Influence of Liposome Bilayer Fluidity on the Transport of Encapsulated Substance into the Skin as Evaluated by EPR

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Purpose. The influence of liposome composition on the bilayer fluidity and on the transport of encapsulated substance into the skin was investigated.

Methods. Multilamellar vesicles (MLV) from dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylcholine (DMPC) with various amounts of cholesterol were prepared by the film method and characterised by photon correlation spectroscopy and electron paramagnetic resonance (EPR) methods. The transport of the hydrophilic spin probe encapsulated in MLV into pig ear skin was investigated by EPR imaging methods. The bilayer domain structure was studied by fitting the lineshape of the experimental EPR spectra with the spectra calculated by the model, which takes into account the heterogeneous structure of the bilayer with several coexisting domains.

Results. Cholesterol strongly influences the entrapped volume of liposomes, the domain structure of the lipid bilayer, and the transport of hydrophilic spin probe into the skin. Transport was not observed for liposomes composed of phospholipid:cholesterol 1:0 or 9:1 (mol:mol), not even above the phase transition temperature from the gel to the liquid crystalline phase of DMPC. A significant delivery of hydrophilic spin probe was observed only if there was 30 or 50 mol% of cholesterol in the liposome bilayer.

Conclusions. It can be concluded that the domain structure of the liposome bilayer is more important for the delivery of encapsulated substance into the skin than the liquid crystalline phase of the pure phospholipids bilayer.

KEY WORDS: liposomes; bilayer fluidity; transport; skin; electron paramagnetic resonance.

INTRODUCTION

Increased delivery of drugs when applied to the skin entrapped in liposomes as compared to the application of the drug in a conventional delivery system is well documented (1–4). However, there is no detailed explanation of the mechanisms by which the liposomes facilitate the transport of the entrapped molecules into the skin (2) and how the lipid composition of liposomes affects this. Some authors believe that defined liposomes can penetrate into the skin (5).

The aim of this study was to investigate the connection between liposome bilayer fluidity and the transport of liposome-entrapped substance into the skin. Bilayer fluidity, which could be described as the reciprocal value of the bilayer's average microviscosity, reflects the ordering and dynamics of phospholipid alkyl chains in the bilayer and is mainly dependent on its composition. When the liposome bilayer is heterogeneous, composed of different phospholipids, cholesterol and/or other additives, in the liposome bilayer regions of the different ordering and dynamics of phospholipid chains are formed. These define the bilayer domain structure. Previous studies in this laboratory performed on liposomes with a heterogeneous phospholipid population indicate that bilayer fluidity and cholesterol content might be of crucial importance for the effective delivery of liposome-entrapped substances into the skin (6). For a better understanding of these results in the present study, the results of the investigation performed on a well-defined liposome system composed of only one sort of phospholipid are reported. The influence of added cholesterol and temperature on bilayer fluidity and on the transport of an entrapped substance into the skin was studied using electron paramagnetic resonance (EPR) methods.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine /DPPC/, dimyristoylphosphatidylcholine /DMPC/ and cholesterol /Ch/ are supplied by Sigma, USA. 0.1mol/l sodium ascorbate /Asc/ (Plivit C ampoules produced by Pliva, Croatia), spin probes N-(1-oxyl-2,2,6,6-tetramethyl-piperidinyl)-N-dimethyl-N-hydroxyethyl ammonium iodide /ASL/ (Fig. 1) and N-oxyl-2-undecyl-2-(3'methoxycarbonyl)-propyl-4,4-dimethyloxaolidine /MeF-ASL (10,3)/ (Fig. 2) (synthesized by Prof. S. Pečar, Faculty of Pharmacy, Ljubljana, Slovenia) were used.

Liposome Preparation

Multilamellar vesicles (MLV) were prepared from DPPC or DMPC with various amounts of Ch (mol:mol) by the film method. The final lipid concentration in liposome dispersions was 48 mg/ml. 0.01mol/l water solution of ASL, which due to its charge does not penetrate the bilayer easily, was incorporated into the liposomes during hydration. The hydration was performed above the phase transition temperature (Tc) of phospholipids. Tc for pure DPPC is 41°C (7) and for pure DMPC 23.8°C (8). In some experiments MLV were extruded through a LiposoFast extruder (manufactured by Avestin, Canada) using polycarbonate membranes with a pore diameter of 800 and 400 nm. Non-entrapped ASL was removed by dialysis at 4°C for 24 hours. The exceptions were preparations from pure DMPC or DMPC:Ch 9:1 (mol:mol) that were dialysed at 30°C, i.e. above Tc, and were kept at this temperature until the measurements were completed in order to prevent the release of ASL from liposomes by-passing the phase transition.

