Biochimica et Biophysica Acta 812 (1985) 688–694
Elsevier

BBA 72425

Effect of cholesterol on Ca^{2+}-induced aggregation and fusion of sonicated phosphatidylserine/cholesterol vesicles

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(Received July 20th, 1984)

Key words: Cholesterol; Phosphatidylserine; Membrane fusion; Ca^{2+}; Vesicle aggregation

Small unilamellar vesicles composed of phosphatidylserine (PS) and cholesterol at various ratios were employed in studying the effect of cholesterol on Ca^{2+}-induced vesicle aggregation and fusion using the Tb/dipicolinic acid assay. The leakage of preencapsulated Tb^{3+} was also measured. The analysis of the data provided estimates for the rate of aggregation, C_{11}, and the rate of fusion per se, f_{11}. An increase in cholesterol contents results in a decrease in C_{11} values. Similarly, aggregation of PS/cholesterol vesicles is slower than that of PS vesicles in the presence of 650 mM NaCl. With 100 or 200 mM NaCl, the overall fusion rate of PS/cholesterol vesicles is slower than that of PS vesicles; the rate being reduced by an increase in cholesterol contents. With 600 mM NaCl, the overall fusion rate of PS/cholesterol 9:1 vesicles is faster than that of PS vesicles, and results are well-simulated by assuming no delay in vesicle aggregation up to dimers. Emerging f_{11} values are larger in PS/cholesterol than in PS vesicles. An analysis of fusion kinetics of several lipid concentrations shows that f_{11} values of PS/cholesterol 3:1 vesicles are 5-times larger than those of PS vesicles, when fusion occurs in a medium containing 200 mM NaCl and 1.5 mM Ca^{2+}. The increase in Na^+ concentration from 100 to 200 mM, or 600 mM results in a 50- or 150-fold reduction in f_{11} values of PS vesicles. It is suggested that incorporation of cholesterol in PS vesicles results in enhancement of Ca^{2+}-induced fusogenic capacity.

Introduction

The role and importance of cholesterol in biological and model membranes have been reviewed [1]. Cholesterol has been demonstrated to affect the fluidity, permeability and organization of lipid bilayers [2–4]. It was suggested that cholesterol reduces the side-chain motion, [5] and that whereas it liquefies the fatty acyl achains below T_t and solidifies them above T_t, it does not affect significantly the mobility of the polar headgroups [6]. By using differential scanning calorimetry, Ladbrooke et al. [7] have found that addition of cholesterol to phosphatidylcholine-containing liposomes results in the reduction of the main phospatidylcholine endotherm.

It was suggested [8] that cholesterol, embedded in several lipid bilayers, may induce destabilization and a reverse hexagonal H_{II} phase. It was shown [9,10] that cholesterol destabilizes membranes composed of lipids of *Acholeplasma laidlawii*. Hydration of phosphatidylcholine (PC)

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

vesicles was reduced by addition of cholesterol at mole fractions higher than 0.22 [11], whereas addition of cholesterol to PC films resulted in enhanced hydration [12]. It was reported that cholesterol causes a small increase in the size of sonicated vesicles [13,14].

Former studies have indicated that in several systems, cholesterol tends to reduce and/or inhibit vesicle-vesicle or cell-to-cell fusion [15–17]. However, in other systems, using oleoylglycerol poly(ethylene glycol) and Sendai virus as fusion agents [18,19], cholesterol was shown to promote fusion. Using a fluorescent assay, based on intermixing of membrane components, Uster and Deamer [20] investigated the effect of the lipid composition on Ca^{2+}-induced fusion of large unilamellar vesicles. They have shown that replacement of 25 mol% PC by cholesterol in mixed PS/PC vesicles facilitates fusion competence. Since there is evidence that PC inhibits and/or reduces fusion competence [21,22], one cannot conclude whether the addition of cholesterol or the reduction in PC content was responsible for the increase in fusion capacity.

