

Size, Electrophoretic Mobility, and Ion Dissociation of Vesicles Prepared with Synthetic Amphiphiles

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Vesicles prepared with synthetic amphiphiles (dioctadecyldimethylammonium bromide and chloride, dihexadecyl phosphate and its sodium salt) were obtained by sonication, ethanol injections, and chloroform injections. The hydrodynamic diameter of vesicles (D_h), estimated from the diffusivity measured by quasielastic light scattering, ranged from 230 to 3000 Å. The electrophoretic mobility (U_m) was measured by free-flow electrophoresis. The zeta potential (ζ) and the degree of counterion dissociation (α) of the vesicles were calculated from U_m and conductivity data. α decreased with increasing D_h of the vesicles, probably due to the decreasing headgroup area and the increasing counterion association needed to relax the surface electrostatic potential. The electrophoretic mobility was also calculated (U_c) according to an impenetrable, nonconducting sphere model with a spherically symmetric charge distribution approximation. Within the limits of the experimental error(s) of the (different) methods employed and the assumptions made in the calculations, the fact that the U_m/U_c ratio ranged from 1.3 to 7.5 was considered to be a good agreement between the calculated and the experimental values.

Introduction

Vesicles prepared with both natural and synthetic amphiphiles are convenient systems for the study of structural and functional properties of bilayers as well as carriers of drugs and genetic information into cells.¹ Particularly, synthetic amphiphile vesicles are extremely efficient catalysts for both ground- and excited-state reactions.¹ The rate of vesicle-modified reactions can be controlled by substrate compartmentalization and changes in the composition of the reaction medium.^{2,3} The size of vesicles can be varied from 250 to 5000 Å in diameter by use of different preparation methods.⁴ Several features, such as solubilization and permeability, are strictly related to vesicle size. Not only the association constants but also the relative positions of bound substrates vary with vesicle size.⁵ These data led us to investigate the degree of counterion dissociation (α) as a function of vesicle size. This parameter is essential for the quantitative analysis of kinetic data using models such as the pseudophase ion exchange.⁶ Moreover, the determination of the parameters needed for the calculation of α of synthetic amphiphile vesicles of different sizes (e.g., hydrodynamic radius, electrophoretic mobility, zeta potential) may be of relevance for the understanding of the (several) differences between lipid and synthetic amphiphile bilayers.

Here we have used free-flow electrophoresis to determine, for the first time, the degree of ion dissociation from vesicles as a function of their size (measured by quasielastic light scattering). The experimental values of electrophoretic mobility of vesicles were lower than those calculated according to theory,⁷ and this discrepancy was analyzed. For both positively and negatively charged amphiphiles the degree of ion dissociation decreased with increasing size of the vesicle.

Materials and Methods

Dioctadecyldimethylammonium chloride (DODAC) (Herga Industrias Químicas, Rio de Janeiro, Brazil) and dioctadecyldimethylammonium bromide (DODAB) (Eastman Kodak) were purified by recrystallization.⁸ Dihexadecyl phosphate (DHPH) (Sigma Chemical Co.) was used as received. The sodium salt of DHPH (DHPNa) was prepared by neutralization of a hexane solution of DHPH with NaOMe in MeOH. After solvent stripping the solid was recrystallized from ethanol:acetone. 1-

[(2-methylphenyl)azo]-2-naphthol (Orange OT) was obtained from *o*-toluidine and 2-naphthol (Aldrich) according to standard procedures.⁹ All other reagents were of analytical grade. Deionized, twice-distilled water was used throughout.

Vesicle preparation. (1) Sonication: Enough amphiphile to give a final concentration of ca. 10 mM was weighed in a sonication tube, water (or aqueous NaCl) was added, and the tube was maintained for 10 min above the phase transition temperature (T_c) of the (corresponding) bilayer. The mixture was then sonicated at the T_c for 1 h (DODAC) or 1.5 h (DODAB) in a bath sonicator (Branson do Brazil, São Paulo, Brazil, MSX-10). Sonication was also performed with a tip sonifier (Braunsonic 1510) for 20 min (above the T_c), and mixtures were later centrifuged at 10000g, for 30 min. (2) Chloroform injection: Large DODAC and DODAB vesicles were prepared by injection of chloroform solutions of DODAX (X = Cl⁻ or Br⁻) into water or aqueous salt as previously described.¹⁰ (3) Ethanol injection: 1.0 mL of an ethanolic solution of DODAC (0.2 M) was slowly injected into 19.0 mL of either water or aqueous NaCl maintained at 50 °C.