Liposome Characterisation

The size of the liposomes was characterised by photon correlation spectroscopy on a Zetasizer 3000 (Malvern Instruments, UK).

The entrapped volume (V_i - average internal aqueous space of liposomes per 100 ml liposome dispersion) was determined by EPR using Asc as a reducing agent which does not penetrate

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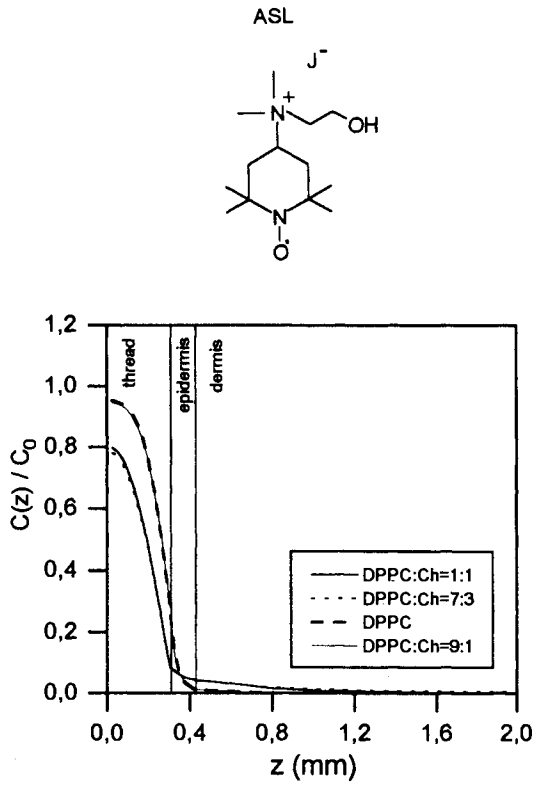

Fig. 1. Influence of cholesterol on the concentration distribution profiles of ASL in skin applied entrapped in DPPC:Ch liposomes. Distribution profiles were calculated using the parameters in Table II. C_0 is the initial concentration of ASL in liposome dispersion.

the bilayer of intact liposomes and therefore reduces only the spin probe in the external aqueous media (6).

Transport Measurements

A thread soaked with liposome dispersion was placed in contact with the stratum corneum of a fresh pig ear skin slice (1×2.5×10 mm) and put into the tissue cell for EPR measurements. The transport of ASL into the skin was measured simultaneously by 1D-EPRI and EPR kinetic imaging method on a Varian E-9, X-band EPR spectrometer.

For 1D-EPRI, the magnetic field gradient 0.25 T/m was applied in a direction perpendicular to the surface of the skin and parallel to the direction of the magnetic field. From the 1D-EPRI spectra, which reflect the concentration profile of the spin probe diffusing into the skin, asymmetry parameter l can be defined as described elsewhere (9). Its alternation with time after the application of liposomes onto the skin was measured ($\Delta l = l_{25\ min} - l_{5\ min}$) as a rough estimate of the formulation transport characteristics. In this way, it is possible to follow continuously the evolution of concentration profiles in the skin of the total amount of the spin probe (entrapped in liposomes and released from liposomes). Only delivery into the skin layers more than 100 μm deep can be observed.