Studies on calcium-phosphate-induced fusion [23] have shown that in mixed PS/cholesterol vesicles, cholesterol reduced the leakage induced by addition of Ca^{2+}. It was also demonstrated that the rate of Ca^{2+}-induced fusion of PS/cholesterol vesicles was increased approx. 1000-fold in the presence of phosphate, whereas the rate of fusion of PS/cholesterol vesicles was lower in comparison with that of PS vesicles in the absence of phosphate. The purpose of this study is to investigate in detail the effect of cholesterol on Ca^{2+}-induced aggregation and fusion of PS/cholesterol sonicated vesicles. In accord with previous studies [24–28], our approach is to separate the overall fusion process into an aggregation followed by the actual fusion event. Correspondingly, we have deviced experiments which yield separately the effect of cholesterol on the rate of aggregation and on the rate of fusion per se of PS/cholesterol vesicles.

As we will show, cholesterol enhances the rate of actual fusion event of Ca^{2+}-induced fusion of these vesicles, but its effect on slowing the rate of aggregation leads, in most cases, to an observation that it reduces the rate of overall fusion process.

Materials and Methods

Bovine brain PS, grade I was purchased from Lipid Products (Nutfield, U.K.) and stored under argon at $-20°C$ in sealed ampules until use. Cholesterol was obtained from Sigma (Tel Aviv, Israel) and recrystalized twice. $CaCl_2$ was obtained from BDH Chemicals. L-Dipalmitoyl phosphatidyl [*N-methyl*-^{3}H]choline ([^{3}H]DPPC) and [^{14}C]sucrose from Amersham, U.K.; $TbCl_3$ from Alfa (Danvers, MA); dipicolinic acid and Tes from Sigma; NaCl from Frutarom (Haifa, Israel); sodium citrate from Matheson, Coleman & Bell (Norwood, Cincinnati,OH); ethylenediaminetetraacetic acid disdoium salt (EDTA), L-histidine monohydrochloride and sodium deoxycholate from Fluka (Switzerland) and Sephadex G-50 medium from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Small unilamellar vesicles were prepared by sonication as described [3,29]. The resulting clear suspension of vesicles was centrifuged at 5°C for 1 h at $120\,000 \times g$ to eliminate any large or multilamellar vesicles. The upper 4/5 of the supernatant was collected for experimental use. The sonicated vesicles were composed of either PS or PS/cholesterol mixtures at molar ratios of 9 : 1, 3 : 1 and 2 : 1.

Entrapped volume of the vesicles was determined by including 20 μCi/ml [^{14}C]sucrose (552 mCi/mmol) with 1000-fold unlabeled sucrose in the Tb and dipicolinic acid encapsulated buffer, and was expressed by liter of encapsulated solution per mole lipid. The inner radius of the vesicles, R was solved numerically from a set of two equations:

$$\frac{4\pi N}{3} R^3 = V$$

$$4\pi N \left[R^2 + (R + d)^2 \right] / A = N_1$$

in which V is the total encapsulated volume, N is number of vesicles per unit volume, $d = 40$ Å is the membrane thickness, A is average area per PS molecule and N_1 is the number of PS molecules per unit volume. The average area per PS molecule in a PS/cholesterol vesicle was determined by taking 93 Å^2 for a PS/cholesterol pair (56 Å^2 + 37 Å^2; see Ref. 36) and 70 Å^2 for PS molecules.

Assay for fusion. Fusion of the vesicles was monitored by means of the assay described previously [30,31] with some slight modifications. The vesicles were prepared in either of the following solutions: (A) 15 mM $TbCl_3$/150 mM sodium citrate/2 mM Tes/2 mM L-histidine (pH 7.4) or: (B) 150 mM sodium dipicolinate/2 mM Tes/2 mM L-histidine (pH 7.4). The vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-50 (column size 1.0 × 30 cm, elution buffer 2 mM Tes/2 mM L-histidine, either with 100 or 200 mM NaCl (NaCl buffer) and 1 mM EDTA (pH 7.4)). Lipid concentrations were determined either by the Bartlett phosphate assay [32] or by detecting the amount of [^{3}H]DPPC which was added in small amounts to the PS or PS/cholesterol mixtures before drying the lipids. For reference, a radioactive lipid mixture of a known weight was submitted to radioactive counting.

