Biochimica et Biophysica Acta, 733 (1983) 172–179
Elsevier

BBA 71805

PREPARATION AND CHARACTERIZATION OF LARGE DIOCTADECYLDIMETHYLAMMONIUM CHLORIDE LIPOSOMES AND COMPARISON WITH SMALL SONICATED VESICLES

A.M. CARMONA RIBEIRO and H. CHAIMOVICH *

Departamento de Bioquímica, Instituto de Química, USP, C.P. 20.780, São Paulo, SP (Brazil)

(Received February 14th, 1983)

Key words: Liposome preparation; Dioctadecyldimethylammonium chloride; Internal volume; Osmotic behavior

Dioctadecyldimethylammonium chloride (DODAC) unilamellar liposomes with a mean external diameter of 0.5 μm and sharp gel-to-liquid-crystalline phase transition temperatures (T_c) were obtained by chloroform vaporization and compared with small sonicated DODAC vesicles. Sucrose, impermeant through large DODAC liposomes and sonicated vesicles, was used for internal volume determinations. The internal volumes for large DODAC liposomes and sonicated DODAC vesicles were 9.0 ± 1.3 and 0.13 ± 0.2 l/mol, respectively. Ideal osmometer behaviour, towards KCl (0–50 mM) and sucrose, was observed only for large DODAC liposomes. Sonicated DODAC vesicles were osmotically non-responsive towards sucrose and flocculated upon addition of KCl. At temperatures near the T_c, a steep increase in the initial shrinkage rate and a minimum for the total extent of shrinkage were observed for large DODAC liposomes. Large DODAC liposomes are proposed as an adequate synthetic membrane model.

Introduction

Much of our present knowledge of membrane properties has been obtained with models prepared with phospholipids [1]. A variety of bilayer structures, formed by dialkyldimethylammonium halides [2] and other synthetic amphiphiles [3–7], have recently been used to mimic membrane properties, since they furnish unique opportunities to investigate structure-function relationships.

Closed-vesicle systems constitute one of the most explored models of membranes. While several methods for the preparation of phospholipid vesicles [8–11] or liposomes [12,13] have been described, synthetic amphiphile vesicles have been obtained mainly by sonication [7]. This latter method yields small unilamellar vesicles with a mean external diameter of approx. 300 Å [14,15] and a corresponding small internal aqueous compartment. Thus incorporation of water-soluble substrates and permeability studies in synthetic amphiphile systems have been restricted.

In this report we describe the preparation and characterization of large DODAC liposomes that: (a) possess a mean external diameter of 0.5 μm; (b) entrap sizeable amounts of sucrose; (c) have a sharp gel to liquid-crystalline phase transition; and (d) exhibit ideal osmometer behaviour over a range of conditions. In addition, we compare the properties of sonicated DODAC vesicles with those of large DODAC liposomes and conclude that this latter system constitutes a model that is more adequate for the investigation of transport properties in membranes.

* To whom correspondence should be addressed.

Abbreviation: DODAC, dioctadecyldimethylammonium chloride.

Materials and Methods

The purification and analysis of dioctadecyldimethylammonium chloride (DODAC) (obtained from Herga Indústrias Químicas do Brasil) have been described [16]. Sephadex G-25 (fine) was obtained from Sigma (St. Louis, MO) and [^{14}C]sucrose from Schwarz-Mann (Orangeburg, NY, spec. act. 480 mCi/mmol). All other reagents were analytical grade. Deionized water, doubly distilled in glass, was used throughout. DODAC concentration was determined by chloride microtitration [17].

Preparation of small DODAC vesicles. Small DODAC vesicles were prepared by sonication (10 min, 50°C) of 60 mg DODAC in 10 ml water using the microprobe of a Cell Disrupter Virsonic Model 150 operated at a nominal output of 90 W. The dispersion was centrifuged (50 min, 10000 × g, 22°C) in order to eliminate residual Ti.