Conductivity was determined (30 °C) in a digital apparatus (Digimed, São Paulo, Brazil) with a cell constant of 1.00. Absorbance was measured in a Beckman M25 spectrophotometer at 495 nm. To minimize light scattering errors, vesicles were destroyed before absorbance measurements by addition of detergent and incubation at 60 °C for 5 min. For DODAC or DODAB we used hexadecyltrimethylammonium bromide (CTAB), and for DHPH or DHPNa we used sodium dodecyl sulfate (SDS). DODAB and DODAC concentrations were measured by halide titration,¹¹ and DHPH was determined, after

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TABLE I

methods and conditions ^a	D_h , Å	$(d\lambda/dc)/f$ ($\times 10^2$)	α
DODAC			
EtOH injection ^b			
no salt (0.92)	230	4.47	0.29
no salt (8.0)	220	4.47	0.35
5 mM NaCl (9.0)	250	2.83	0.23
sonication			
no salt (5.0)	530 (484 ^c)	3.32	0.21
no salt (1.0)	530	3.32	0.22
0.93 mM NaCl (5.0)		2.80	0.20
2.42 mM NaCl (5.0)		2.35	0.17
4.76 mM NaCl (5.0)	380	1.73	0.13
9.27 mM NaCl (5.0)	400	1.40	0.11
CHCl ₃ injection			
no salt (0.3)	2850 (5000, ^d 3000 ^e)	0.61	0.044
4.4 mM NaCl (0.3)	3500		
9.0 mM NaCl (0.3)	3500		
DODAB			
sonication			
no salt (5.0)	650	1.64	0.13
CHCl ₃ injection (0.3)	2750	0.58	0.043
DHPNa			
sonication (5.0)	673 (508 ^f)	2.50	0.22
DHPH			
sonication (4.0)	1162 (500, ^g 900 ^f)	2.92	0.068
CTAB			
NaBr 0.025 M, 30 °C	61 (64.4 ^h)	2.73	0.203
SDS			
25 °C	51 ⁱ	2.52	0.26
30 °C		2.69	0.24

^a Amphiphile concentrations (mM) in parentheses. ^b 5% (v/v) EtOH. ^c Reference 28. ^d Reference 10. ^e Reference 19. ^f Reference 29. ^g Reference 30. ^h Reference 31. ⁱ Reference 32.

hydrolysis, by measuring inorganic phosphate.¹²

Electrophoretic mobility (U_m) was determined according to the procedure described by Hoyer et al.¹³ as modified by Sepulveda and Cortes.¹⁴ The agar (2%) bridge contained 0.2 M NaCl. Labelling was done by adding 20 μ L of an acetonitrile solution of Orange OT (0.025 M) to vesicle preparations to yield 5×10^{-5} M final dye concentration. Current was measured with a Keithley 197 multimeter. The applied voltage (300 V) was maintained with a Shandon SAE 2761 source. The silver electrodes (tapes $1 \times 10 \times 0.1$ cm, generously supplied by Degussa S. A. Brasil) were immersed in NaCl (0.2 M). During electrophoresis the temperature was maintained at 30.0 ± 0.02 °C with a thermostating-circulating pump (Quimis Brasil) in a 50 L water bath. U_m was calculated from the equation¹³

$$U_m = \frac{\lambda(A_i - A_f)v}{itA_i} \quad (1)$$

where A_i and A_f are the initial and final absorbances, respectively, of the Orange OT labeled vesicle solution contained in a capillary of volume (v) = 3.71 cm³; t is the time (in seconds) during which the current (i , amperes) was measured; λ is the conductivity of the sample (mho per centimeter).

Dynamic light scattering measurements were made at 25 °C in an apparatus equipped with a Coherent Innova 90-5 argon ion laser operating at 488 nm and a Thorn EMI 9863Q-35 phototube with an APED-II amplifier for single photon detection, connected to a Brookhaven PI-2030 digital correlator. Vesicle solutions were passed through nylon filters before quasielastic light scattering (QELS); for sonicated and ethanolic vesicles the filter cutoff was 2200 Å and for chloroformic vesicles it was 8000 Å.

