.20 mM $CaCl_2$	1.80 mM $CaCl_2$
Liposome composition				
C_{12}/PC/Chol (8:12:10)	1.17 (0.18)	1.31 (0.15)	1.98 (0.38)	2.00 (0.34)
C_{16}/PC/Chol (8:12:10)	1.63 (0.38)	2.49 (0.41)	2.86 (0.44)	2.82 (0.48)
C_{18}/PC/Chol (8:12:10)	1.48 (0.11)	2.19 (0.43)	3.13 (0.39)	2.87 (0.56)
C_{12}/Chol (20:10)	0.71 (0.11)	1.51 (0.25)	2.18 (0.33)	2.18 (0.35)
C_{16}/Chol (20:10)	0.78 (0.14)	1.78 (0.35)	1.38 (0.12)	1.25 (0.14)
C_{18}/Chol (20:10)	0.84 (0.12)	1.49 (0.35)	1.42 (0.40)	1.15 (0.36)
C_{16}/DSPC/Chol (8:12:10)	1.71 (0.33)	2.68 (0.35)	2.63 (0.49)	2.54 (0.50)

Values were corrected for dilution and each value is the mean of at least four separate experiments (SD of each mean value is presented in parentheses).

calcium ions. It is interesting thereby to investigate the morphology of these structures.

ζ-Potential Measurements

To explain the aggregation results, the effect of $CaCl_2$ on vesicle ζ-potential values was evaluated. In general, as the ζ-potential of the vesicles decreases, it is logical to have better chances for aggregation between vesicles, because of the decrease of repulsive forces.

The results of the ζ-potential measurements are presented in Figure 3A and B. In Figure 3A, it is observed that, for mixed arsonoliposomes (Ars/PC/Chol), the vesicles composed of C_{12} arsonolipid are affected minimally in respect to the modification of their surface charge by increasing concentrations of Ca^{2+} ions. Conversely, the ζ-potential of the C_{16} arsonolipid vesicles is decreased (the negative value) in a linear dependency with $CaCl_2$ concentration. This observation is in good agreement with the higher increase in turbidity of mixed arsonoliposomes composed of arsonolipids with longer acyl chain length ($C_{18} > C_{16} > C_{12}$) (as observed in Fig. 1A). Conversely, from Figure 3B, in which the effect of Ca^{2+} ions on the ζ-potential of plain arsonoliposomes (Ars/Chol) is presented, it is obvious that the C_{12} and C_{18} arsonolipid vesicles are affected similarly in terms of surface charge modification, in agreement with the similar effect of $CaCl_2$ on the turbidity of these arsonoliposomes (Fig. 1B). Therefore, in general, increases in vesicle turbidity correlate well with the effect of Ca^{2+} ions on the vesicle surface charge.

