ELSEVIER

Chemistry and Physics of Lipids
109 (2001) 75–89

CPL
CHEMISTRY AND PHYSICS OF LIPIDS

www.elsevier.com/locate/chemphyslip

Preparation and properties of arsonolipid containing liposomes

D. Fatouros [a], O. Gortzi [a], P. Klepetsanis [a], S.G. Antimisiaris [a,*], M.C.A. Stuart [b], A. Brisson [b], P.V. Ioannou [c]

[a] *Laboratory of Pharmaceutical Technology, Department of Pharmacy, University of Patras, Patras 26500, Greece*
[b] *Department of Biophysical Chemistry, Groningen Biomolecular Biotechnological Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*
[c] *Department of Chemistry, University of Patras, Patras 26500, Greece*

Received 3 March 2000; received in revised form 16 October 2000; accepted 18 October 2000

Abstract

Arsonolipids are analogs of phosphonolipids which have a chemically versatile head group. In preliminary cell culture studies, liposomes composed solely of arsonolipids or of phosholipid–arsonolipid mixtures, demonstrate a specific toxicity against cancer cells (Gortzi et al., unpublished results). The possibility of using such formulations as an alternative of arsenic trioxide with or without combination of other cytostatic agents (encapsulated in their aqueous interior) prompted the investigation of their physicochemical characteristics. Herein we compared the characteristics of arsonolipid containing vesicles with different lipid compositions. Experimental results and morphological observations reveal that non-sonicated formulations have different structures and stability (when both membrane integrity and aggregation are taken into account) depending on the acyl chain length of the arsonolipid. When phospholipids and especially cholesterol are included in their membranes almost all arsonolipids studied produce more stable vesicles. An interesting aspect of these arsonolipid containing vesicles is also their negative surface charge, which may be modulated by mixing phospholipids with arsonolipids. Sonicated vesicles have smaller sizes and profoundly higher stability, especially when containing cholesterol and phosphatidylcholine mixed with arsonolipids. The only exception is that of the arsonolipid with the C_{12} acyl chain which was observed to produce long tubes which break down to cubes by sonication. In conclusion, these initial studies demonstrate that sonicated vesicles composed of arsonolipid and phospholipid mixtures mixed with cholesterol posses the stability required to be used as an arsonolipid delivery system. In addition, although cryo-electron microscopy demonstrated that the sonicated vesicles are elliptical in shape, their encapsulation efficiency is not significantly lower than sonicated phospholipid liposomes. Thereby, these vesicles may be also used for the delivery of other drug molecules which can be sufficiently retained in their aqueous interior.

Keywords: Arsonolipids; Arsonoliposome structure; Liposome; Size; Properties

* Corresponding author. Tel.: +3-061-997725; fax: +3-061-997728.
E-mail address: s.antimisiaris@upatras.gr (S.G. Antimisiaris).

0009-3084/01/$ - see front matter
PII: S0009-3084(00)00221-8

1. Introduction

Arsenic containing analogues of phosphonolipids, the *rac*-2,3-diacyloxypropylarsonic acids, were recently prepared and characterized (Tsivgoulis et al., 1991a,b; Serves et al., 1992, 1993; Rogers et al., 1996). The ability of arsonolipid As(V) to be reduced to As (III) by thiols (Timotheatou et al., 1996) is an interesting aspect of these lipids. In vivo, such interactions with membrane-bound or cytoplasmic thiols may alter the biochemistry of the cell, rendering the arsonolipids active against hematological and/or solid tumor cells when the latter contain elevated levels of thiols.

In order to explore the above hypothesis, the preparation of vesicles containing these lipids was attempted. The results of preliminary toxicity studies performed in cell cultures, with some of the initial arsonolipid dispersions prepared, were very promising. Arsonolipid containing lipid dispersions were demonstrated to be toxic against several cancer cell types in culture, while nontoxic under identical conditions, against normal cells (Gortzi et al., unpublished results). In view of these preliminary results in conjunction with recent reports that As_2O_3 is effective in vitro against leukemia (Huff et al., 1999; Waxman et al., 1999) and solid tumor cells (Akao et al., 1999) and also demonstrated to cure several clinical cases of relapsing promyelocytic leukemia (Sun et al., 1992; Zhang et al., 1996; Shen et al., 1997; Soignet et al., 1998), we decided to study the preparation of arsonolipid containing vesicles in order to gain knowledge on the possibility of attaining stable preparations for possible applications as therapeutics or/and drug targeting systems. These vesicles could be used for the delivery and possibly targeting of arsonolipids to cancer cells. In addition, other agents, as cytostatics, may be encapsulated in the aqueous phase of such vesicles and possibly enhance any arsonolipid anticancer activity if released from the vesicles in the close vicinity of cancer cells. In this respect, the ability of arsonolipids to form plain liposomes or mixed liposomes with phospholipids (natural phosphatidylcholine or synthetic distearoylphosphatidylcholine) and/or cholesterol was investigated. Arsonolipids with four different acyl chains C_{12}–C_{18} were used for liposome preparation. The in vitro stability of these vesicles in buffered aqueous media in the absence and presence of serum proteins was measured, and the prepared vesicles were characterized by size distribution and surface charge measurements. Furthermore, morphological studies using optical, scanning electron and transmission electron microscopy were performed in order to verify vesicle formation and to provide knowledge on their shape, structure, size and lamellarity.

2. Materials and methods

2.1. Materials

Egg L-α-phosphatidylcholine [PC] (grade 1) and distearoyl-phosphatidylcholine [DSPC] (synthetic, grade 1), were obtained from Lipid Products, Nutfield, UK. The 99% purity of the lipids was verified by thin layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany), as described previously (New, 1990). Both lipids gave single spots. Cholesterol [Chol] (pure), and all buffer salts and other reagents used throughout the study were purchased from Sigma-Aldrich (O.M), Athens, Greece. 5,(6)-Carboxyfluorescein [CF] was purchased from Eastman Kodak, and was used after purification (New, 1990). 8-Hydroxypyrenetrisulfonic acid trisodium salt [HPTS] (Molecular Probes, Eugene, OR) was a kind gift from the late Professor Demetrios Papahadjopoulos. Gels used for gel chromatography were obtained from Pharmacia, Sweden. The water used was deionized and then distilled. *rac*-Arsonolipids [Ars] (C_{12}, C_{14}, C_{16}, and C_{18}), were synthesized as described previously (Tsivgoulis et al., 1991b; Serves et al., 1992, 1993).

2.2. Liposome composition

Using the arsonolipids C_{12}–C_{18}, Chol, DSPC, and egg PC, we prepared liposomes from: (a) Ars alone (plain arsonoliposomes); (b) Ars/Chol (1:1 and 1:2 mol/mol); (c) Ars/DSPC (1:1 mol/mol); (d) PC/Ars/Chol 12:8:10 [mol/mol/mol] (40% of

total lipid is Ars) and 17:3:10 [mol/mol/mol] (15% of total lipid is Ars); and (e) DSPC, PC and PC/Chol 1:1 [mol/mol], for comparison.

