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Note

The effect of pH on the electrophoretic behaviour of a new class of liposomes: arsonoliposomes

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Abstract

Herein we report the effect of pH on the surface charge of a new class of liposomes: arsonoliposomes. Plain or mixed arsonoliposomes with cholesterol (Chol) and distearoyl-phosphatidylcholine (DSPC) in 1:1 molar ratio were prepared with lauryl-(C12), myristoyl-(C14) and palmitoyl-(C16) acyl side chain arsonolipids. The one step hydration method was used for vesicle preparation and zeta potential measurements were performed in the pH range from 3 to 9.

The results revealed that these lipids hold a negative surface charge at all pH values investigated. The presence of cholesterol in 1:1 molar ratio results in higher zeta potential compared with plain arsonoliposomes with the exception of palmitoyl-(C16) acyl chain arsonolipids in neutral and slightly basic pH values. Oppositely, the DSPC (1:1 molar ratio) containing arsonoliposomes had lower values of zeta potential compared with plain arsonoliposomes.

Concluding, the experimental results reveal that zeta potential of arsonoliposomes is indeed modified when the vesicles are incubated in environments with different acidity. In most cases these changes are in accordance with the ionization pattern of the arsonolipid headgroup, while some peculiar deviations may be connected with the known difference in the structure between some of the vesicle types studied.

Keywords: Arsonoliposomes; Liposome size; Zeta potential; pH

The introduction of charge on the lipid surface modifies the biological (Allen et al., 1990) and physicochemical properties of liposomes (Crommelin, 1984), since electrostatic phenomena play a crucial role in many biological processes.

Recently we have prepared arsonolipid-containing liposomes (Fatouros et al., 2001). Arsonolipids (Fig. 1) are analogues of phosphonolipids in which P has been replaced by As in the head group (Tsivgoulis et al., 1991; Serves et al., 1993). The morphology and the characteristics of these liposomes are affected by the

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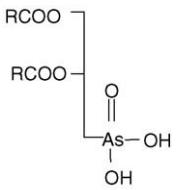


R=$C_{11}H_{23}$ (C12)

R=$C_{13}H_{27}$ (C14)

R=$C_{15}H_{31}$ (C16)

Fig. 1. Chemical structure of arsonolipids.

acyl chain length of the specific arsonolipid used for their preparation and the inclusion of cholesterol in their membrane, as demonstrated recently (Fatouros et al., 2001). The ability of these lipids to transport Ca^{2+} and Mg^{2+} through an organic phase has been proven (Gortzi et al., 2001a). Furthermore, and most important, promising preliminary activity results have been obtained with some of the arsonoliposomes prepared which, demonstrate a different toxicity towards cancer and normal cells (Gortzi et al., 2001b, 2003) as well as substantial anti-parasitic activity (Antimisiaris et al., 2003).

The aim of this study is to monitor, by means of zeta potential measurements, the electrophoretic behaviour of these charged vesicles in presence of media with different pH values.

Our attempt is to gain useful information about the physical stability and behaviour of these vesicles. The understanding of their physicochemical behaviour is of great importance since the stability of new formulations is highly dependent on their properties. Furthermore, our aim in the future, under the light of these findings, is to correlate their properties with the results obtained so far from their interactions with normal and cancer cells and their anti-parasitic activity.

Potassium chloride and glycine were obtained from Sigma-Aldrich Ltd. (Athens). Distearoyl L-a phosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids (USA) and cholesterol (Chol) from Sigma-Aldrich Ltd. (Athens). Arsonolipids with lauric C12, myristic C14 and palmitic C16 acid side chains were synthesized, as reported elsewhere (Tsivgoulis et al., 1991; Serves et al., 1993).

Plain arsonolipid vesicles and also mixed vesicles with arsonolipid and DSPC or cholesterol in a 1:1 molar ratio were prepared in 1 mM KCl containing 1 mM glycine by the one step method (Fatouros et al., 2001). The phase transition temperatures of the arsonolipids used in this study were C12:72 °C, C14:59 °C and C16:74 °C, respectively. Briefly the lipid or lipids (powders) were mixed with 1 mM KCl, 1 mM glycine and stirred magnetically for at least 4 h. In all cases the hydration and the annealing of the formulation were carried out at temperatures between 60 and 80 °C above the phase transition of the arsonolipids (depending on the arsonolipid used). The final lipid concentration was 6.5 μM. After formation the multilamellar vesicles were placed for 1 h above their phase transition temperature in order to anneal any structural defects.