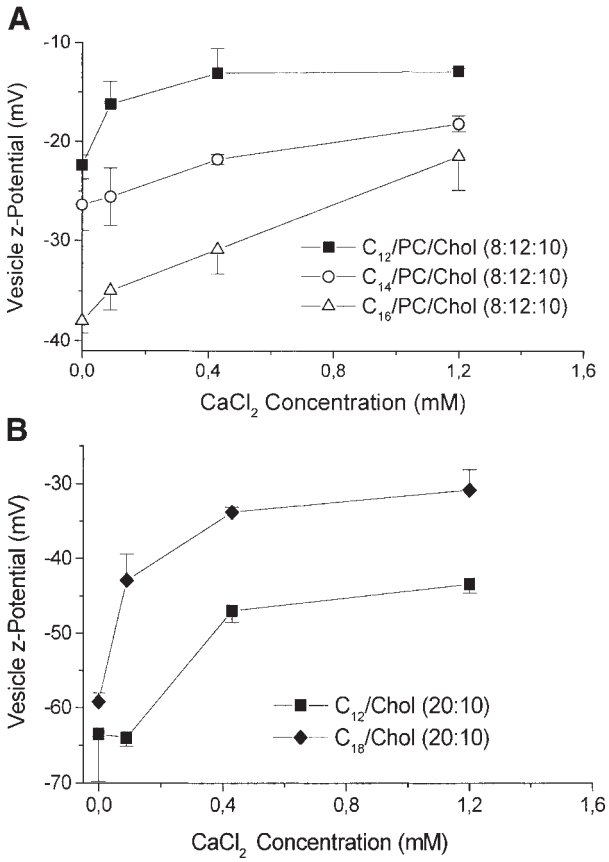


Figure 3. ζ-Potential values of sonicated arsonoliposomes in the absence and presence of various concentrations of $CaCl_2$ (at time 0—immediately after incubation). Each value is the mean of at least five measurements from three different samples. Bars represent standard deviations. Experiments were performed as presented in detail in the Materials and Methods section. (A) Mixed arsonoliposomes consisting of Ars/PC/Chol (8:12:10, mol/mol/mol). (B) Plain arsonoliposomes Ars/Chol (20:10, mol/mol). Key: as presented in the inserts.

Cryo-Electron Microscopy

Samples of the Ars/PC/Chol arsonoliposomes composed of C_{12} and C_{16} arsonolipids, as well as the C_{16}/DSPC/Chol arsonoliposomes, were evaluated morphologically before and after 6 and 24 h of incubation in the presence of 1 mM $CaCl_2$. These compositions were selected for a morphological evaluation of the effect of Ca^{2+} ions, because of the values of relative turbidity measured after addition of EDTA, which indicate that increase in size is irreversible. Results are presented in Figure 4 for C_{12}/PC/Chol (Fig. 4A–C), C_{16}/PC/Chol (Fig. 4D–F), and C_{16}/DSPC/Chol (Fig. 4G–I) arsonoliposomes, respectively. Left-side micrographs show vesicles in absence of Ca^{2+} ions, middle micrographs show vesicles incubated in $CaCl_2$ for 6 h, and right-side micrographs show the same vesicles after 24 h. In all cases, it is evident that vesicle diameter is significantly increased after incubation with $CaCl_2$, with some very large vesicles present (the structures mainly presented in the micrographs shown here). In some cases (marked by arrows), there is evidence of fusion between two vesicles, in correlation to a theory published previously.[22–24] According to this theory, during vesicle fusion, a hemifusion intermediate is formed, in which two fusing compartments are separated by one mutual bilayer membrane, the "bilayer diaphragm." In our micrographs, such "diaphragms" are observed, as well as cases in which it looks as the diaphragm has been opened (forming a fusion pore).