2.3. Preparation of liposomes

For the preparation of arsonolipid containing liposomes we used the 'one step method' (Talsma et al., 1994). In brief, lipid or lipids (as powders) were mixed with water (or in phosphate-buffered saline (PBS), pH 7.40, or a solution containing 100 mM CF or 35 mM HPTS, both with pH 7.4 and adjusted with NaCl to an osmolarity of 280 mOsm/l) and magnetically stirred vigorously on a hot plate for 6–12 h at 70–90°C, depending on the transition temperature of the arsonolipid used (Serves et al., 1993). In some cases, depending on the initial temperature of the aqueous phase and the stirring applied, vesicles were observed to form significantly faster (in a few hours). In the cases in which PC was used, a much lower temperature was applied, due to its low transition temperature. After formation of liposomes, the samples were left to anneal for at least 1 h at the liposome preparation temperature used in each case.

In order to reduce liposome size, the large liposome suspension initially produced was sonicated using a vibra cell sonicator (Sonics and Materials, UK), equipped with a tappered micro tip, for at least two 10 min cycles. In all cases the initially turbid liposomal suspension was well clarified after sonication. Following sonication, the liposome suspensions were left to stand for 2 h at a temperature higher than the transition temperature of the lipid used in each case, in order to anneal any structural defects. The titanium fragments and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at $10\,000 \times g$ for 15 min.

Non-encapsulated CF or HPTS was separated from liposomes on a Sephadex G-50 column (1×35 cm) eluted with PBS, pH 7.40, which rendered the liposomes osmotically stable (Cleland and Allen, 1981). The column was presaturated with the lipid used in each case and therefore the lipid recovery in all cases was well over 95%, as calculated by a colorimetric assay for phosholipids (Stewart, 1980), assuming no Ars was adsorbed on the column. The final lipid concentration in the incubations was adjusted to 1.0 mM, unless otherwise stated.

2.4. Cryogenic and electron microscopy

Cryo-electron microscopy was performed according to standard procedures (Dubochet et al., 1988). A small droplet of the liposome suspension was applied onto a glow-discharged honey carbon film on a copper grid. After blotting the excess of liquid, grids were plugged into liquid ethane and stored under liquid nitrogen until observation. The vitrified specimens were observed in a Philips CM 10 or CM 120 cryo-electron microscope equipped with a Gatan cryo stage (model 626). Pictures were recorded under low-dose conditions (less than two electrons/square angstrom).

2.5. Determination of CF or HPTS release by fluorescence

The leakage of small water-soluble dyes encapsulated in the aqueous interior of liposomes during their preparation is often used as a method to study their membrane integrity during incubation under various conditions (temperature, pH, presence of serum proteins, etc.). In this study we used CF and also HPTS which is a dye that is recently being used in liposome cell interaction studies due to its pH-dependent fluorescence (Straubinger et al., 1983; Daleke et al., 1990). The release of CF or HPTS was taken as a measure of the liposome membrane integrity, and was estimated after incubating the liposomes in buffer or in presence of serum proteins [80% FCS] at 37 ± 0.5°C under mild agitation.

In the case of HPTS, after incubation under the conditions studied for appropriate time periods, liposomes were separated from free dye by gel filtration as described above. Subsequently, liposome retained and free dye were calculated by measuring HPTS fluorescence in the column fractions by a Shimatzu Fluorescence spectrophotometer at EM 413 nm and EX 518 nm. At this emission wavelength the fluorescence of HPTS is pH independent (Straubinger et al., 1983). In addition, no quenching of the dye was detected.

In the case of CF, which was encapsulated in the vesicles in a quenched concentration, release was calculated without separation of free and liposomal dye, as reported elsewhere (Senior and Gregoriadis, 1982). In brief, 10 μl were drawn out from each incubation tube and diluted with 4 ml of PBS, pH 7.40. The fluorescence intensity of the samples was then measured (EM 470 nm, EX 520 nm), before and after the addition of Triton X-100 at a final concentration of 1% v/v.

The percent of CF Latency (% Latency) was determined at the experimental conditions, from the equation:

$$\%\text{Latency} = \frac{F_T - F_I}{F_T} \times 100$$

where F_I and F_T are the CF fluoresence of the sample in the absence and presence of 1% Triron X-100 (final concentration), respectively (values obtained after mixing the samples with triton were corrected accordingly for dilution).

2.6. Liposome sizing and aggregation measurements

A total of 50 μl of the liposome dispersion were diluted with 20 ml of filtered buffer (0.22 μm pore size, polycarbonate filters, Millipore, UK) and sized immediately by photon correlation spectroscopy (Malvern Instruments, Model 4700C), which enabled the mass distribution of particle size to be obtained, according to manufacturers. Measurements were made at 25°C with a fixed angle of 90° and sizes quoted are the z-average mean (dz) for the liposomal hydrodynamic diameter. The size of liposomes was also confirmed by image analysis of electron micrographs using a LEICA Q500MC image analysis system.

In some cases the size of liposomal dispersions was re-measured after incubation in appropriate media and temperature for 24 and/or 48 h, in order to confirm aggregation.

2.7. Liposomal surface charge

Liposome dispersions were diluted with PBS pH 7.40 and their electrophoretic mobility was measured at 25°C by photon correlation spectroscopy (Zetasizer 5000, Malvern Instruments, UK). Zeta potentials of the dispersions were calculated by the instrument according to the Smolowkovski equation.

3. Results

3.1. Preparation of arsonolipid containing liposomes

Several conventional liposome preparation methods (New, 1990) (e.g. the lipid thin film hydration method, the direct sonication method, etc.) were employed in order to prepare liposomes composed only of arsonolipids (arsonoliposomes), but failed to succeed. One of the problems encountered during liposome preparation, which was a great obstacle preventing the usage of several conventional liposome preparation techniques, was the high foaming observed during sonication. The inability to use these conventional techniques could be, at least partly, attributed to the very high transition temperatures of arsonolipid, which have been measured previously (Serves et al., 1993), and range between 70 and 90°C according to the acyl chain of the lipid. This high temperature is difficult to preserve during conventional liposome preparation procedures. In order to overcome the problems encountered, the one step method (Talsma et al., 1994) was successfully applied. Indeed, liposomes consisting solely of arsonolipids with diameters ranging between 250 and 350 nm were easily prepared in distilled water. In Tris buffer pH 7.40, very large arsonoliposomes or liposomal aggregates (10–20 μm), were formed. Nevertheless, this phenomenon must be attributed to the specific buffer salt, since vesicles of smaller size were easily prepared in PBS or borate buffers (at the same pH value). In addition, mixed liposomes composed of arsonolipids and phospholipids were easily prepared using the same method in water or PBS.

3.2. Morphology of arsonolipid containing dispersions

Liposomes prepared by the one step method (without sonication) and composed of mixtures of

C_{12}, C_{14}, C_{16} or C_{18} arsonolipids with phospholipids (PC or DSPC) and/or Chol, were found by transmission electron microscopy to be largely composed of round, multilamellar vesicles (not shown). The same was also observed in the case of samples prepared only with C_{14}, C_{16} or C_{18} arsonolipids, but those of C_{12} arsonolipid contained many tube-like long structures (not shown). Light microscopy also revealed that while the C_{14}, C_{16} and C_{18} arsonolipids in water formed round-shaped vesicles, the arsonolipid with the C_{12} acyl chain formed peculiar dispersions when prepared by the one step method. These peculiar shaped long structures present in the C_{12} arsonolipid dispersions could also be viewed after sonication (not shown).