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Results and Discussion

Results are presented in Tables I and II. Vesicles of various sizes have been prepared with lipids (for a review see ref 4), and some of the methods used for lipids have been adapted for synthetic amphiphiles.^{8,10} In particular, dialkyldimethylammonium (DODA) amphiphiles form aggregates of different architecture (e.g., micelles or vesicles) depending not only on the preparation method but also on the alkyl chain length and counterion type.¹⁵ Size determinations using QELS depend on several assumptions, including corrections for particle-particle interactions.¹⁶ The reported hydrodynamic diameters (D_h) were estimated from the diffusivity measured by QELS using the Stokes-Einstein relation.¹⁷ We note that for all reported D_h 's the polydispersity index, Q ,¹⁸ ranged from 0.1 to 0.2. In the case of micelles, extrapolation to the critical micelle concentration (cmc) generally affords consistent sizes from QELS data.¹⁶ The apparent sizes of sonicated vesicles varied little with concentration up to 10 mM amphiphile (not shown). Hence, for the purpose of this work, we elected to report all D_h 's at a single amphiphile concentration (Table I). The micellar D_h of CTAB was extrapolated to the cmc and agrees with published data.¹⁶

Ethanol injection (DODAC) produced aggregates with an D_h of 230 Å with or without added salt. Sonicated DODAC vesicles exhibited a D_h of 520 Å when prepared in water. Sonication of DODAC in salt ($(5-10) \times 10^{-3}$ M) produced smaller vesicles ($D_h = 380-400$ Å). For vesicles obtained by chloroform injection the presence of salt in the preparation medium led to an increase in D_h from 2850 Å (no salt) to 3500 Å ($(4-9) \times 10^{-3}$ M NaCl). Comparison of the values obtained here for D_h with published data (presented in Table I) show good agreement for sonicated vesicles. In the case of large (chloroformic) vesicles the present values are smaller than those obtained by electron microscopy¹⁰ but similar to those obtained by QELS.¹⁹ Since these vesicles are salt-sensitive,¹⁰ it is probable that the difference in the sizes reflects the effect of the staining salt used for contrast in electron microscopy. A limited study of the size of DHPH vesicles was also undertaken (Table I). It should be noted that the size of DHPH vesicles was extremely sensitive to the nature of the counterion. Sonicated DHPH vesicles exhibited a D_h of 1162 Å while for DHPNa D_h was 673 Å. This difference is probably related to the formation of intrabilayer hydrogen bonds²⁰ in the DHPH bilayer which could prevent the formation of small vesicles.

Measured mobilities are sensitive functions of both amphiphile and salt concentrations.²¹ Without added salt the values of U_m of large vesicles were 30-50% lower than those of small (sonicated) vesicles irrespective of the nature of the counterion. The mobility of positively charged vesicles was similar to those of DHPNa and DHPH vesicles and to micelles (Table II). In spite of the remarkable size difference between the amphiphilic structures, ranging from 40 to 5000 Å in diameter, the maximum difference in U_m was ca. 2-fold. The data in Table I suggest that there is no relationship between particle size and electrophoretic mobility. This lack of relationship could be expected for particles in which the size increases were accompanied by a decrease in ion dissociation, e.g., a decrease in effective surface potential (vide infra).

From the electrophoretic mobility (U_m) we estimated the streaming potential (ξ) of the particles using a simple Smoluchovski model. The ξ value calculated by using the (uncorrected) Smoluchovsky expression is known to yield a minimum estimate of the streaming potential.²² There are serious doubts that ξ values can be calculated from U_m data even using more rigorous data-

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TABLE II

methods and conditions ^a	U_m ($\times 10^4$), cm ² /(V s)	U_c ($\times 10^3$), cm ² /(V s)	U_c/U_m	U_c/U_m^b	ξ , ^c mV
DODAC					
EtOH injection ^d					
no salt (0.92)	8.2	4.3	5.2	2.7	96.4
no salt (8.04)	4.5	3.4	7.5	6.6	52.9
5 mM NaCl (9.42)	4.0	1.4	3.4	3.0	47.0
sonication					
no salt (1.0)	7.2				85.0
no salt (5.0)	7.8	4.1	5.3	3.3	91.5
0.93 mM NaCl (5.0)	6.2				72.6
2.42 mM NaCl (5.0)	5.9				70.1
4.76 mM NaCl (5.0)	4.9	0.92	1.3	1.1	57.9
9.27 mM NaCl (5.0)	4.6	0.86	1.9	1.6	54.1
CHCl ₃ injection					
no salt (5.0)	5.4	2.8	5.2	4.2	63.6
DODAB					
sonication					
no salt (5.0)	6.7	3.0	4.5	3.2	78.4
CHCl ₃ injection					
no salt (5.0)	5.1	2.4	4.5	3.8	60.4
DHPNa					
sonication (4–10)	6.6	4.6	5.8		77.9 (77–127 ^e)
DHPH					
sonication (4.0)	8.3	2.9	3.6	1.8	97.3
CTAB					
25 mM NaBr (5.0)	5.0 (2.45, ^f 4.75 ^g)	0.94	1.9	1.6	58.9
SDS					
25 °C (16)	4.8 (4.5 ^h)	0.8	1.7	1.4	56.2
30 °C (16)	5.6	0.8	1.5	1.1	66.2