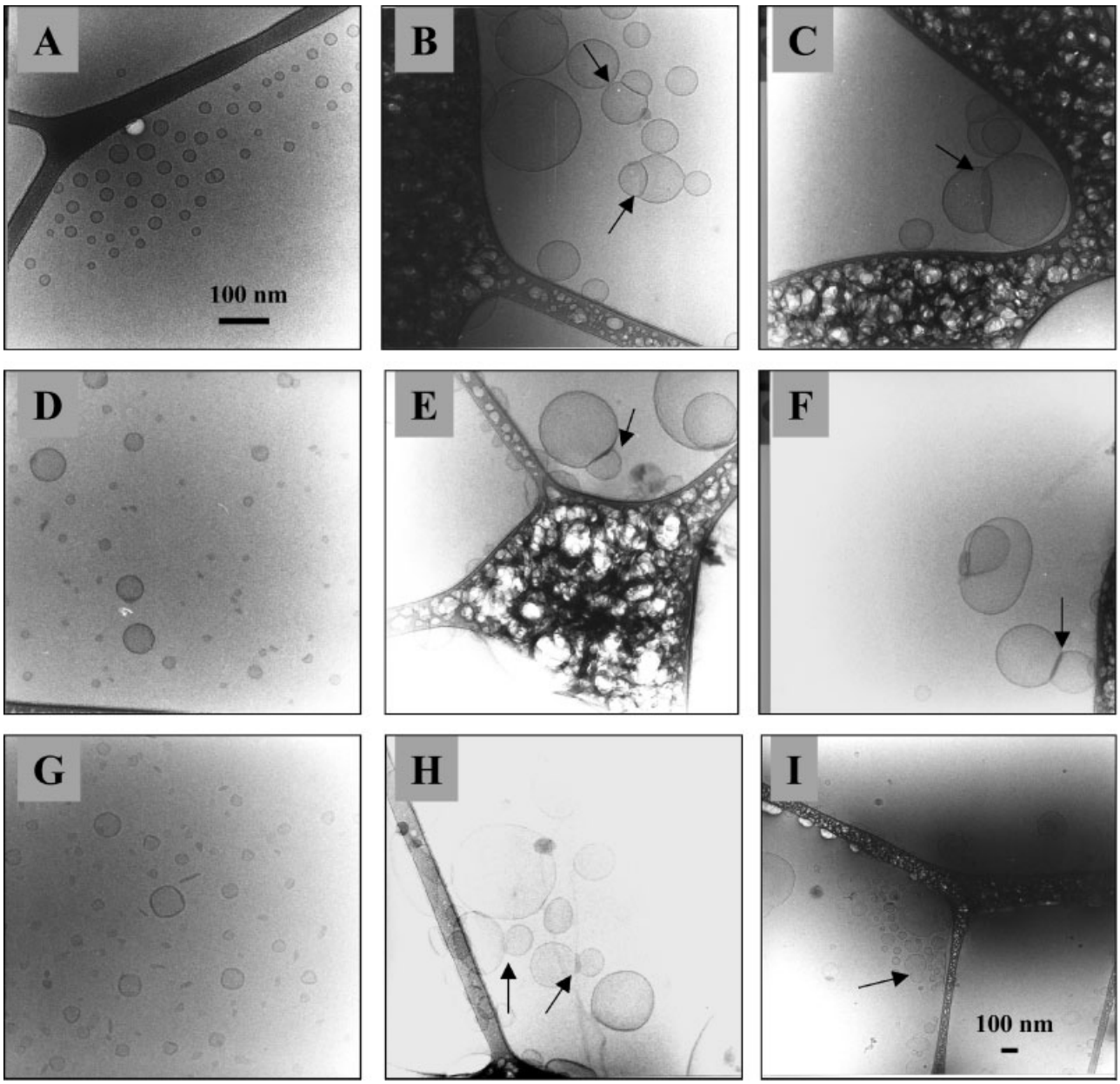


Figure 4. Cryo-electron micrographs of the vesicles observed in samples of C_{12}/PC/Chol (8:12:10, mol/mol/mol) arsonoliposomes (top panels), C_{16}/PC/Chol (8:12:10, mol/mol/mol) arsonoliposomes (middle panels), and C_{16}/DSPC/Chol (8:12:10, mol/mol/mol) arsonoliposomes (bottom panels) as they are dispersed in H_2O (A, D, and G) after they have been incubated in $CaCl_2$ (1 mM final concentration) for 6 h (B, E, H) or for 24 h (C, F, I). Magnification is always 206500× except I where the magnification is 45500×. Bar is 100 nm.

Regardless of the mechanism of fusion, it is evident that vesicle size distribution of the vesicles present in the samples in all the arsonoliposome compositions studied is substantially higher after incubation in the presence of $CaCl_2$, whereas no difference between the 6- and 24-h incubation is observed. However, to avoid misinterpretation, it should be mentioned that the micrographs shown here are not representative of the whole picture obtained after incubation of arsonoliposomes with calcium, because in all cases, a number of vesicles was still of small size (comparable to the initial size

Table 2. Mean Diameters of Arsonoliposomes before and after Incubation in the Presence of 1 mM $CaCl_2$, for 6 and 24 h

Sample	Vesicle mean diameter (SD)
C_{12}/PC/Chol (8:12:10), control	36 (13)
C_{12}/PC/Chol (8:12:10) + 1 mM $CaCl_2$ (6 h)	158 (67)
C_{12}/PC/Chol (8:12:10) + 1 mM $CaCl_2$ (24 h)	130 (23)
C_{16}/PC/Chol (8:12:10), control	35 (15)
C_{16}/PC/Chol (8:12:10) + 1 mM $CaCl_2$ (6 h)	70 (21)
C_{16}/PC/Chol (8:12:10) + 1 mM $CaCl_2$ (24 h)	91 (52)

For each sample, the mean diameter and standard deviation (in parentheses) were calculated after measuring the diameter of at least 300 vesicles from the micrographs obtained by cryo-electron microscopy, as discussed in detail in the Materials and Methods section.

of the vesicles, not shown). From the total number of micrographs obtained, we calculated the mean diameter of vesicles for each case studied (of the Ars/PC/Chol arsonoliposomes from C_{12} and C_{16} arsonolipids) by measuring the diameter of at least 300 vesicles. As presented in Table 2, the mean vesicle size of the arsonoliposomes in all cases is significantly increased after incubation in the presence of $CaCl_2$; however, even the highest mean diameter is $<$200 nm. Anyhow, these mean diameter values can only be used for comparison between the different samples and are not accurate measurements of the vesicles sizes, because a relatively low number of vesicles was measured.