By using cryo-electron microscopy, it was clarified that when the C_{12} arsonolipid was dispersed in aqueous media by the one step method, it produced very long tubes (>5 μm) with a diameter of ~ 50 nm, Fig. 1(a–b), which after sonication were observed to 'break' into cubes or barrels (20×20–100×100 nm^2). These smaller particles were transformed into the long tubes again, as demonstrated by re-examining the samples after a few hours. Although we do not know the exact rate of this re-organization of cubes into tubes, we have observed tubes under a light microscope only a few minutes after sonication, thereby demonstrating that this process is probably of high velocity.

Under the same conditions the C_{14}, C_{16} and C_{18} arsonolipids formed only disk-like liposomes, with diameters of ~ 80 nm (Fig. 1(c–d)). Upon addition of Chol to C_{16}, flattened liposomes and some large sheets were formed (Fig. 1(e)), while elliptical unilamellar liposomes were formed from PC/C_{16}/Chol with 8:12:10 molar ratio (Fig. 1(f)).

3.3. Size distribution of arsonoliposomes

As demonstrated by the sizing experiments, the mean diameters of liposomes prepared solely of arsonolipids (plain arsonoliposomes) by the one step method, without sonication, range between 250 and 330 nm, Table 1. The polydispersity index for each preparation was also measured (not shown) and found to range between 0.09 and 0.22, demonstrating that all liposomal preparations were significantly homogenous in size. From these results it is observed that vesicle size is slightly influenced by the length of the fatty acyl chain of the specific arsonolipids, but not changed when liposomes are prepared in PBS, pH 7.4, instead of water. Indeed, arsonolipids C_{12}, C_{16} and C_{18} form smaller liposomes, while arsonolipid C_{14} forms the largest vesicles, slightly larger than the other lipids. However in the case of the C_{12} arsonolipid we should keep in mind that these size measurements are possibly not highly accurate, due to the large proportion of tube-like structures present in dispersions of this lipid, as demon-

Table 1
Diameter of liposomes containing arsonolipids and prepared by the one step method (plain arsonoliposomes, and mixed Ars/Chol 1:1 and Ars/DSPC 1:1 arsonoliposomes)[a]

Lipid composition	Diameter[b] (nm)			
	Ars		Ars/Chol (1:1 mol/mol)	Ars/DSPC (1:1 mol/mol)
Arsonolipid	d.d H_2O	PBS pH 7.4	d.d H_2O	d.d H_2O
C_{12}	251 ± 6[c]	270 ± 10[c]	247 ± 13	346 ± 19
C_{14}	329 ± 22	374 ± 27	384 ± 36	–
C_{16}	273 ± 41	282 ± 17	362 ± 18	247 ± 13
C_{18}	265 ± 23	–	427 ± 61	290 ± 22

[a] Liposomes were prepared in H_2O or PBS, pH 7.4 as indicated, and measured immediately after their preparation.
[b] Each value is the mean ± SD (standard deviation) of measurements from at least four different samples.
[c] The accuracy of these measurements is questionable, since C_{12} arsonolipid was demonstrated to form tubular structures in a high proportion (Fig. 1).

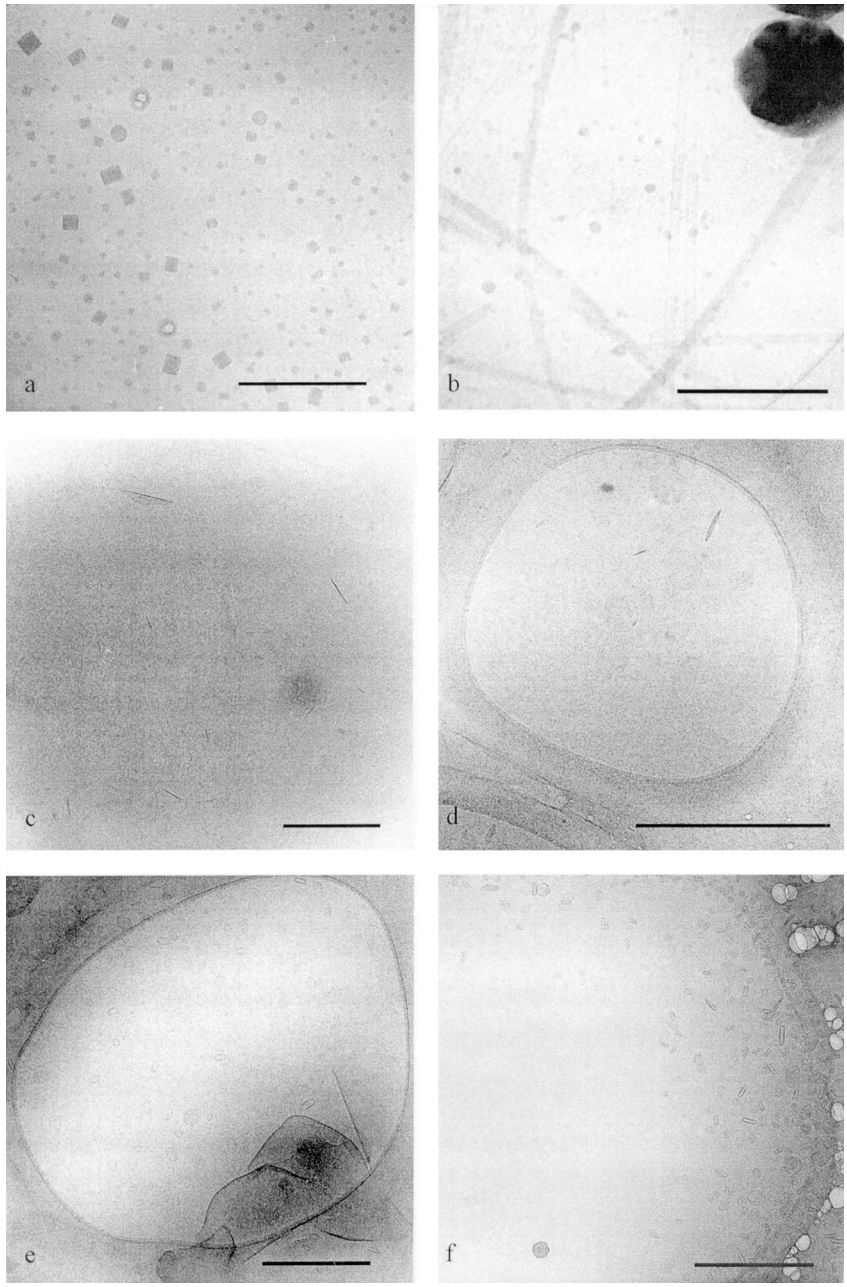

Fig. 1. Cryo-electron microscopy of plain and mixed arsonoliposomes. (a) Plain C_{12} arsonoliposomes made by the one step method followed by sonication. Cube or barrel shaped structures were found directly after sonication. (b) The cubes and barrels progressively disappear and transform into very long tubes (several μm). (c–d) Flat, disk-like, liposomes of plain C_{16} (c) and C_{18} (d) arsonolipids made by the one step method followed by sonication. The disks appear as thin stripes when viewed edge-on. The contrast is very low when they are viewed face-on and their disk shape is better recognized at high defocus values. (e) C_{16} arsonolipid, mixed with cholesterol (2/1). Flattened liposomes and some large sheets were found. This might be an indication for incomplete solubilization of the arsonolipid or phase separation. (f) Mixed liposomes of PC, arsonolipid (Ars) and Chol (12:8:10 mol/mol/mol). Small unilamellar liposomes were found. Upon tilting the specimen it becomes clear that all observed liposomes are elliptical in shape. Bar represents 500 nm.