^a Amphiphile concentrations (mM) in parentheses. ^b Corrected U_c values; see text. ^c Calculated from U_m ; see text. ^d 5% v/v EtOH. ^e Reference 33. ^f Reference 14a. ^g Reference 14b. ^h Reference 21.

analysis models. Nevertheless, the calculations were considered to be illustrative for comparative purposes. ξ was calculated by using the expression²²

$$\xi = \frac{\eta U_m}{\epsilon_r \epsilon_0} \quad (2)$$

where η is the water viscosity (7.975×10^{-3} dyn s/cm²), ϵ_0 the permittivity of free space (8.85717×10^{-7} dyn/V²), and ϵ_r the dielectric constant of water (76.55) at 30 °C. The calculated ξ values (Table II) ranged from 50 to 90 mV, well within the range published for similar supramolecular aggregates of this type at low salt concentration.²²

From U_m and conductance data we calculated the relative counterion dissociation of the supramolecular aggregate (α)²³ using the relationship

$$\alpha = \frac{10^3 d\lambda/dc}{F(U_m + U_x)f} \quad (3)$$

where the Faraday constant (F) is equal to 96 500, U_x is the mobility of the counterion, and $d\lambda/dc$ is the slope of the conductance vs total amphiphile concentration plot. For micelles $d\lambda/dc$ was taken above the cmc.¹⁴ In the case of vesicles $d\lambda/dc$ was corrected for the fraction of amphiphiles residing in the external surface (f). The dissociation of ions from the internal surface should not contribute to the measured conductance.²⁴ Hence f is 1.0 for micelles and was taken as 0.7, 0.6, and 0.5 for vesicle diameters up to 250 Å, from 250 to 700 Å, and greater than 700 Å, respectively.¹⁰ The conductances of solutions of large vesicles were significantly lower than those containing smaller structures. In some cases a deviation from linearity in the λ vs

c plots was observed. The values of $d\lambda/dc$ are presented in Table I. Vesicles of DHPNa and DHPH exhibited similar $d\lambda/dc$. However, since the specific conductance of H⁺ is larger than that of Na⁺, the calculated value of α for DHPH was much lower than that of DHPNa. Function-dependent changes due to replacement of H⁺ in DHPH monolayers have been described for intra- and interlayer forces.²⁰

As previously observed for micelles,¹⁴ the degree of counterion dissociation (α) from small DODAX vesicles was larger with Cl⁻ ion than with Br⁻ ion. The difference in α , however, is negligible when Cl⁻ ion and Br⁻ ion are compared in large DODAX vesicles (Table I). Ion dissociation from the large vesicles was very small, and the detection of minor differences in α (between DODAC and DODAB) in large vesicles would be outside the sensitivity of the present method. The salt dependence of α , especially in the small DODAC vesicles, suggests that ion dissociation in vesicles is affected by ionic strength more strongly than in micelles. It is attractive to speculate that the effective size of the ion pair at the surface¹⁵ affects both ion dissociation and vesicle size. The size dependence of α is probably partially due to the difference in headgroup packing in the bilayer as a function of changing radius of curvature. Abuin and Lissi⁹ have observed marked changes in the partition coefficients of neutral substrates as a function of vesicle size and interpreted their data in terms of a lesser monomer packing in the small vesicles. Lesser packing should reduce the electrostatic repulsion at the surface and permit a larger extent of ion dissociation as observed. The values of α presented here were utilized to analyze quantitatively vesicle size dependent catalytic effects on chemical reactions and resulted in excellent fits.²⁵ The large differences in α , varying by as much as a factor of 8 (Table I), cannot be wholly explained by changes in headgroup area. The latter is not expected to exceed a factor of about 2 in going from a vesicle diameter of 220 Å to 2850 Å.²⁶

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The surface potential (ψ) of the vesicle should be roughly proportional to

$$\frac{\alpha R}{(1 + \kappa R)}$$

where κ is the Debye-Hückel inverse screening length and R is taken as half of D_h . At very low salt concentration the interaction energy between an ion and the surface would increase with increasing vesicle size to levels well above kT unless some counterions condense back to the headgroups; i.e., α must decrease with increasing vesicle size in order to maintain the thermal distribution of bound and free counterions.