Preparation of large DODAC liposomes. The vaporization method of Deamer and Bangham [10] was adapted for DODAC after experimenting with several solvent combinations, temperatures and DODAC concentrations. 1.0 ml DODAC (0.02 M) in $CHCl_3$ was injected (0.2 ml/min) into 4.0 ml of water maintained at 70.0 ± 0.5°C. The experimental arrangement for injection was that described by Deamer and Bangham [10]. Aliquots (1.0 ml) of the DODAC dispersion were filtered through a Sephadex G-25 column (1.8 × 14.0 cm) previously saturated with DODAC. DODAC recoveries were higher than 90% in all cases. The $CHCl_3$ contents [18] of the chloride-containing fractions eluting at the void volume (V_0) were less than 0.5 mol% ($CHCl_3$/DODAC).

Electron microscopy. Aliquots of DODAC dispersed by chloroform vaporization and filtered on Sephadex G-25 were mixed with equal volumes of ammonium molybdate (1%). After drying, the sample was examined in a Zeiss EM-9 S2 Electron Microscope operated at 60 kV. Mean external diameter were calculated from histograms obtained from several electron micrographs.

Determination of apparent internal volume (V). Aliquots of DODAC dispersion, prepared in water containing [^{14}C]sucrose, were filtered through a Sephadex G-25 column. The [^{14}C]sucrose entrapped/free ratio (R) was expressed as R = cpm(V_0)/cpm(V_i) where cmp(V_0) is the total radioactivity eluting in the void volume (V_0), and cpm(V_i), the total radioactivity eluting in the internal volume (V_i). The apparent internal volume (V) was expressed as $V = R/[\text{DODAC}]$, where [DODAC] is the concentration of the DODAC dispersion applied to the filtration column. The extent of sucrose adsorption to preformed DODAC vesicles or liposomes was determined as follows. Small DODAC vesicles (or large DODAC liposomes) were prepared in water as described. To these preparations was added [^{14}C]sucrose, and the dispersions were filtered through a Sephadex G-25 column. The extent of sucrose adsorption per mol DODAC was expressed as an apparent volume (V_a) (see above).

The true internal volume (V_{in}) was defined as

$$V_{in} = V - V_a$$

Determination of osmotic properties. DODAC dispersions were added to aqueous solutions of KCl or sucrose (or aqueous solutions of KCl or sucrose were added to DODAC dispersions) and the time-dependent scatter-derived absorbance changes were registered in a Cary-14 Spectrophotometer at either 400 or 280 nm. The time lag between mixing and the start of the register was usually less than 6 s. The initial shrinking rate (v%) was defined [19] as:

$$v\% = \frac{1}{A_0} \cdot \frac{\Delta A}{\Delta t} \cdot 100 \qquad (1)$$

where $\Delta A/\Delta t$ represents the initial slope of the curve absorbance vs. time and A_0, the initial absorbance. The reciprocal of the total absorbance change ($1/\Delta A$) was taken to be:

$$\frac{1}{\Delta A} = \frac{1}{(A_f - A_0)} \qquad (2)$$

where A_f is the final measured absorbance. The extent of shrinkage (S) was defined as:

$$S = (1/A_0 - 1/A_f) \cdot A_0 \cdot 100 \qquad (3)$$

Mean values for these parameters were obtained from at least two independent experiments and errors are expressed as mean square deviations.

Phase transition determinations. The changes of absorbance as a function of temperature were obtained in a Cary-14 Spectrophotometer equipped with a thermostatically controlled cell compartment (Haake E-12 Circulating Bath). Heating or cooling rates were generally 2 K/min. Temperatures were measured inside the cuvette with a copper-constantin thermocouple connected to a millivoltimeter. The response of this measuring system is linear between 0 and 100°C with a relative error of less than 0.01%.