A total amount of 50 μl of the vesicle dispersion were diluted with 20 ml of filtered buffer and sized immediately by photon correlation spectroscopy. Measurements were conducted at 25 °C with a fixed angle of 90° and sizes quoted are the *z*-average mean (dz) for the liposomal hydrodynamic diameter. The refractive index of water 1.330 was used for these measurements. All experiments in this study were performed in triplicate. The instrument used for the particle analysis was a Zetasizer 5000.

Plain and mixed arsonoliposomes were prepared in appropriately buffered solutions of various pH values from 3.0 to 9.0 containing 1 mM KCl and 1 mM glycine. The pH was adjusted with 0.1 M HCl or KOH accordingly. The vesicle electrophoretic mobility was measured at 25 °C by photon correlation spectroscopy (Zetasizer 5000, Malvern Instruments, UK). The instrument calculates the zeta potential values of the dispersion by the Smoluchowski equation.

The mean diameters of the arsonoliposomes prepared in 1 mM KCl/1 mM glycine, by the one step method without sonication are presented in Table 1. In most cases the size of liposomes ranged from 250 to 390 nm (Table 1). The polidispersity index (a measure of the homogeneity of vesicle dispersions) ranged from 0.10 to 0.20, indicating that the dispersions are quite homogeneous.

From the results it is obvious that there is no clear correlation between the vesicle size and the length of the acyl chain of the arsonolipid used. When cholesterol was added into the liposome bilayers, no changes in the size of the liposomes prepared from the C12 ar-

Table 1
Diameter and polydispersity indexes (PI) of liposomes containing arsonolipids prepared by the one step method (plain arsonoliposomes, and mixed Ars/Chol 1:1 and Ars/DSPC 1:1 arsonoliposomes) in KCl 1 mM containing 1 mM glycine

z-average mean diameter (nm)			
Lipid composition	Ars (PI)	Ars:DSPC 1:1 (PI)	Ars:Chol 1:1 (PI)
C12	260 ± 17 (0.123)	352 ± 32 (0.176)	256 ± 36 (0.101)
C14	342 ± 21 (0.134)	299 ± 13 (0.206)	390 ± 18 (0.143)
C16	280 ± 26 (0.107)	278 ± 29 (0.215)	352 ± 25 (0.105)

Values are mean ± S.D. (standard deviation) of three experiments.

sonolipid were observed, but the size of the vesicles prepared by longer side chain arsonolipids (C14 and C16) significantly increased. These observations are in good agreement with the measurements obtained previously from similar formulations prepared in distilled water (Fatouros et al., 2001). When comparing plain arsonoliposomes with the DSPC/arsonolipid liposomes a significant ($p < 0.05$) increase in size was only demonstrated in the case of the vesicles prepared by the C12 arsonolipid, in agreement again with our previous observations for the same vesicles prepared in distilled water (Fatouros et al., 2001).

The size of vesicles formed from plain arsonolipids are much smaller with a tighter size distribution compared with the vesicles prepared from arsonolipids with DSPC and cholesterol (Table 1). This could be attributed to changes in the degree of the headgroup dissociation of arsonolipids. But more studies are required (e.g., DSC) to gain information about the miscibility of these mixtures.

In Fig. 2, the zeta potential values of plain C12, C14 and C16 arsonoliposomes as a function of pH, are presented. From the results it is obvious that these lipids hold a negative surface charge as demonstrated before (Fatouros et al., 2001).

In comparison with the C14 and C16 arsonoliposomes, which have a similar behaviour, with an initial sharp increase in their zeta potential value as pH increases, followed by a stabilization (or a much lower gradual increase) of the zeta potential at pH values higher than 6, the C12 arsonoliposomes demonstrate a peculiar behaviour at pH values higher than 7, after which, the negative zeta potential decreases sharply. Perhaps this may be correlated with the different (in comparison with the more conventional vesicular structures formed by the other arsonolipids tested) tubular structures that are formed by C12 arsonolipids, as demonstrated recently (Fatouros et al., 2001).