For the fusion assay, the Tb vesicles and the dipicolinate-containing vesicles were mixed in a 1 : 1 molar ratio at final lipid concentrations of 10, 25, 50 and 100 μM in 2 ml NaCl buffer, containing 0.2 mM EDTA.

Fluorescence was measured in a Perkin-Elmer MPF 44 spectrofluorometer by excitation at 276 nm wavelength and the emission was measured at a wavelength of 545 nm with the addition of a less than 520 nm cutoff filter to eliminate the scattering contribution to the fluorescence which in all cases was less than 5% of the full scale fluorescence.

Full scale (100%) fluorescence was set as described [31].

The fusion reaction was initiated by injecting to the vesicle mixture an aliquot of 100 mM $CaCl_2$ or adding a larger volume of smaller $CaCl_2$ concentration with required NaCl concentrations. The working solution was stirred constantly, and temperature was kept at 24°C. When high vesicle concentrations were involved, the fusion reaction was initiated by adding a large volume of mixed populations of the vesicles to a Ca^{2+}-containing working solution in order to avoid high local Ca^{2+} concentrations, since the fusion reaction is much more dependent on Ca^{2+} concentration than on the lipid concentration [33,34].

The leakage experiment. The leakage out of the vesicles was monitored by means of the Tb leakage induced by $CaCl_2$, measuring the build-up of the Tb (dipicolinic acid)$_3^{3-}$ fluorescent complex by using 20 μM dipicolinic acid in a medium without EDTA [35]. In leakage experiments, Ca^{2+} concentrations were smaller by 0.2 mM than those used in fusion experiments.

Lipid concentrations in each leakage measurement were set to be the same as in the parallel fusion experiment.

Vesicle aggregation. Aggregation was monitored by measuring changes in absorbance at 550 nm in an Aminco DW-2a spectrophotometer.

Results

The experiments of Ca^{2+}-induced fusion were designed to see the effect of cholesterol on the rate constants of aggregation, C_{11}, and rate constants of fusion, f_{11}. The kinetic equations and analysis

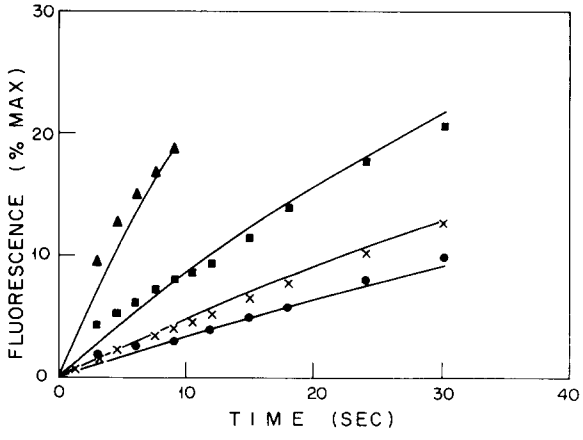

Fig. 1. Effect of Ca^{2+} concentration and of cholesterol content on Ca^{2+}-induced fusion of small unilamellar vesicles. The theoretical curves for the time development of the Tb fluorescence are shown by solid lines. The corresponding experimental results (corrected for the release of vesicle contents) are given as data points in which half of the leakage percentage is included. The experiments were carried out as described in Materials and Methods, in 100 mM NaCl/2 mM Tes/2 mM L-histidine/0.2 mM EDTA (pH 7.4) at 24°C. The leakage experiments were carried out without EDTA. In all cases, the fluorescence scale was calibrated to the total amount of Tb present and the lipid concentration was 25 μM. The calculations are described in Refs. 25–27,37. The values of the parameters C_{11} and f_{11} are given in Table I. ▲, PS/ cholesterol (9 : 1), 2.2 mM Ca^{2+}; ■, PS/cholesterol (9 : 1), 1.7 mM Ca^{2+}; ●, PS/cholesterol (9 : 1), 1.4 mM Ca^{2+}; ×, Ps/cholesterol (2 : 1), 1.7 mM Ca^{2+}.