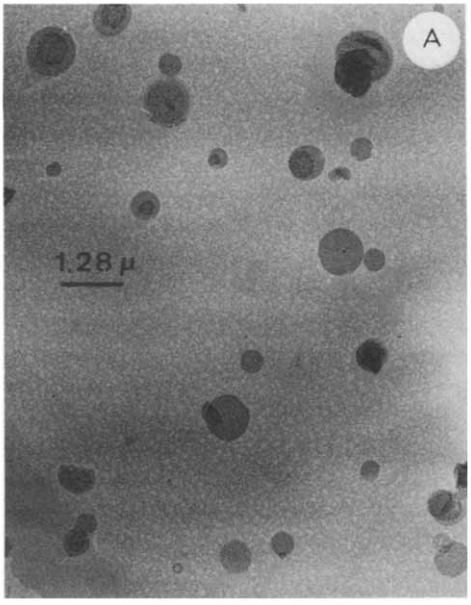
Results

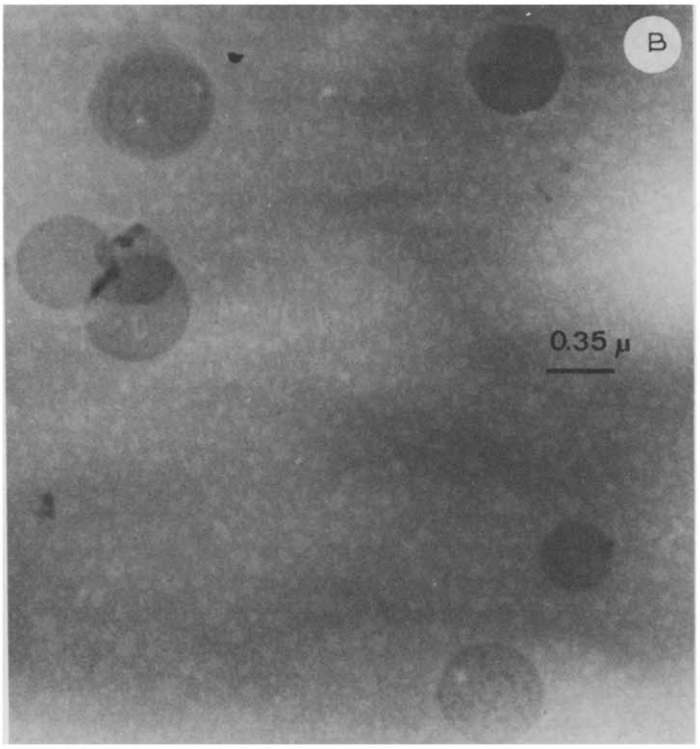
Physical characterization

DODAC dispersions, obtained by chloroform vaporization, eluted at the void volume of a Sephadex G-25 filtration column. The apparent absorptivity (400 nm) of the chloride-containing fractions was 300 $M^{-1} \cdot cm^{-1}$.

These latter fractions examined by electron microscopy with both negative and positive staining, exhibited closed vesicular structures (Fig. 1) with a mean external diameter of 0.51 μm (range 0.2–1.0 μm, Fig. 1C). Thus, the chloroform vaporization-Sephadex filtration preparation procedure yielded large DODAC liposomes which will thereafter be referred to as such.

DODAC liposomes exhibited a gel-to-liquid-crystalline phase transition temperature range narrower than sonicated vesicles, no pretransition being observed in either system (Fig. 2). The midpoints of the phase transition (T_c) for DODAC liposomes were 38.6°C (heating) and 34.8°C (cool-





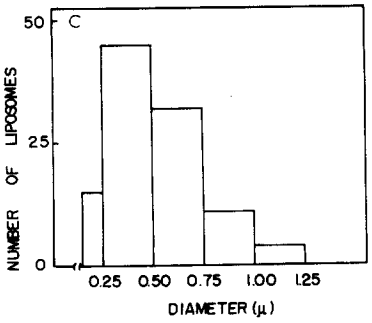


Fig. 1. Electron micrographs of large DODAC liposomes positively stained with 2% ammonium molybdate, 6630× (A); 24225× (B). The distribution of liposome sizes can be seen in (C).