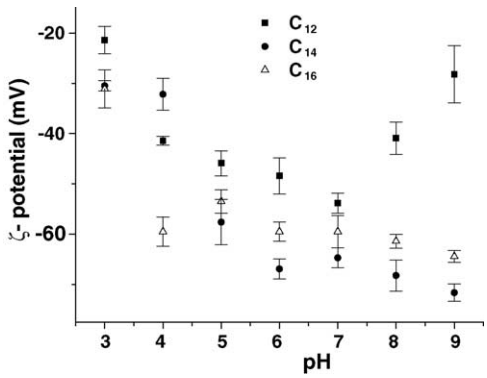
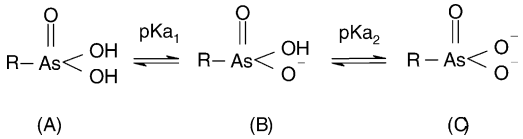


Fig. 2. The effect of pH on the zeta potential values of arsonoliposomes with different length of fatty acyl chains (C12, C14 and C16), prepared by the one step method in KCl 1 mM/1 mM glycine. Values are mean ± S.D. (standard deviation) of five measurements per batch out of three different batches carried out at the stationary level.

In general, the effect of pH on zeta potential of vesicles can be quantitatively explained by just assuming that an increase in HO^- adsorption on the liposome surface is taking place. Such adsorption would result in increasingly negative surface charge on the liposomal surface as the pH value of the surrounding medium increases. However, when the surface charge of lipidic-based vesicles is under investigation, the ionization of the component-lipid head groups should be accounted for. Indeed, aliphatic arsonic acids are ionized (in the pH range studied) as shown below,



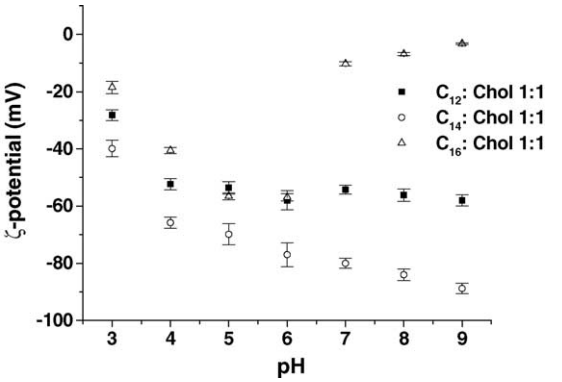


Fig. 3. The effect of pH on the zeta potential values of arsonoliposomes from C12/Chol 1:1, C14/Chol 1:1 and C16/Chol 1:1 mol/mol, prepared by the one step method in KCl 1 mM/1 mM glycine. Values are mean ± S.D. (standard deviation) of five measurements per batch out of three different batches carried out at the stationary level.

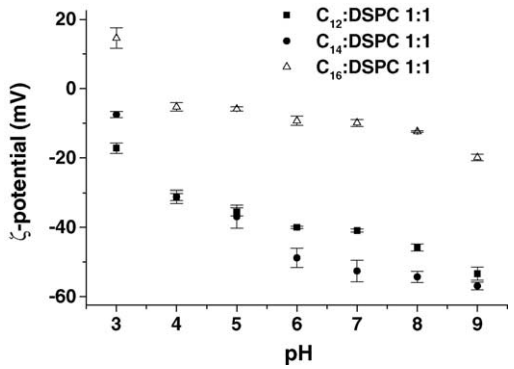


Fig. 4. The effect of pH on the zeta potential values of arsonoliposomes from C12/DSPC 1:1, C14/DSPC 1:1 and C16/DSPC 1:1 mol:mol, prepared by the one step method in KCl 1 mM/1 mM glycine. Values are mean ± S.D. (standard deviation) of five measurements per batch out of three different batches carried out at the stationary level.

with pK_a ∼4 and pK_a ∼9. It should be stated however, that these pK_a values might be somewhat higher due to specific adsorption of H^+ on the negatively charged bilayer, as was demonstrated for phosphatidic acid (McIntosh et al., 1989). In accordance with the ionization pattern presented above, one should expect the zeta potential of plain and mixed (with neutral lipids or cholesterol) arsonoliposomes to become more negative as the pH increases, unless other phenomena contribute to oppose such a trend. This is observed for all the plain arsonoliposomes studied, (Fig. 2) up to pH 7. The sharp decrease of the negative zeta potential of the plain C12 arsonoliposomes after pH 7 may indicate specific counter ion adsorption facilitated by a change in the shape or structure of the particle, because such a decrease is not seen for the C14 and C16 arsonoliposomes. The latter, however, have an almost constant zeta potential after pH 4 (or pH 5 in the case of C14 arsonoliposomes), implying that the effect of deprotonation to give (B) and (C) is almost exactly counter-balanced by specific cation adsorption, as previously demonstrated for phosphatidylserine and cardiolipin liposomes in the 7–9 pH region (Lis et al., 1982).