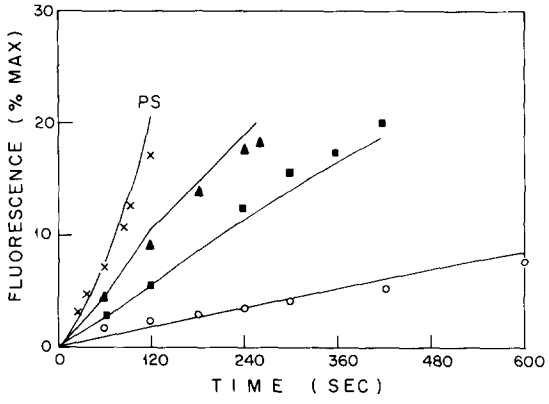

Fig. 2. Effect of lipid concentration and of cholesterol content on Ca^{2+}-induced fusion of small vesicles. The theoretical curves for the time development of the Tb fluorescence are shown by solid lines. The encapsulated volumes ranged between 0.18 and 0.24 l/mol in PS vesicles and from 0.18 to 0.28 in PS/cholesterol (3:1) vesicles. ×, PS, 100 μM lipid; ▲, PS/cholesterol (3:1) 100 μM lipid; ■, PS/cholesterol (3:1),50 μM lipid; ○, PS/cholesterol (3:1), 12 μM lipid. The medium contained 200 mM NaCl and 1.5 mM $CaCl_2$.

of data have been described before [25–27,37]. Parallel experiments provide fusion curves [30,31], F, which represent percent of maximal fluorescence of the Tb-dipicolinate complex, and leakage curves, L, which represent leakage of preencapsulated Tb^{3+} [35].

The experimental results in Figs. 1 and 2 give the quantity $I = F + 0.5\ L$, which represents the corrected percent of maximal fluorescence during the initial fusion stages [24–27]. The procedure of data analysis consists of first determining the rate of aggregation, C_{11}, from I values in dilute lipid suspensions, in which aggregation is rate-limiting to the overall fusion process, so that the condition $K = f_{11}/(C_{11}X_0) \gtrsim 100$, is satisfied, where X_0 is the molar concentration of vesicles. Then, f_{11} values are determined from more concentrated vesicle suspensions, where K is smaller, i.e., there is a delay due to the fusion reaction itself. In practice, f_{11} can be determined when $K \lesssim 10$. The use of concentrated lipid suspensions, satisfying the condition $K \lesssim 10$, caused difficulties, because the mixing of suspensions was not rapid enough. In an attempt to slow down the rate of overall fusion reaction, we reduced Ca^{2+} concentrations. However, as it turns out, a reduction in Ca^{2+} concentrations resulted in a more significant reduction in C_{11} values than f_{11} values (see Table I and Fig. 3). Hence, this procedure enabled a determination of C_{11} values, but it provided only a lower bound on f_{11} values, from which their dependence on cholesterol contents could not be deduced. We resolved this difficulty by using a large NaCl concentration, e.g., 200 mM. Under these conditions, f_{11} values are reduced [28,37] much more than C_{11} values, and both C_{11} and f_{11} values could be determined from fusion experiments employing three lipid concentrations of 10, 50 and 100 μM.