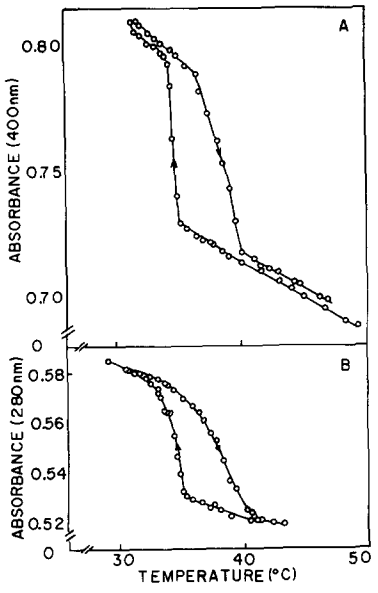


Fig. 2. Absorbance of 4.96 mM DODAC as a function of temperature. Arrows indicate the direction of temperature variation. (A) Large DODAC liposomes. (B) Sonicated DODAC vesicles.

ing). For the sonicated DODAC vesicles, the t_c values were 37.5°C (heating) and 34.4°C (cooling).

Functional characterization

Sucrose impermeability, entrappment and adsorption. The apparent internal volume (V) of sonicated DODAC vesicles, estimated using

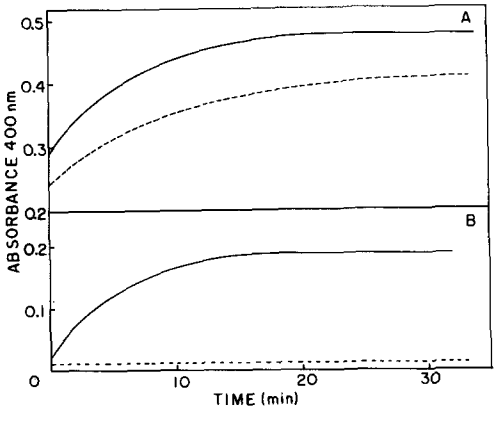


Fig. 3. Time-dependent absorbance changes after addition of: (A) 0.05 M KCl (———) or 0.10 M sucrose (- - - - -) to large DODAC liposomes and (B) 0.05 M KCl (———) or sucrose (- - - - -) to sonicated DODAC vesicles.

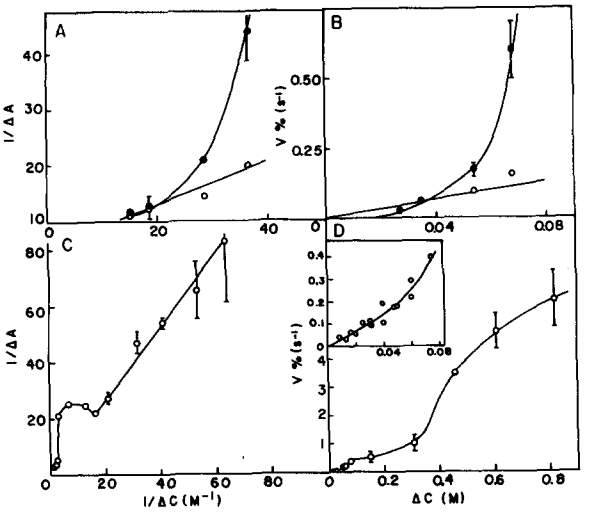


Fig. 4. Tests of osmometer behaviour towards KCl for large DODAC liposomes (○) and sonicated DODAC vesicles (●). The reciprocal of the total absorbance change ($1/\Delta A$) was plotted against the reciprocal of the concentration gradient of KCl ($1/\Delta C$) and the initial shrinking rate (v%), against the concentration gradient of KCl (ΔC). These parameters were calculated from the registered time-dependent absorbance changes obtained as follows: (A) and (B) 0.1 ml sonicated DODAC (4.96 mM) vesicles or large DODAC (4.96 mM) liposomes (prepared in water) were added to KCl solution (0.9 ml); (C) and (D) 0.2 ml large DODAC liposomes (3 mM) (prepared in water) were added to 0.8 ml KCl solutions. The insert in (D) contains an amplified plot of v% against ΔC for the lowest range of [KCl].