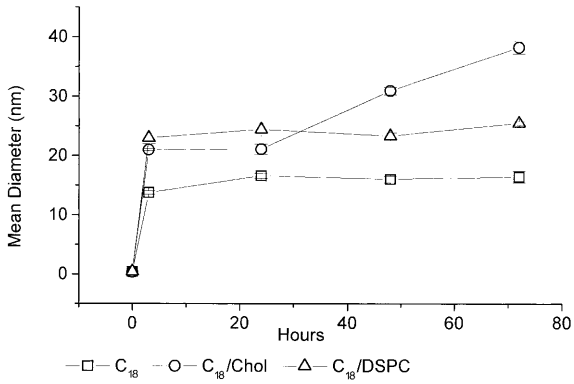

Fig. 2. Mean diameters of non-sonicated arsonolipid vesicles prepared and incubated in water. Vesicles were composed solely of arsonolipid C_{18} (squares) or of C_{18}/Chol (1:1, mol/mol) (circles) or of C_{18}/DSPC (1:1 mol/mol) (triangles), measured initially, and at different time periods after preparation. Each point is the mean of at least three experiments, and bars represent SD values.

strated by our morphological studies (Fig. 1). The C_{18} arsonoliposomes were found by light microscopy, and by measurements of size by laser scattering under agitation (Malvern Mastersizer) (Fig. 2), to quickly form large aggregates which slowly precipitate out and therefore the small diameter shown in Table 1, possibly represents only the size of suspended vesicles.

In addition to vesicle size, the length of the fatty acyl chain of the specific arsonolipid, also seems to influence the effect of cholesterol or DSPC incorporation on the size of the liposomes produced, Table 1. Indeed, the mean diameter of arsonoliposomes is unchanged when cholesterol is added in liposomes from arsonolipid C_{12}, there is a small increase for C_{14}, but there is a significant increase in liposomes of arsonolipids with longer side chains (C_{16} and C_{18}). However when DSPC is added to arsonoliposomes the size is unchanged for the longer side chain arsonolipid liposomes, but increases in those containing arsonolipid C_{12}.

When the 'one step' multilamellar liposomes are probe-sonicated their mean diameter is considerably reduced in all cases studied, Table 2. By comparing the mean diameters of MLV and sonicated preparations (Tables 1 and 2), it is easily observed that in all cases arsonoliposomes are reduced in size after sonication by $\sim 50\%$. Indeed, the mean diameters of all sonicated Ars liposomes range between 116 and 130 nm with no significant difference from the Ars/Chol 2:1 liposomes (for which diameters are ranging between 103 and 121 nm). In both of the former cases there is practically no effect of the fatty acyl chain length, on the size of the sonicated vesicles. When a percentage of arsonolipid is substituted with natural PC, the size of the liposomes produced decreases as the PC content increases, for all arsonolipids studied. Again, liposome mean diameter does not seem to be influenced by the acyl chain length of the arsonolipid, and there is no difference in size of liposomes prepared in water or PBS, pH 7.40 (Table 2). Considering the lamellarity of these sonicated vesicles, it has been clearly demonstrated by cryo-electron microscopy (Fig. 1) that they are unilamellar. Therefore they may be referred as LUV.

A measure of physical stability of vesicles is their tendency for aggregation. In order to investigate this, vesicle size measurements were performed after 24 or 48 h, or even 72 h of incubating liposomes at 37°C, for both types of liposomes prepared (MLV and LUV). As revealed by the results of this study (Fig. 2 and Table 2), the MLV arsonoliposomes containing arsonolipid C_{18} are highly aggregated during incubation in water (Fig. 2). On the other hand, for the sonicated liposomes there is a very slight, in some cases statistically significant (at $P = 0.05$) increase in the mean diameter for the Ars/Chol compositions, while the PC/Ars/Chol liposomes, in general, had the same size (Table 2). However, after sonicated mixed C_{14} arsonoliposomes were incubated in the presence of PBS, pH 7.40 (37°C) for 48 h, their mean diameter was further increased for all lipid compositions studied (Table 2). Nevertheless, the per cent increase in liposome mean diameter even after this long incubation period was between 8 and 23% of their initial size, demonstrating that these vesicles are relatively stable when their size is considered.

3.4. Encapsulation efficiency and membrane integrity of arsonoliposomes

In addition to physical stability, when liposomes are to be used as carrier or targeting sys-

Table 2
Mean diameter of sonicated mixed (PC/Ars/Chol) arsonoliposomes, immediately after preparation ($t = 0$) and after 24 h ($t = 24$ h) of incubation in H_2O at 37°C[a]

Lipid composition	Ars		Ars/Chol (0:210 mol/mol)		PC/Ars/Chol (12:8:10 mol/mol/mol)		PC/Ars/Chol (17:3:10 mol/mol/mol)	
Ars	Diameter[b] nm ($t = 0$ h)	Diameter[b] nm ($t = 24$ h)	Diameter[b] nm ($t = 0$ h)	Diameter[b] nm ($t = 24$ h)	Diameter[b] nm ($t = 0$ h)	Diameter[b] nm ($t = 24$ h)	Diameter[b] nm ($t = 0$ h)	Diameter[b] nm ($t = 24$ h)
C_{12}	116 ± 10	119 ± 8.9	102.5 ± 8.5	110.9 ± 5.9[c]	72.6 ± 8.4	78.8 ± 2.4[c]	62.8 ± 7.5	61.8 ± 2.2
C_{14}	118 ± 15	119 ± 11	107.9 ± 8.7	115.4 ± 4.3[c]	87.0 ± 6.8	86.2 ± 4.1	76.4 ± 3.1	76.8 ± 1.9
			116.6 ± 7.4	118.8 ± 6.9	73.5 ± 6.5	87.5 ± 6.5[c]	68.3 ± 8.1	77.2 ± 9.8
				(133 ± 13)[d]		(99.2 ± 6.9)[d]		(82.4 ± 9.1)[d]
C_{16}	130.2 ± 9.6	136 ± 10	110.8 ± 8.7	117.5 ± 4.0[c]	90.9 ± 6.6	94.2 ± 5.6	78.0 ± 5.7	79.4 ± 2.4
			116 ± 11	–	83.1 ± 6.9	–	74.8 ± 7.3	
C_{18}	–	–	121.3 ± 6.8	131.8 ± 5.5[c]	93.4 ± 5.4	101.5 ± 6.7[c]	74.8 ± 6.7	92.6 ± 2.9[c]
			120.2 ± 8.9	–	80.8 ± 7.1	–	60.1 ± 7.9	–