The results in Figs. 1 and 2, (which represent a fraction of the data), show that the overall fusion rates of PS/cholesterol are slower than those of

TABLE I

EFFECT OF CHOLESTEROL CONTENT, AND Ca^{2+} AND Na^+ CONCENTRATION ON RATE OF AGGREGATION, C_{11}, AND ON RATE OF FUSION, f_{11}, IN PS/CHOLESTEROL VESICLES

C_{11} values are expressed in $M^{-1}\cdot s^{-1}$, and f_{11} values in s^{-1}.

Ca^{2+} (mM) + 100 mM NaCl	Membrane composition (molar ratios)							
	PS		PS/cholesterol (9:1)		PS/cholesterol (3:1)		PS/cholesterol (2:1)	
	C_{11}	f_{11}	C_{11}	f_{11}	C_{11}	f_{11}	C_{11}	f_{11}
1.1	$(1.9–2.1)\cdot10^6$	≥1	$(1.1–1.8)\cdot10^6$	≥1				
1.4	$(1.5–3)\cdot10^6$	≥1	$(1.4–1.6)\cdot10^6$	≥1	$(0.9–1.1)\cdot10^6$	≥1		
1.7	$(4–6)\cdot10^6$	≥5	$(4–4.2)\cdot10^6$	≥5	$(2–2.3)\cdot10^6$	≥5	$(1.9–2.2)\cdot10^6$	≥5
2.2	$(4–6)\cdot10^7$	≥5	$(1.3–2)\cdot10^7$	≥5	$(1.1–1.4)\cdot10^7$	≥5		
1.7	$(4–5)\cdot10^5$	0.01			$(0.6–0.9)\cdot10^5$	0.05		
(200 mM NaCl)		(0.005–0.015)				(0.05–0.1)		
2.5		0.003		0.0034				
(600 mM NaCl)		(0.0025–0.0031)		(0.0032–0.0036)				

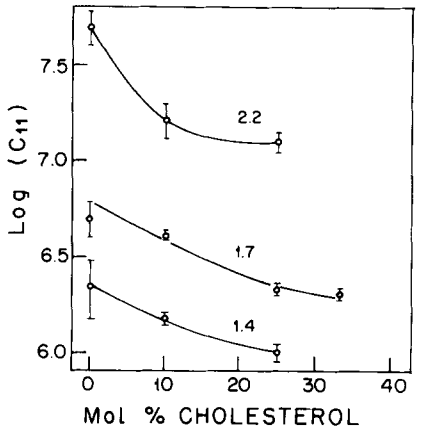

Fig. 3. Effect of cholesterol content and Ca^{2+} concentration on values of rate of aggregation, C_{11}. Ca^{2+} concentrations used were 1.4, 1.7 and 2.2 mM.