[^{14}C]sucrose as a water-soluble marker, was 0.33 ± 0.20 l/mol (n = 4). Vesicles containing [^{14}C]sucrose did not leak this marker when incubated up to 24 h in water at room temperature. The extent of external sucrose adsorption (see

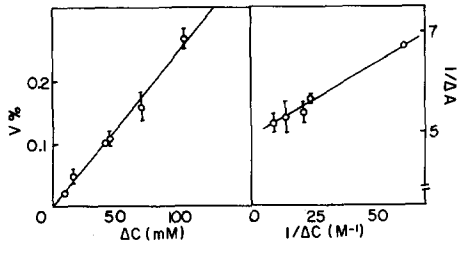


Fig. 5. Osmometer behaviour of large DODAC liposomes towards sucrose. Large DODAC liposomes (0.1 ml, 2.1 mM) were added to 0.9 ml sucrose solutions and time-dependent absorbance changes registered for calculations of $1/\Delta A$ (total absorbance change) or v% (initial shrinking rate).

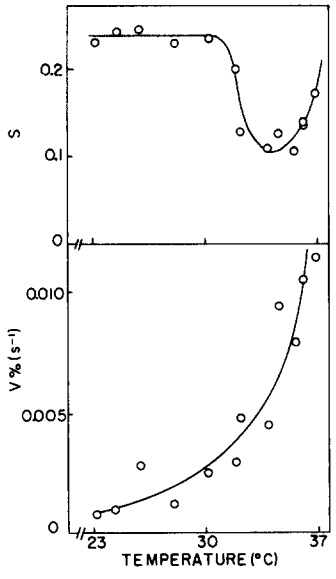


Fig. 6. Effect of temperature on the total extent of shrinkage and on the initial shrinking rate for large DODAC liposomes. A suspension of large DODAC liposomes (0.2 ml, 4.96 mM) was added to a solution of KCl (0.8 ml, 30.4 mM).

Materials and Methods) corresponded to a volume (V_a) of 0.20 ± 0.08 l/mol ($n = 6$). This latter value did not change when the unlabelled vesicles were incubated with externally added marker up to 24 h.

For large DODAC liposomes, an apparent internal volume of 9.66 ± 1.30 l/mol ($n = 3$) was determined. Equilibrium dialysis and filtration on Sephadex G-25 columns demonstrated (not shown), that no leak-out of entrapped [^{14}C]sucrose or leak-in of added [^{14}C]sucrose occurred over a period of 48 h. The extent of marker adsorption was 0.64 ± 0.30 l/mol ($n = 14$).

Osmotic properties

The time-dependent absorbance changes of DODAC sonicated vesicles (Fig. 3A) or DODAC liposomes (Fig. 3B) were registered for various osmotic gradients generated with KCl or sucrose. When KCl (0–50 mM) was the impermeant solute, linear dependences of v% as a function of KCl concentration gradient or of $1/\Delta A$ on the reciprocal of the KCl concentration gradient were obtained only for the large DODAC liposomes (Fig. 4). No changes in absorbance with time were detected for sonicated DODAC vesicles upon establishing osmotic gradients with sucrose. For DODAC liposomes, linear functions relating v% and ΔC or $1/\Delta A$ and $1/\Delta C$ were obtained with sucrose osmotic gradients (Fig. 5).

The effect of temperature on the osmometric behaviour of large DODAC liposomes was characterized by a steep increase in the initial shrinkage rate and a minimum for the total extent of shrinkage near the T_c (33–37°C) (Fig. 6).