Finally, from this morphological study, we can conclude that in the presence of Ca^{2+} ions, Ars/PC/Chol arsonoliposomes fuse, forming larger round vesicles, whereas no differences can be observed between the different arsonolipids studied here, in terms of the mechanism of fusion or the extent and rate of the fusion process. In addition, it should be stressed that these results cannot provide accurate information about the extent of aggregation or fusion of arsonoliposomes in serum. Indeed, because there is no protein present in the vesicle suspensions studied here, Ca^{2+} effects in the test tube might be more dramatic than in the blood stream, where much Ca^{2+} is bound to serum proteins.

CONCLUSIONS

Herein, we studied the physical stability of sonicated arsonoliposomes, in the absence and presence of various concentrations of $CaCl_2$. The results of the experiments performed reveal that the vesicle lipid composition has a significant effect on the self-aggregation (physical stability) of arsonoliposomes. Indeed, plain arsonoliposomes are more stable compared with mixed ones composed of mixtures of PC with arsonolipids; however, when PC is replaced by DSPC in C_{16}-containing arsonoliposomes, the physical stability of these mixed arsonoliposomes is comparable to that of plain arsonoliposomes. When comparing the physical stability of the mixed arsonoliposomes studied, it is evident that as the acyl chain length of the arsonolipid used for vesicle preparation increases, physical stability of vesicles decrease. Arsonoliposomes prepared by the C_{18} arsonolipid are the least stable.

In the presence of Ca^{2+} ions, vesicle aggregation is substantially enhanced for plain arsonoliposomes as well as for mixed arsonoliposomes (no difference between mixed arsonoliposomes with PC or DSPC). However, although the arsonolipid acyl chain length seems to have an influence on the behavior of mixed arsonoliposomes (higher aggregation as arsonolipid acyl chain increases); for the plain arsonoliposomes, a statistically significant effect was demonstrated only for initial turbidity, at time 0.

The most interesting difference between mixed and plain arsonoliposomes in terms of Ca^{2+}-induced vesicle aggregation is that, in all cases of mixed arsonoliposomes (even the DSPC-containing ones), this aggregation is not EDTA-reversible, a fact that indicates that fusion is the mechanism of increase of vesicle size. This indication was confirmed after morphological evaluation of the effect of calcium on the mixed arsonoliposomes which revealed that vesicles are fusing into larger vesicles, possibly by the previously reported[21–24] "stalk-pore" model. Concluding, we should mention that this last finding may suggest that, when arsonolipids are incorporated in liposomal membranes with phospholipids, they may enhance the

possibility of fusion between vesicles and cells, depending on the composition of the specific cell membrane. Perhaps this may be linked to the interesting results obtained previously with this type of liposomes[5,6] in terms of their interactions with different cell types in culture.

Finally, further experiments are needed to demonstrate whether the findings of this *in vitro* study can be used for the prediction of the relative *in vivo* behavior of different types of arsonoliposomes, whereas the results obtained here may be used as a basis for the design of future *in vivo* studies.