[a] The second row, when applicable, are values measured after preparation in buffer and also incubation in PBS buffer, pH 7.4, at 37°C.
[b] Each value is the mean ± SD (standard deviation) of five subsequent measurements from at least four different samples.
[c] Diameters are significantly different (at the 0.05 level) from the equivalent diameter values measured immediately after sample preparation (Diameter 0).
[d] Values in parenthesis are mean diameters of the corresponding samples measured after 48 h of incubation in the same conditions.

tems, their ability to efficiently encapsulate drug molecules and to retain them until they reach their site of action is of great importance. In order to gain such knowledge about arsonolipid containing liposomes, we initially studied the membrane integrity of liposomes prepared by the one step method, for all lipid compositions used, after encapsulating CF in their aqueous interior. The percentage of CF retained in arsonoliposomes from C_{12}, C_{12}/Chol and C_{12}/DSPC during incubation in buffer at 37°C is presented in Fig. 3(A). Arsonoliposomes (plain or mixed) composed of arsonolipids C_{14}, C_{16} and C_{18}, follow similar CF release kinetics (not shown), however arsonoliposomes from C_{14} and C_{16} are significantly more stable than those from C_{12} and C_{18} (for which $< 10\%$ of the initially encapsulated CF remains in vesicles after 24 h of incubation) (Fig. 3(B)). The addition of Chol in the liposomal membrane significantly increases the stability of all arsonoliposomes, while the addition of DSPC stabilizes only the less stable C_{12} (significantly) and C_{14}, C_{18} (slightly), but slightly destabilizes the C_{16} arsonoliposomes (Fig. 3(B)). Plain DSPC liposomes prepared by the same method were studied for comparison and although their size was significantly larger (~ 5 μm) than that of arsonoliposomes (~ 250 nm, see Table 1) it is demonstrated (Fig. 3(B)) that they are equivalent in terms of CF leakage with Chol containing mixed arsonoliposomes.

In the presence of serum proteins the membrane integrity of these arsonoliposomes is profoundly decreased (Fig. 4). Indeed, most of the entrapped CF is released even from the Chol containing vesicles, after a few hours of incubation. Thereby, we continued studies using sonicated liposomes, which as unilamellar and smaller in size would be preferred for drug delivery purposes.

For these latter studies we decided to use the C_{14} arsonolipid which was earlier demonstrated to easily produce liposomes of higher stability by the one step method, which were not highly aggregated (as the C_{18} liposomes) nor contained other, tube-like, structures (as the C_{12} arsonolipid). Since, cryo EM studies revealed that sonicated arsonoliposomes composed of C_{14} with PC and/or Chol are quite elliptical in shape, we initially calculated their encapsulation efficiency using CF, as well as HPTS. As presented in Table 3, the encapsulation efficiencies calculated with both CF and HPTS are similar and although low, absolutely comparable with the encapsulation values usually calculated for sonicated unilamellar liposomes of this size (Gregoriadis, 1993; Batzri and Korn, 1973). In addition, the retention of CF in

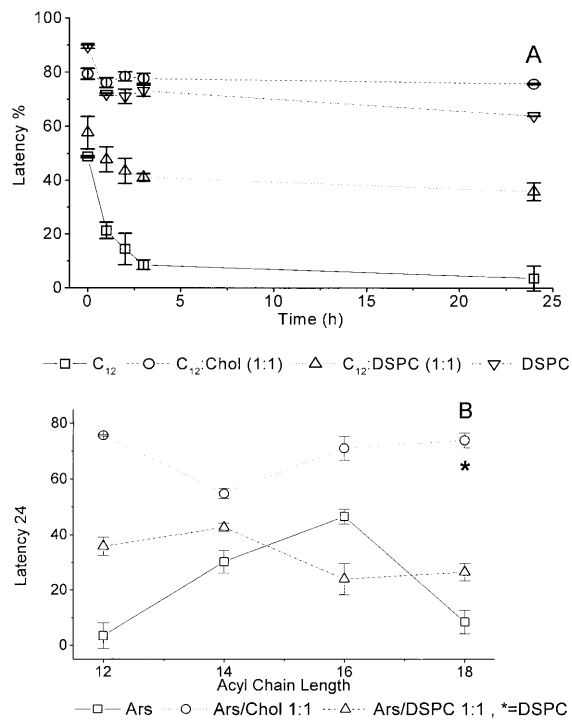

Fig. 3. (A) % Latency of encapsulated CF in non-sonicated, plain arsonolipid C_{12} vesicles (squares) or mixed arsonoliposomes with C_{12} and DSPC (1:1 mol/mol) (up triangles) or cholesterol (1:1 mol/mol) (Circles), after incubation in isotonic Tris buffer, pH 7.40, at 37°C. DSPC liposomes prepared and tested under identical conditions were studied as control vesicles (down facing triangles). Each point is the mean of at least four different experiments, and bars represent SD values. (B) % Latency of encapsulated CF after 24 h (Latency-24) of incubation in isotonic Tris buffer, pH 7.40, at 37°C (Latency-24), of non sonicated, arsonoliposomes prepared from arsonolipids C_{12}–C_{18}. Key: Squares, plain arsonolipid liposomes; circles, Ars/Chol 1:1 mol/mol; up triangles, Ars/DSPC 1:1 mol/mol; star, DSPC. Each point is the mean of at least four different experiments, and bars represent SD values.

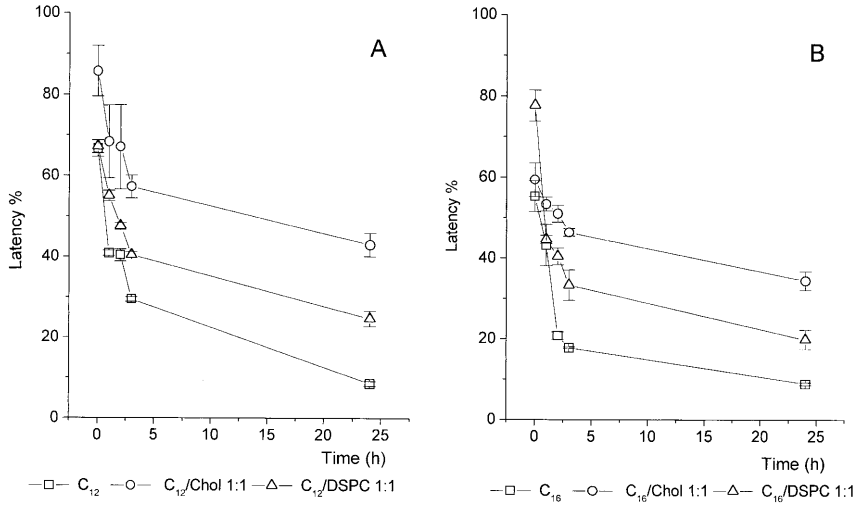

Fig. 4. % Latency of encapsulated CF in non-sonicated arsonoliposomes composed of Ars (squares) or Ars/Chol (1:1 mol/mol) (Circles) or Ars/DSPC (1:1 mol/mol) (up triangles), after incubation in serum proteins (FCS), at 37°C. One volume of vesicles was mixed with five volumes of FCS in each incubate (83.3% FCS). (A) Vesicles from arsonolipid C_{12}. (B) Vesicles from arsonolipid C_{16}. Each point is the mean of at least four different experiments, and bars represent SD values.

these liposomes is very high when liposomes are incubated in buffer, while HPTS retention is even higher (Fig. 5(A)), possibly due to the fact that this latter dye is highly ionised in physiological pH. HPTS retention in sonicated PC/C_{14}/Chol (12:8:10) arsonoliposomes was also followed during incubation in presence of serum proteins (Fig. 5(B)). Results reveal that they are highly stable under these conditions as well. Indeed, $< 40\%$ of the initially encapsulated dye is released after 24 h of incubation at 37°C.