When cholesterol is incorporated into these vesicles at a 1:1 molar ratio (Fig. 3), similar profiles of zeta potential values versus pH are observed (compared to plain arsonoliposomes), with the exception of the sharp decrease in potential observed in the case of the C16/Chol arsonoliposomes, and the more predictable behaviour of the vesicles formed by the C12 arsonolipid.

Cholesterol is known to alter liposome structure and lipid hydration. Furthermore, the addition of cholesterol can cause changes in the degree of the headgroup dissociation.

In the case of the C12/Chol arsonoliposomes, this behaviour could be again related to the structural difference of the vesicles formed by this particular arsonolipid (Fatouros et al., 2001), as mentioned also above. In fact, the structures formed by C12 when cholesterol is added, are not tubular but discoid shaped closed vesicles, similar to those formed by the other arsonolipids, therefore, a more predictable behaviour is expected.

The effect of incorporation of equimolar amounts of a saturated phospholipid DSPC on the zeta potential values of arsonoliposomes as a function of pH is illustrated in Fig. 4. In all cases the arsonolipid/DSPC arsonoliposomes have zeta potential values that increase as the pH of the dispersion medium increases. The contribution of DSPC to the overall surface charge of arsonoliposomes (when compared to the plain arsonoliposomes) was more pronounced in C16 vesicles. Indeed, in the case of the C16/DSPC arsonoliposomes in the pH range between 3 and 9 the zeta potential

values of the vesicles range from +14.6 to −19.9, values that are more than three times lower, compared to those of the plain C16 arsonoliposomes (that range from −31.3 to −64.4). In addition, these values are highly shifted compared to the zeta potential values obtained for the C12/DSPC and C14/DSPC arsonoliposomes which have a very comparable (almost the same) behaviour, with zeta potential values that are statistically equal in some cases (pH 4.0, 5.0, and 9.0, $p < 0.05$). Although we cannot provide any explanation for this difference in behaviour between C12, C14 and C16 arsonolipid containing vesicles, one can speculate that the higher acyl chain length of the C16 arsonolipid which is closer to that of DSPC (C18) permits a stronger interaction between the headgroups of the two lipids, resulting thus in a minimization of the vesicle zeta potential. When the zwitterionic DSPC is incorporated into the arsonoliposomes at 1:1 molar ratio, then the negative zeta potential should decrease more than in the case of Chol, due to dilution, because the areas occupied by PC and Chol are ∼0.70 and 0.28 nm^2, respectively (Small, 1967; Szoka and Papahadjopoulos, 1980), and this was indeed found, (Fig. 4). The decrease is more pronounced for C16/DSPC liposomes, reaching a positive value at pH 3.0, at which, the PC phosphate should be half protonated, the arsonate group well protonated to (A) and the surface dominated by the -N^+Me_3 moiety, leading to positive zeta potential values in the absence of Cl^- adsorption. This adsorption, however, seems to take place for the C14/DSPC and the C12/DSPC liposomes, and is possibly related with weaker hydrophobic interactions of the shorter C12 and C14 arsonolipids with the C18 acyl-chains of DSPC. When the pH is increased there is a gradual deprotonation at both P–OH– and $-AsO_3H_2$ groups, which results in a negatively charged surface.

On the basis of the results presented herein, we can conclude that the surface charge of arsonoliposomes is indeed affected by pH, especially in the range of pH between 3 and 5, which perhaps is not very interesting for their in vivo behaviour. Nevertheless, the rather unpredictable behaviour of some of the mixed arsonoliposomes studied, and especially that of the C16/Chol (that demonstrate a high shift in zeta potential between pH 6 and 7) and the C16/DSPC (that have low zeta potential values compared to the vesicles prepared by the other two arsonolipids studied) may have interesting physiological implications for these vesicles that can be further investigated.

Considering the mechanisms of the modulation of the vesicle surface charge, for plain arsonoliposomes differences between zeta potentials could be due to a different degree of ionization of the different arsonolipids. In addition, it is clear that morphology (structural differences) of the vesicles has a definite effect on their zeta potential values (case of C12 arsonoliposomes). Conclusions related with the length of fatty acyl chain are difficult to be drawn out. When cholesterol and DSPC are included in arsonoliposome bilayer, the zeta potential differences could be related to the different degree of ionization of cholesterol and DSPC or a pH-dependent modulation of the lipid distribution in the bilayers.

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