Discussion

In this section, we show that DODAC liposomes, in contrast to small sonicated DODAC vesicles, are suitable as models for permeability and transport studies through bilayers composed of synthetic amphiphiles.

A fundamental property of a closed membrane model is to act as a barrier towards some substances entrapped in its internal aqueous compartment. Sucrose was shown here to be a suitable impermeant marker for internal volume determinations [20]. The apparent internal volume of DODAC liposomes (9.7 ± 1.3 l/mol), obtained from sucrose entrappment and adsorption data, was comparable to that of large unilamellar vesicles prepared by diethyl ether vaporization of phospholipids [10].

The internal volume of DODAC liposomes can also be calculated from the mean diameter measured by electron microscopy. The mean area per DODAC monomer in the bilayer is approx. 40 Å^2 [21] and the number of monomers in the inner and outer monolayer of DODAC liposomes can be considered equal. Assuming that DODAC liposomes (0.5 μm mean external diameter) are unilamellar, one calculates an internal volume of 10 l/mol, a value that is in very good agreement with that obtained experimentally by measuring sucrose entrapment.

The absence of typical multilamellar structures in electron microscopy (see, for example, Ref. 2), the similarity of the preparation method with that of large unilamellar phospholipidic vesicles [10] and the close agreement between the calculated and measured internal volumes indicate strongly that DODAC liposomes are unilamellar.

An identical calculation can be made for

DODAC sonicated vesicles. Taking a mean radius of 150 Å [14], an area per monomer of 40 Å^2 [21] and a 2 : 1 ratio of monomers in the outer and inner monolayer, one calculates an internal volume of 0.15 l/mol for these vesicles. From our sucrose data, subtracting the volume corresponding to adsorption (V_a) from the apparent internal volume (V), one obtains an upper limit of 0.13 l/mol for the internal volume (V_{in}) of sonicated DODAC vesicles. This value is comparable to the calculated internal volume and identical to that measured by Papahadjopoulus and co-workers [20] for sonicated phosphatidylserine vesicles.

Sonicated DODAC vesicles are osmotically non-responsive towards sucrose concentration gradients. Osmotic inactivity of small phospholipid vesicles has been described [23] specially for vesicles of external diameters smaller than 400 Å [24]. Absence of osmotic response for small sonicated vesicles has been associated with a rigid-sphere behaviour derived from the high radius of curvature of these structures [24,25]. Osmotic non-response in small sonicated vesicles can also be rationalized in terms of the organization of water in the internal aqueous compartment. Taking 0.13 l/mol as the internal volume and a 2 : 1 ratio for the number of monomers in the outer and inner monolayer, a simple calculation shows that there are only approx. 22 water molecules per DODAC monomer in the internal aqueous compartment of sonicated DODAC vesicles. This latter value is comparable to the number of water molecules undergoing slow exchange with bulk water in phosphatidylserine (Na^+) bilayers [26]. Thus, the osmotic non-responsiveness could be related not only to the rigid structure of the bilayer but also to the nature of the tightly bound water existing in the small compartment. In other restricted environments such as that of inverted micelles there is firm experimental evidence for motion-restricted water under a variety of conditions [27].