Vesicles, because they can be prepared with a number of different monomers in varied sizes and charge distributions, are interesting systems to test theories for particle movement as well as particle-particle interactions in solution. Although a completely satisfactory model for the relationship between electrophoretic mobility, polyion parameters, and solution parameters does not exist, the impermeable sphere model should be a better approximation for vesicles and micelles than for polymeric polyelectrolytes. In the approximation of an impenetrable, nonconducting sphere with a spherically symmetric charge distribution, the leading term for the expression of electrophoretic mobility is given by⁷

$$U_c = z\epsilon X(kR) / [(6\pi\eta R)(1 + kR)] \quad (4)$$

Here k is the Debye-Hückel parameter, R is the radius of the sphere, and $X(kR)$ is a sigmoidal function which varies from 1.0 to 1.5 over the range of kR from zero to infinity. The hydrodynamic radii were used for R in the above equation.

In order to estimate the mobility according to eq 4, it was necessary to make approximations for the values of z and k in the cases of no added electrolyte. Two assumptions were used to estimate z : First, the headgroup area per monomer in all the vesicles was taken as 75 \AA^2 . Literature values for the surface areas of monomers in bilayers range from 40 to 100 \AA^2 for amphiphiles as different as DODAC to (a number of) naturally occurring lipids.²⁷ Thus, while headgroup area is expected to decrease with increasing radius, the (constant) value chosen should not affect z by more than a factor of 2 in any of the cases. Second, the value of α was used to compute the net charge via

$$z = \alpha(\pi D_h^2) / 75 \quad (5)$$

where D_h is measured in angstroms. This equation implicitly assumes that only the dissociated headgroups on the external surface contribute to the vesicles' net charge and mobility.

In the cases of added NaCl the usual prescription for the inverse Debye-Hückel screening length was used:

$$k = (I)^{1/2} / (3.04) \quad (6)$$

where I is the ionic strength in terms of moles and k has the units of inverse angstroms.

In the case where no added salt was present, the value of k was estimated by assuming the ionic strength to be due to the concentration of (external) unassociated counterion, e.g., to αf [amphiphile]/2. No attempt is made to justify this procedure since in the absence of added salt the applicability of the assumptions of Debye-Hückel theory is uncertain. It is important to note that by this estimation even low effective free counterion concentrations, on the order of 0.1 mM lead to ionic atmosphere radii on the order of the vesicles' radii and hence to appreciable values for kR in eq 4.

The shortest interparticle distance (800 \AA) was calculated for ethanol-injected vesicles (8 mM DODAC, Table I), and even in this case, the screening length (100 \AA) was lower than the intervesicle distance. As the size of the particle increases, at the amphiphile concentrations used, the interparticle distances increase. Thus, for the large DODAC vesicles (0.3 mM DODAC, Table I) the intervesicle distance was ca. $1.36 \times 10^4 \text{ \AA}$ and the screening length was 409 \AA . It is therefore clear that the assumption of no interparticle repulsions, in the calculation of α , was adequate.

Table II shows the values for the calculated mobilities (U_c) and the ratio U_c/U_m . The latter values range from 1.3 to 7.5. Even this level of agreement is surprising given the number of approximations. In the case of all vesicles the U_c values are larger than U_m values. Booth⁷ carried out the expansion in terms of zeta potential to third order for the electrophoretic mobility of impenetrable, uniformly charged, nonconducting spheres in symmetric electrolytes and found that for potentials above about 40 mV the mobility begins to deviate downward from the linear relation of eq 4. For a potential of 100 mV the linear fit overestimates the mobility by a factor of 2. In light of this correction, all of the U_c values in Table II can be revised downward. Table II shows the values of U_c/U_m with U_c corrected to third order using Booth's graphical summary.⁷ Since Booth's theory also only applies in the case of added symmetric electrolyte, it is most interesting to see the effect of the correction on the case of 5 mM salt for sonicated DODAC in the Table II, using the associated estimate for the zeta potential. The revised value was within 10% of the measured value (Table II). The good agreement between measured and calculated values for the micelles is probably due to the fact that the error inherent in the approximation of k has a relatively small effect on the value of kR , since the micellar radius is quite small. The larger deviations for the ethanolic vesicle preparations may have to do with differences in dielectric constant around the vesicles due to the (unknown) distribution of the ethanol between the bulk and vesicular pseudophases.

Given the good agreement between experiment and theory in the case of the sonicated DODAC vesicles and added NaCl, it would be interesting to conduct more extensive tests on vesicles or liposomes that are stable at high ionic strengths.

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