pure PS vesicles. This conclusion applies to media containing 100 or 200 mM NaCl. The results indicate that overall fusion rates of PS vesicles are much faster than those of PS/cholesterol vesicles, particularly at lower lipid concentrations, where aggregation is rate-limiting to the overall fusion process, and thus, alluding to the possibility that an increase in cholesterol contents in the vesicles brings about a reduction in aggregation rates. The analysis of fusion studies on large vesicles (Bental, M., Wilschut, J. and Nir, S., unpublished data) has indicated that an increase in cholesterol contents in PS/cholesterol vesicles causes an increase in f_{11} values. We present results of an experiment which demonstrates this conclusion directly. Table II shows fusion results of PS and PS/cholesterol 9 : 1 vesicles in the presence of 600 mM NaCl, i.e., under conditions where the vesicles are capable of aggregating without Ca^{2+}, and hence, the actual fusion rather than aggregation is rate-limiting to the overall fusion process. The results in Table II indicate that, in the presence of 600 mM NaCl, the overall fusion rate of PS/cholesterol vesicles becomes faster than that of PS vesicles. The results in Fig. 4 show that PS/cholesterol vesicles aggregate slower than PS vesicles in large Na^+ concentrations. Hence, it is evident that indeed, the difference in overall fusion rate must reflect the fact that f_{11} values of PS/cholesterol vesicles are larger than those of PS vesicles. The experimental results in Table II are well-simulated by the equation $I(t) = 50 \quad (1 - \exp - f_{11}t)$, which was employed by Bentz et al. [28,37] in similar systems where the delay in fusion due to aggregation could be ignored. The values of f_{11} which yield this simulation are 0.003 and 0.0034 s^{-1} in PS and PS/cholesterol 9 : 1 vesicles, respectively. More extensive difference in f_{11} values is obtained from the analysis of the results presented in Fig. 2 (see Table I). This analysis shows that in the presence of 200 mM NaCl and 1.5 mM Ca^{2+}, f_{11} values are 0.05 and 0.01 s^{-1}, in PS/cholesterol 3 : 1, and PS vesicles, respectively. This significant difference in f_{11} values is above the experimental uncertainty. Furthermore, PS/cholesterol 3 : 1 vesicles have radii slightly larger (up to about 8%) than those of PS vesicles. Hence, the larger f_{11} values in PS/cholesterol vesicles must be interpreted to indicate that incorporation of cholesterol into PS (or PS/cholesterol) vesicles enhances their tendency to undergo Ca^{2+}-induced fusion.

TABLE II

FUSION OF PS AND PS/CHOLESTEROL VESICLES INDUCED BY 2.5 mM Ca^{2+} IN THE PRESENCE OF 600 mM NaCl

In experimental results, half of the leakage percentage is included. The experiments were carried out as described in Materials and Methods, in 600 mM NaCl/2 mM Tes/2 mM L-histidine/0.2 mM EDTA (pH 7.4) at 24°C. The leakage experiments were carried out without EDTA. The lipid concentration was 25 μM. The calculations employ the equation $I(t) = 50(1 - \exp - f_{11}t)$ [28,37] and f_{11} values are given in Table I. exp., experimental; calc., calculated.

Time (s)	PS		PS/cholesterol (9 : 1)	
	$I(t)$ exp.	$I(t)$ calc.	$I(t)$ exp.	$I(t)$ calc.
15	1.7	2.2	1.9	2.5
30	3.4	4.3	4	4.8
60	7.5	8.2	8.4	9.2
120	16.1	15.1	17.4	16.8
150	18.9	18.1	20.6	20

The results in Fig. 4 show that PS/cholesterol vesicles aggregate slower in 650 mM NaCl than PS vesicles. These results also show that dispersed PS/cholesterol vesicles give 2-fold larger turbidity values than PS vesicles. Part of this effect may be due to the fact that PS/cholesterol vesicles have a larger refractive index than PS vesicles [38], in addition to a small difference in size.

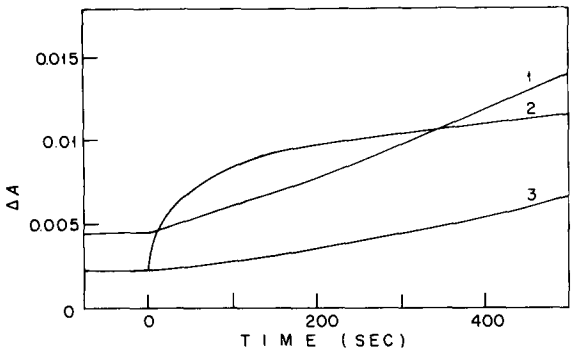

Fig. 4. Effect of cholesterol content and lipid concentration on Na^+-induced vesicle aggregation. Aggregation of vesicles was initiated by introducing concentrated NaCl solution into the vesicle suspension. Final solution concentration was 650 mM NaCl/2 mM Tes/2 mM L-histidine (pH 7.4). Temperature was kept at 24°C and suspension was stirred constantly. Final solution volume was in all cases 3 ml. Aggregation was monitored by measuring changes in absorbance at 550 nm in an Aminco DW-2a spectrophotometer. (1) PS/cholesterol (3:1), 200 μM lipid, 650 mM NaCl; (2) PS, 200 μM lipid, 650 mM NaCl. (3) PS/cholesterol (3:1), 100 μM lipid, 650 mM NaCl.