3.5. Zeta potential of liposomes

Zeta potential measurements of arsonoliposomes (Table 4), demonstrate that incorporation of arsonolipids in liposomes results in a high negative surface charge a fact which was also confirmed by electrophoresis (not shown). After sonication, the z-potential values are increased (in absolute value) in comparison to non-sonicated preparations of the same arsonolipid.

Sonicated arsonoliposomes mixed with PC and/or Chol at pH 7.40 have zeta potentials ranging from -69 to -28 mV which is influenced slightly by the acyl chain length of the arsonolipid but mainly by the liposomal lipid composition (Table 4). Indeed, the z-potential values are highly reduced when part of the arsonolipid is substituted with PC (PC/Ars/Chol 12:8:10 and 17:3:10). On the other hand arsonolipid acyl chain length does not have a high impact on the z-potential of the arsonoliposomes, although a slightly higher — not always significant — z-potential was measured in all cases in arsonoliposomes from C_{16}. Furthermore, the ionic strength of the solution, as anticipated, influences the z-potential of these liposomes, since values measured in water were significantly lower in comparison to those in buffer (PBS, pH 7.40), for the same arsonoliposome compositions.

4. Discussion

A new class of lipids, called arsonolipids, has been previously synthesized (Tsivgoulis et al., 1991a,b; Serves et al., 1992, 1993). These lipids helped in elucidating the mechanism of action of phospholipase A_2 (Rogers et al., 1996) and were proven to be potent non-competitive inhibitors of carbonic anhydrase isozyme II (Supuran et al., 1996). The ability of arsonolipid As(V) to be reduced to As(III) by thiols (RSH) is an interest-

ing aspect of these lipids, which we started to explore by investigating the interaction of liposomes consisting of arsonolipids, with cancer and normal cells in culture (Gortzi et al., unpublished results). As stated above, these preliminary results demonstrated that arsonolipid dispersions (containing possibly liposomes), were toxic against the cancer cell lines tested while non-toxic (in the same concentration range) against the normal cells used.

Under this perspective, some basic vesicle characteristics which are known to have a great impact on the ability of homing devices to deliver and/or target drugs, as well as on the way they interact with cells, like membrane integrity (Papahadjopoulos et al., 1972; Senior and Gregoriadis, 1982), surface charge (Langner and Kubica, 1999), size distribution and aggregation (Shaw, 1970), were measured for the vesicles prepared. The final aim of this study was to prepare the best possible arsonolipid containing particles and characterize them, in order to continue cell culture and perhaps, at a later stage, in vivo studies.

Studies were performed with four different arsonolipid molecules containing different acyl chain lengths, C_{12}, C_{14}, C_{16} and C_{18}, while liposomes were prepared using arsonolipids only, or arsonolipids mixed with Chol, which is known to enhance liposomal membrane integrity in the presence of plasma proteins (Papahadjopoulos et al., 1973; Jonas and Maine, 1979; Senior and Gregoriadis, 1982), and it is thus widely used in liposomal drug preparations. In addition, liposomes were prepared by mixtures of arsonolipids with phospholipids (saturated DSPC or unsaturated, PC), at various molar ratios, in order to investigate if, and in which mode, arsonolipids modify the characteristics of conventional liposomes. Concerning the preparation method, the one step method was used (Talsma et al., 1994), since due to the high transition temperatures and foaming of arsonolipids no other conventional liposome preparation technique was found efficient. The difficulty encountered in dispersing arsonolipids in aqueous media may possibly be attributed to the fact that the pKa values of the simple aliphatic arsonic acids are between ~ 4 and 9 (Doak and Freedman, 1970). Therefore the arsonolipids are expected to be easily dispersed in buffer solutions with pH values ≥ 8 because their head group becomes more polar (Serves et al., 1992), but not in water or buffers with pH 7.4. Undoubtedly the size, polarity and acidity of the head group of lipids determine their ability to be dispersed in aqueous media and this may be linked with the peculiar structures formed by the C_{12} arsonolipid in contrast to the more conventional liposomes formed by the other arsonolipids used. In some cases liposomes were sonicated after initial formation, in order to study the effect of sonication on the preparations formed.

In all cases the arsonolipid containing vesicles formed have a high negative z-potential value. This should correspond to very stable preparations, however this was not the case for most of the vesicles formed without applying sonication. Considering the non-sonicated formulations, the results of the studies performed show that the acyl chain length of arsonolipids has a profound effect on several liposome characteristics, basically their morphology, size and ability to retain encapsulated molecules.

From the preparations consisting solely of arsonolipids, the C_{12} arsonolipid does not form vesicles — as already mentioned above — but tubes which by sonication are 'broken' into cubes, while all the other arsonolipids tested form more conventional liposomes which are multilamellar, with mean diameters ranging between 200 and 300 nm (Table 1). From the later liposomes, the

Table 3
Percent encapsulated CF in sonicated plain and mixed arsonoliposomes from arsonolipid C_{14}, prepared in isotonic PBS pH 7.40

Lipid composition	PC: C_{14}: Chol (17:3:10)	PC: C_{14}: Chol (12:8:10)	C_{14}: Chol (20:10)
% CF encapsulated	3.31 ± 0.57	4.15 ± 0.83	1.45 ± 0.65
% HPTS encapsulated	2.7 ± 0.9	4.54 ± 0.78	1.73 ± 0.60