REFERENCES

1. Fatouros D, Gortzi O, Klepetsanis P, Antimisiaris SG, Stuart MCA, Brisson A, Ioannou PV. 2001. Preparation and properties of arsonolipid containing liposomes. Chem Phys Lipids 109:75–89.
2. Bode AM, Dong Z. 2002. The paradox of arsenic: Molecular mechanisms of cell transformation and chemotherapeutic effects. Crit Rev Oncol Hematol 42:5–20.
3. Gregoriadis G. 1993. Liposome technology. 2nd ed. Boca Raton, FL: CRC Press.
4. Lasic DD, Papahadjopoulos D. 1998. Medical applications of liposomes. Amsterdam: Elsevier Science.
5. Gortzi O, Papadimitriou E, Kontoyannis C, Antimisiaris SG, Ioannou PV. 2002. Arsonoliposomes, a novel class of arsenic-containing liposomes: Effect of palmitoyl-arsonolipid-containing liposomes on the viability of cancer and normal cells in culture. Pharm Res 19:79–86.
6. Gortzi O, Papadimitriou E, Antimisiaris SG, Ioannou PV. 2003. Cytotoxicity of arsonolipid containing liposomes towards cancer and normal cells in culture: Effect of arsonolipid acyl chain length. Eur J Pharm Sci 18:175–183.
7. Antimisiaris SG, Ioannou PV, Loiseau PM. 2003. *In vitro* antileishmanial and trypanocidal activities of arsonoliposomes and preliminary *in vivo* distribution. J Pharm Pharmacol 55:647–652.
8. Bentz Z, Nir S. 1981. Aggregation of colloidal particles modeled as a dynamic process. Proc Natl Acad Sci USA 78:1634–1637.
9. Crommelin DJA. 1984. Influence of lipid composition and ionic strength on the physical stability of liposomes. J Pharm Sci 73:1559–1565.
10. Mosharraf M, Taylor KMG, Craig D. 1995. Effect of calcium ions on the surface charge and aggregation of phosphatidylcholine liposomes. J Drug Target 2:541–545.
11. Armengol X, Estelrich J. 1995. Physical stability of different liposome compositions obtained by extrusion method. J Microencapsul 12:525–535.
12. Chauhan A, Chauhan VPS, Brockerhoff H. 1986. Effect of cholesterol on Ca^{2+} induced aggregation of liposomes and calcium diphosphatidate membrane traversal. Biochemistry 25:1569–1573.
13. Wilschut J, Duzgunes N, Hong K, Hoekstra D, Papahadjopoulos D. 1983. Retention of aqueous contents during divalent cation-induced fusion of phospholipid vesicles. Biophys Biochim Acta 734: 309–318.
14. Menikh A, Fragata M. 1993. Fourier-transform infrared spectroscopic study of ion-binding and intramolecular interactions in the polar head of digalactosyldiacylglycerol. Eur Biophys J 22:249–258.
15. Stewart RJ, Boggs JM. 1993. A carbohydrate interaction between galactosylceramide-containing liposomes and cerebroside sulfate-containing liposomes: Dependence on the glycolipid ceramide composition. Biochemistry 32:10666–10674.
16. New RRC. 1990. Liposomes: a practical approach. In: New RRCR, New RR, editors. Oxford: Oxford University Press, pp 134, 255.
17. Tsivgoulis GM, Sotiropoulos DN, Ioannou PV. 1991. *rac*-1,2-Diacylo-xypropyl-3-arsonic acids: Arsonolipid analogues of phosphonolipids. Phosphorus Sulfur Silicon 63:329–334.
18. Serves SV, Sotiropoulos DN, Ioannou PV, Jain MK. 1993. One pot synthesis of arsonolipid via thioarsenite precursors. Phosphorus Sulfur Silicon 81:181–190.
19. Stewart JCM. 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 104:10–14.
20. Frederik PM, Burger KNJ, Stuart MCA, Verkleij AJ. 1991. Lipid polymorphism as observed by cryo-electron microscopy. Biochim Biophys Acta 1062: 133–141.
21. Ravoo BJ, Stuart MCA, Brisson A, Weringa WD, Engberts JBFN. 2001. Electron microscopic investigation of the morphology and calcium-induced fusion of lipid vesicles with an oligomerised inner leaflet. Chem Phys Lipids 109:63–74.
22. Siegel DP. 1993. Energetics of intermediates in membrane fusion: Comparison of stalk and inverted micellar intermediate mechanisms. Biophys J 65: 2124–2140.
23. Siegel DP. 1999. The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion. Biophys J 7:291–313.
24. Chernomordik LV, Zimmerberg J. 1995. Bending membranes to the task: Structural intermediates in bilayer fusion. Curr Opin Struct Biol 5:541–547.