The results (Figs. 2, 3 and Table I) also show that the effect of Ca^{2+} on an increase in the rate of aggregation. The values of C_{11} increase by at least an order of magnitude as Ca^{2+} concentration increases from 1.1 to 2.2 mM. Results in Fig. 2 and Table II demonstrate that enhanced Na^+ concentrations inhibited the overall fusion reaction. In Table I, we see that an increase in Na^+ concentration from 100 to 200 mM, or 600 mM, results in a respective 50- or 150-fold reduction in f_{11} values of PS vesicles. This reduction in f_{11} values in media containing large Na^+ concentrations has been explained as due to a significant reduction in the binding ratio Ca^{2+}/PS [22,27,28, 37,39] which in turn very sharply affects f_{11} values [28,37].

Discussion

The dramatic increase of C_{11} values with Ca^{2+} concentration has been explained before, as due to Ca^{2+} binding to PS molecules [22,24,39–41]. However, we note that the addition of a neutral component, such as cholesterol, to PS membranes, results in a reduction in their rate of aggregation. This reduction is evident both in Ca^{2+}-induced fusion as well as in Na^+-induced aggregation. It is worth while to mention that the addition of cholesterol to phospholipid membranes is expected to increase somewhat the magnitude of the attractive Van der Waals interaction between vesicles [42]. This effect of cholesterol in retarding cation-induced aggregation of PS or PS/cholesterol vesicles, is significantly less than the effect of another neutral component, such as PC, whose action in inhibiting fusion and aggregation of PS/PC vesicles [22] has been attributed to repulsive hydration forces [43]. It cannot be excluded that cholesterol may contribute to similar short-range repulsive interaction. It was found that at least in DPPC/cholesterol multilamellar structure, cholesterol causes a significant increase in bilayer separation [44]. If we allow for the possibility that a similar effect may also occur in PS/cholesterol membranes, then the somewhat smaller values of C_{11} that arise with an increase in cholesterol content, may be attributed to larger repulsive hydration forces [44].

In contrast to the action of PC, cholesterol enhances significantly the capacity of PS or PS/cholesterol to undergo Ca^{2+}-induced fusion. Here the effect is more dramatic than in larger vesicles (Bental, M., Wilschut, J. and Nir, S. unpublished data) and may be correlated with the asymmetry of packing between inner and outer monolayers in smaller vesicles [45].

Enhancement of fusion capacity of PS vesicles by the addition of cholesterol may be related to the existence of phase separations on a macroscopic scale, or in the form of micro domains, in PS/cholesterol vesicles. As proposed before [4,29], the molecular arrangements are less ordered in domain boundaries, and therefore regions in membranes where such boundaries occur are more susceptible to fusion.

A recent study [46], which employed a fluorescent assay monitoring mixing of membrane components, revealed that the addition of Ca^{2+} (but not Mg^{2+}), resulted in phase separations in PS/cholesterol small unilamellar vesicles (1.5 : 1).

A recent calorimetric study [47] on multilamellar PS/cholesterol has demonstrated increasing degrees of phase separations with an increase in the molar ratio of cholesterol at ratios of 7 : 3 and above. Our study shows some degree of enhancement in fusogenicity at a 9 : 1 ratio, but it cannot be excluded that a certain degree of micro do-

mains exists without being detected by the calorimetric measurements.

Acknowledgements

This work was partially supported by the Shainbrun Funds of the Hebrew University of Jerusalem (S.N.) and by a grant in aid of the Israel Academy of Sciences and Humanities – Basic Research Foundation (P.I.L.). We thank Dr. Jan Wilschut for his guidance in the experimental procedures.

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