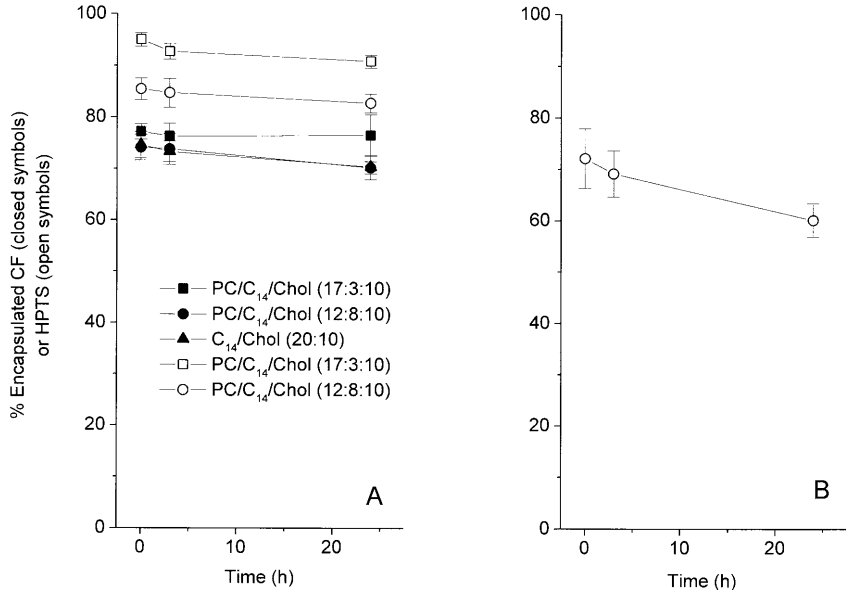

Fig. 5. Percent of encapsulated molecules retained in sonicated arsonoliposomes consisting of $PC/C_{14}/Chol$ (17:3:10) (squares) or $PC/C_{14}/Chol$ (12:8:10) (circles) or $C_{14}/Chol$ (20:10) (triangles) during incubation in: A. PBS buffer, pH 7.4, or B. FCS (serum/sample 5/1). The values for CF are presented as closed symbols and for HPTS as open symbols. Each point is the mean of at least four different experiments, and bars represent SD values.

ones containing C_{18} are very unstable and rapidly aggregate (Fig. 2). The reason why these unsonicated (multilamellar) arsonoliposomes aggregate, while the sonicated (unilamellar) ones do not, is not easily explained. Increased planarity of unsonicated vesicles has been proposed as a reason for aggregation (Frokjaer et al., 1982) but in our case the sonicated plain and mixed arsonoliposomes are disk-like (Fig. 1) but do not aggregate (Table 2). A likely explanation of the observed aggregation is that non sonicated particles have a small surface charge density due to heavy hydration of the $-AsO_3H^-$ group. Thereby, the chances of productive collision of these charged particles increase, leading to aggregation due to a number of water molecules released into the solution. In other words the aggregation is entropically driven. However this is a hypothesis and no conclusive proof can be derived from the experiments performed.

The morphological observations and aggregation studies (Figs. 1 and 2) are nicely correlated with the results of the membrane integrity studies performed with these non-sonicated liposomes (Figs. 3 and 4). Indeed, the structures formed by C_{12} arsonolipid and the rapidly aggregating C_{18} liposomes were seen to rapidly loose their contents during incubation at 37°C. On the other hand the addition of Chol and/or phospholipids in these liposomes was demonstrated to produce more conventional liposomes with properties that are closer to those of phospholipid liposomes almost in all cases studied. However, in all cases the vesicles prepared without sonication were not sufficiently stable judging from their membrane integrity (especially in the presence of serum proteins) and considering the fact that as multilamellar vesicles they should retain encapsulated molecules for longer time periods. Therefore, not only the plain arsonolipid liposomes (especially the ones from arsonolipids C_{12} and C_{18}), but also the ones which contain phospholipids and/or Chol, are probably not sufficiently stable for in vivo use.

As already mentioned above, sonication seems to produce vesicles that are more stable and possibly could be used for therapeutic applications. Indeed, sonicated arsonoliposomes are in almost

all cases (with the exception of C_{12} particles) elliptical disk-like vesicles which can encapsulate water soluble molecules in their aqueous interior and retain them for long time periods, without highly aggregating. In the case of the C_{14} arsonoliposomes encapsulation efficiencies are comparable with those reported for LUV liposomes composed of phospholipids (Table 3). Furthermore their stability is sufficient for usage as delivery systems of arsonolipids and also other cytotoxic agents, considering their membrane integrity as well as their low tendency for aggregation (Table 2, Fig. 5). In the case of sonicated liposomes the arsonolipid acyl chain length does not seem to have a very pronounced effect on the vesicle characteristics as size and z-potential. The addition of arsonolipids in higher amounts in sonicated PC/Chol liposomes results in a increase in vesicle size, which is linearly correlated with the percent of arsonolipid added in the vesicles (R 0.9987, 0.9988, 0.9921 and 0.9909 for C_{12}, C_{14}, C_{16} and C_{18} respectively, when the percent of arsonolipid is plotted against liposome size). This observation is opposite to that observed when charged phospholipids (like phosphatidylserine) are added (Roy et al., 1998) in PC liposomes, in which case there is a reduction in vesicle size. This implies that, the non-isosteric arsonolipid molecule, irrespective of its chain length, requires more head group space than the PC molecule.

Also, when phospholipids are mixed with arsonolipids the vesicles formed have lower z-potential values, as anticipated since the arsonolipids are negatively charged lipids.

The only exception to the conclusion that sonicated arsonolipid liposomes are more stable is, as mentioned above, the particles consisting solely of C_{12} arsonolipid which form lipid tubules. Although not applicable as conventional homing devices, such structures are interesting (Fuhrhop and Helfrich, 1993) and will be further studied. Indeed, lipid tubules have recently attracted special attention both from a theoretical point of view (Fuhrhop and Helfrich, 1993), and for potential applications (Archibald and Mann, 1993).

Although this study was performed having in mind to simply characterize different arsonolipid containing liposomal preparations with the final aim of attaining the best possible formulations in order to continue our cell culture studies, some general conclusions have been extracted. These conclusions mainly concern the effect of arsonolipid properties on the characteristics of the vesicles they form. Nevertheless, focused on the perspective of developing an arsonolipid, or arsonolipid and drug, delivery system, we may conclude that sonicated arsonoliposomes composed of arsonolipids and phospholipids mixed with cholesterol, which have a substantial negative surface charge and may retain encapsulated molecules for sufficient periods of time (in presence of serum proteins), are the best choice.

However, in order to clarify the physicochemical behavior of these structures, more studies

Table 4
Zeta potential values of sonicated mixed arsonoliposomes prepared in PBS buffer, pH 7.40, or in d.d. water

Lipid composition	PC/Ars/Chol (17:3:10)	PC/Ars/Chol (12:8:10)	Ars/Chol (2:1)	Ars	Ars
	LUV	LUV	LUV	LUV	MLV
Arsonolipid	z-Potential (mV)[a]	z-Potential (mV)[a]	z-Potential (mV)[a]	z-Potential (mV)[a,b]	z-Potential (mV)[a,b]
C_{12}	-23.9 ± 1.9	-42.0 ± 2.8	-63.5 ± 6.3	-50.8 ± 0.6	-46.4 ± 1.3
C_{14}	-28.2 ± 1.1	-43.0 ± 2.6	-65.4 ± 1.5	-51.3 ± 0.8	-44.1 ± 2.7
C_{16}	-42.1 ± 2.9	-50.3 ± 1.0	-69.5 ± 2.3	-57.2 ± 1.3	-57.7 ± 1.1
C_{18}	-32.1 ± 2.3	-48.4 ± 1.2	-59.2 ± 3.1	-40.1 ± 2.1	-38.5 ± 2.0

[a] Each value is the mean $\pm$ SD (standard deviation) of five subsequent measurements from at least four different samples.
[b] Liposomes were prepared and measured in d.d. H_2O.

should be performed, e.g. studies conducted at different temperatures and in solutions of different pH.