A large part of this work has dealt with permeability of DODAC liposomes and sonicated vesicles towards water. Osmotic properties of some phospholipidic systems have been characterized extensively [28,29] and the demonstration of ideal osmometer behaviour of phosphatidylcholine liposomes was based on the verification that this latter system follows Boyle-Van't Hoff's law for solutes such as glucose, manitol and salts [28]. In the present work, we have taken advantage of the linear relationship between the final liposome volume after shrinkage and the reciprocal of the final absorbance [19,28,29] to obtain some of the osmometric parameters. Our criteria for osmometric behaviour was also Boyle-Van't Hoff's law, which predicts a linear relationship between $1/\Delta A$ and $1/\Delta C$ and also between v% and ΔC. DODAC liposomes display the expected linear relationships between 0 and 50 mM KCl (Fig. 6). Above this salt concentration, the liposome preparation was seen to flocculate readily. Correspondingly, the linearity of the osmometric parameters with the concentration, or the reciprocal of the concentration, is not maintained. For sonicated vesicles, ideal osmometric behaviour was not observed under any set of conditions. In fact, visible aggregation and/or flocculation were noticed at most of the concentration gradients used here. The published osmometric behaviour of sonicated DODAC dispersions [14,30] is probably due to aggregation phenomena. The fact that sucrose gradients do not elicit an osmotic response with sonicated vesicles (vide supra) further establishes the difficulty of expecting ideal osmometric behaviour in the sonicated DODAC system. The marked difference of behaviour towards salts between the DODAC liposomes and the sonicated vesicles may have several explanations. One should notice, however, that intervesicular interactions are more probable in sonicated vesicles since the total exposed area is larger than that in large liposomes. Moreover, for equal amphiphile concentrations, the encounter probability is larger for the smaller particles. In view of the lack of data concerning surface potential of these systems, it is premature to offer definitive rationalizations that might account for the observed difference.

The pronounced hysteresis obtained for the phase transition temperature curves (Fig. 3) was expected, since the molar volume of surfactant in the liquid crystalline phase is greater than that in the gel phase and a domain of liquid crystalline phase will be under compression, whereas a domain of gel will be under tension [31]. McDonald et al. [32] have indeed shown that hysteresis is more likely to occur for charged lipids and that aqueous dispersions of phosphatidylserine do not

have superimposable heating and cooling curves. It is also well established that if the sharpness of the transition increases, so does the co-operativity [31,33]. Accordingly, the phase transition of large DODAC liposomes was more cooperative than that of sonicated DODAC vesicles (Fig. 2).

Phospholipid liposomes are permeable to water even below the T_c, being osmotically-sensitive structures both above and below the T_c [31]. In the vicinity of the T_c, a markedly reduced change in absorbance after an osmotic shock may occur due to increased permeability to water or to solute [34–38]. Large DODAC liposomes, in particular, behaved near the T_c as dimyristoylphosphatidylcholine liposomes [19], attaining a minimum for their total extent of shrinking and abruptly increasing the initial shrinking rate (Fig. 6). Increased permeability near the T_c to water and KCl is probably due to the formation of statistical pores between the gel and liquid-crystalline domains.

In conclusion, the two most interesting features of this work are: (i) the introduction of a confident criteria for stability of synthetic amphiphile vesicles towards salt, i.e., the determination of salt concentration ranges in which osmometric behaviour occurs; and (ii) the perspective of employing methods available for phospholipids to obtain new surfactant systems in which functional properties of membranes can be systematically checked.

Acknowledgements

This work was supported in part by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), The Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP B-76-81-295-00-00). We gratefully acknowledge Dr. Antonio Sesso and Mr. H. Brandão for the electron micrographs.