Acknowledgements

This work was supported by the General Secretariat of Research and Technology, Athens, Greece (PENED 95).

References

Akao, Y., Nakagawa, Y., Akiyama, K., 1999. Arsenic trioxide induces apoptosis in neuroblastoma cell lines through the activation of caspase 3 in vitro. FEBS Lett. 455, 59–62.

Archibald, D.D., Mann, S., 1993. Structural studies of lipid fibers formed by sphingosine. Biochim. Biophys. Acta 1166, 154–162.

Batzri, S., Korn, E.D., 1973. Single layer liposomes prepared without sonication. Biochim. Biophys. Acta 298, 1015–1019.

Cleland, M., Allen, T., 1981. Serum induced leakage of liposome contents. Biochim. Biophys. Acta 597, 418–426.

Daleke, D.L., Hong, K., Papahadjopoulos, D., 1990. Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay. Biochim. Biophys. Acta 1024, 352–366.

Doak G.O, Freedman L.D., 1970. Organometallic Compounds of Arsenic, Antimony, and Bismuth, Wiley, New York, pp. 26–32.

Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., Mc Dowall, A.W., Schultz, P., 1988. Cryo-electron microscopy of vitrified specimens. Q. Rev. Biophys. 21, 129–228.

Frokjaer, S., Hjorth, E.L., Worts, O. In: Bundgraard, H., BaggerHausen, A., Kofod, H. (Eds.), Optimization of Drug Delivery, Munkgaard, Copenhagen, 1982.

Fuhrhop, J.-H., Helfrich, W., 1993. Fluid and solid fibers made of lipid molecular bilayers. Chem. Rev. 93, 1565–1582.

Gregoriadis, G. (Ed.), 1993. Liposome Technology, vol. 1, second ed. CRC Press, Boca Raton, FL.

Huff, J., Waalkes, M., Nyska, A., Chan, P., 1999. Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. J. Natl. Cancer Inst. 91, 1690–1691.

Jonas, A., Maine, G.T., 1979. Kinetics and mechanism of phosphatidylcholine and cholesterol exchange between single bilayer vesicles and bovine serum high-density lipoprotein. Biochemistry 18, 1722–1728.

Langner, M., Kubica, K., 1999. The electrostatics of lipid surfaces. Chem. Phys. Lipids 101, 3–35.

New, R.R.C. (Ed.), 1990. Liposomes: A Practical Approach, Oxford University Press, Oxford, pp. 134, 255.

Papahadjopoulos, D., Cowden, M., Kimelberg, H., 1973. Role of cholesterol in membranes. Effects on phospholipid–protein interactions, membrane permeability and enzymatic activity. Biochim. Biophys. Acta 330, 8–26.

Papahadjopoulos, D., Nir, S., Oki, S., 1972. Permeability properties of phospholipid membranes: effect of cholesterol and temperature. Biochim. Biophys. Acta 266, 561–583.

Rogers, J., Yu, B.-Z., Serves, S., Tsivgoulis, G.M., Sotiropoulos, D.N., Ioannou, P.V., Jain, M.K., 1996. Kinetic basis for the substrate specificity during hydrolysis of phospholipids by secreted phospholipase A_2. Biochemistry 35, 9375–9384.

Roy, M.T., Gallardo, M.L., Estelrich, J., 1998. Influence of size on electrokinetic behavior of phosphatidylserine and phosphatidylethanolamine lipid vesicles. J. Coll. Inter. Sci. 206, 512–517.

Senior, J., Gregoriadis, G., 1982. Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. Life Sci. 30, 2123–2136.

Serves, S.V., Sotiropoulos, D.N., Ioannou, P.V., Jain, M.K., 1992. Synthesis of (*R*) — and (*S*)-1,2-diacyloxypropyl-3-arsonic acids: optically active arsonolipids. Phosphorous Sulfur Silicon 71, 99–105.

Serves, S.V., Sotiropoulos, D.N., Ioannou, P.V., Jain, M.K., 1993. One pot synthesis of arsonolipid via thioarsenite precursors. Phosphorous Sulfur Silicon 81, 181–190.

Shaw, D.J., 1970. Introduction to Colloid and Surface Chemistry, second ed., University Press, London.

Shen, Z.X., Chen, G.Q., Ni, J.H., Li, X.S., Xiong, S.M., Qiu, Q.Y., Zhu, J., Tang, W., Sun, G.L., Yang, K.Q., Chen, Y., Zhou, L., Fang, Z.W., Wang, Y.T., Ma, J., Zhang, P., Zhang, T.D., Chen, S.J., Chen, Z., Wang, Z.Y., 1997. Use of arsenic trioxide in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood 89, 3354–3360.

Soignet, S.L., Maslak, P., Wang, Z.G., Jhanwar, S., Calleja, E., Dardashti, L.J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D.A., Pandolfi, P.P., Warrell, R.P., 1998. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N. Engl. J. Med. 339, 1341–1348.

Stewart, J.C.M., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10–14.

Straubinger, R.M., Hong, K., Friend, D.S., Papahadjopoulos, D., 1983. Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles. Cell 64, 1069–1079.

Sun, H.D., Ma, L., Hu, X.C., Zhang, T.D., 1992. Treatment of acute promyelocytic leukemia by Ailing-1 therapy with use of syndrome differentiation of traditional Chinese medicine. Chin. J. Comb. Trad. Chin. Med. West. Med. 12, 170–171.

Supuran, C.T., Serves, S.V., Ioannou, P.V., 1996. Carbonic anhydrase inhibitors. Part 33. Isozyme inhibition with 2,3-dihydroxypropylarsonic acids and arsonolipids. J. Inorg. Biochem. 62, 207–212.

Talsma, H., Van Steenbergen, M., Borchert, J., Crommelin, D., 1994. A novel technique for the one-step preparation of liposomes and non-ionic surfactant vesicles in a continuous gas stream: the 'bubble method'. J. Pharm. Sci. 83, 276–280.

Timotheatou, D., Ioannou, P.V., Scozzafava, A., Briganti, F., Supuran, C.T., 1996. Carbonic anhydrase interaction with lipothioarsenites: a novel class of isozymes I and II inhibitors. Metal-Based Drugs 3, 263–268.

Tsivgoulis, G.M., Sotiropoulos, D.N., Ioannou, P.V., 1991a. 1,2-Dihydroxypropyl-3-arsonic acid: a key intermediate for arsonolipids. Phosphorus Sulfur Silicon 57, 189–193.

Tsivgoulis, G.M., Sotiropoulos, D.N., Ioannou, P.V., 1991b. *rac*-1,2-Diacyloxypropyl-3-arsonic acids: arsonolipid analogues of phosphonolipids. Phosphorous Sulfur Silicon 63, 329–334.

Waxman, S., Jing, Y.-K., Chen, Z., Chen, G.-Q., 1999. Response to Re: Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. J. Natl. Cancer Inst. 91, 1691.

Zhang, P., Wang, S.Y., Hu, X.H., 1996. Arsenic trioxide treated 72 cases of acute promyelocytic leukemia. Chin. J. Hematol. 17, 58–62.