References

1 Gomperts, B.D. (1977) The Plasma Membrane: Models for Structure and Function, 1st Edn., Academic Press, London.
2 Kunitake, T., Okahata, Y., Tamaki, K., Kumamaru, F. and Takayanagi, M. (1977) Chem. Lett., 387–390
3 Hargreaves, W.R., and Deamer, D.W. (1978) Biochemistry 17, 3759–3768
4 Mortara, R.A., Quina, F.H. and Chaimovich, H. (1978) Biochem. Biophys. Res. Commun. 81, 1080–1086
5 Czarniecki, M.F. and Breslow, R. (1979) J. Am. Chem. Soc. 101, 3675–3676
6 Murakami, Y., Nakano, A. and Fukuya, K. (1980) J. Am. Chem. Soc. 102, 4253–4254
7 Fendler, J.H. (1982) Membrane Mimetic Chemistry: Characterization and Applications of Micelles, Microemulsions, Monolayers, Bilayers, Vesicles, Host-Guest Systems and Polyions, 1st edn., Ch. 6, Wiley-Interscience, New York
8 Huang, C. (1969) Biochemistry 8, 344–352
9 Batzri, S. and Korn, F.D. (1973) Biochim. Biophys. Acta 298, 1015–1019
10 Deamer, D. and Bangham, A.D. (1976) Biochim. Biophys. Acta 443, 629–634
11 Barenholz, Y., Amselem, S. and Lichtenberg, D. (1979) FEBS Lett. 99, 210–214
12 Bangham, A.D. and Horne, R.W. (1964) J. Mol. Biol. 8, 660–668
13 Kinsky, S.C. (1974) Methods Enzymol. 32, 501–513
14 Kano, K., Romero, A., Djermouni, B., Ache, H. and Fendler, J.H. (1979), J. Am. Chem. Soc. 101, 4030–4037
15 Fendler, J.H. (1980) Acc. Chem. Res. 13, 7–13
16 Cuccovia, I.M., Aleixo, R.M.V., Mortara, R.A., Filho, P.B. Bonilha, J.B.S., Quina, F.H. and Chaimovich, H. (1979) Tetrahedron Lett. 3065–3068
17 Schales, O. and Schales, S.S. (1941) J. Biol. Chem. 140, 879–884
18 Snell, F.D. and Snell, C.T. (1937) Colorimetric Methods of Analysis, 2nd. Edn., Vol. 2, pp. 557–558, D. Van Nostrand Company, Inc., New York
19 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1976) Biochim. Biophys. Acta 433, 1–12
20 Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta 266, 561–583
21 Herrmann, U. and Fendler, J.H. (1979) Chem. Phys. Lett. 64, 270–274
22 Schreier, S., Polnaszek, C.F. and Smith, I.C.P. (1978) Biochim. Biophys. Acta 515, 375–436
23 Johnson, S.M. and Buttress, N. (1973) Biochim. Biophys. Acta 307, 20–26
24 Lichtenberg, D., Freire, E., Schmidt, C.F., Barenholz, Y., Felgner, P.L. and Thompson, T.E. (1981) Biochemistry 20, 3462–3467
25 Schullery, S.E., Schmidt, C.F., Felgner, P., Tillack, T.W. and Thompson, T.E. (1980) Biochemistry 19, 3919–3923
26 Finer, E.G. and Darke, A. (1974) Chem. Phys. Lipids 12, 1–16
27 El Seoud, O.A., Chinelatto, A.M. and Shimizu, M.R. (1982) J. Colloid Interface Sci. 88, 420–427
28 Bangham, A.D., De Gier, J. and Greville, G.D. (1967) Chem. Phys. Lipids 1, 225–246
29 Rendi, R. (1967) Biochim. Biophys. Acta 135, 333–346
30 Tran, C.D., Klahn, P.L., Romero, A. and Fendler, J.H. (1978) J. Am. Chem. Soc. 100, 1622–1624
31 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237–281
32 McDonald, R.C., Simon, S.A. and Baer, E. (1976) Biochemistry 15, 885–891

33 Takemoto, H., Inoue, S., Yasunaga, T., Sukigara, M. and Toyoshima, Y. (1981) J. Phys. Chem. 85, 1032–1037
34 Haest, C.W.M., De Gier, J., Van Es, G.A., Verkleij, A.J. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 288, 43–53
35 Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330–348
36 Inoue, K. (1974) Biochim. Biophys. Acta 339, 390–402
37 Nichols, P. and Miller, N. (1974) Biochim. Biophys. Acta 356, 184–198
38 Blok, M.C., Van der Neut-Kok, E.C.M., Van Deenen, L.L.M. and De Gier, J. (1975) Biochim. Biophys. Acta 406, 187–196