The rate of reduction of spin probe molecules by oxy-redoxy systems in the skin, which had been released after the destruction of liposomes was measured by the EPR kinetic imaging method. The rate of reduction was measured from the EPR spectra intensity decreased with time for the whole sample

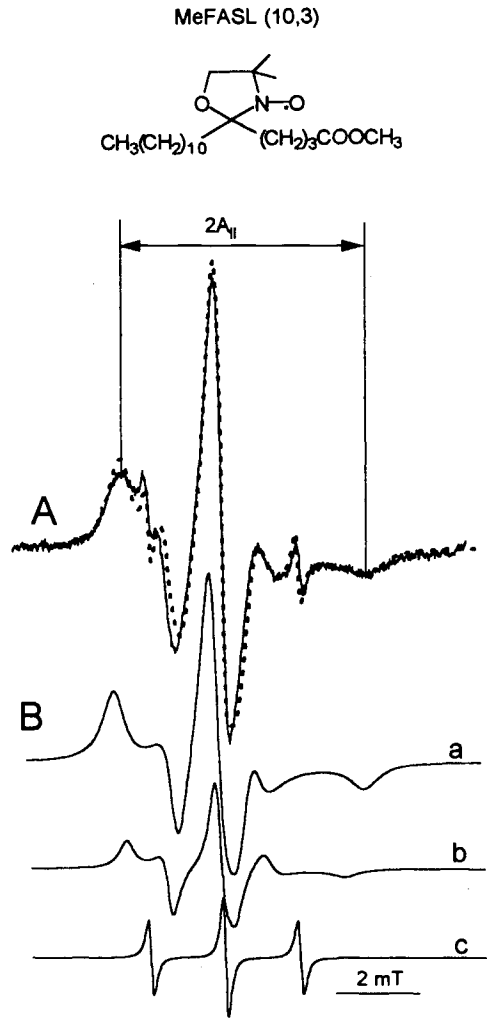

Fig. 2. Typical EPR spectra of MeFASL(10,3) in the bilayer of DPPC:Ch (9:1 mol:mol) liposomes at 20°C. A. Full line: experimental spectra, dotted line: the best fit to the experimental spectra, taking into account the superimposition of spectra of three coexisting domains shown in Fig. 1B. B. Computer simulation of specific EPR spectra of three coexisting domains: a; domain a (S=0.75, τ = 0.6 ns, W=0.67), b; domain b (S=0.48, τ=1 ns, W=0.30) and c; domain c (S=0.01, τ=0.05 ns, W=0.02).

Table I. Characterisation of DPPC:Ch (5:5 mol:mol) liposomes, before (MLV) and after extrusion through polycarbonate membranes with pore diameter 800 nm and 400 nm

Liposome type	d(nm)[a]	P.I[b]	V_i(%)[c]	Δl[d]
MLV	1183 ± 125	—	8.0 ± 1.0	0.11 ± 0.02
800 nm	664 ± 13	0.71 ± 0.25	8.6 ± 1.4	0.10 ± 0.01
400 nm	355 ± 9	0.25 ± 0.06	7.5 ± 2.0	0.07 ± 0.03

[a] Average diameter.
[b] Polidispersity index.
[c] Entrapped volume.
[d] Transport parameter.

Table II. The influence of cholesterol on entrapped volume (V_i), transport parameter (Δl), and on the parameters used in calculating the kinetics of the EPR spectra amplitudes decrease in the DPPC liposomes: k_2=rate of liposome disintegration, D=diffusion coefficient

	Preparation/ compartment	ASL in water	ASL in DPPC MLV	ASL in MLV DPPC:Ch=9:1 (mol:mol)	ASL in MLV DPPC:Ch=7:3 (mol:mol)	ASLin MLV DPPC:Ch=1:1 (mol:mol)
V_i (%)	—	—	0.5 ± 0.1	0.6 ± 0.2	5.2 ± 2.0	8.0 ± 1.0
Δl	—	0.00	0.00	0.00	0.08 ± 0.02	0.11 ± 0.02
	Thread	—	0.0	0.0	0.0	0.0
$k_2(10^{-3}s^{-1})$	Epidermis	—	3.8 ± 1.5	3.0 ± 1.5	7.2 ± 0.8	7.2 ± 0.8
	Dermis	—	<0.1[a]	<0.1[a]	<0.1[a]	<0.1[a]
	Thread	10.0 ± 3.0	0.6 ± 0.2	0.6 ± 0.2	1.6 ± 0.2	1.7 ± 0.2
$D(10^{-2}cm^2s^{-1})$	Epidermis	0.3 ± 0.06	0.8 ± 0.2	0.8 ± 0.2	13.0 ± 4.0	13.0 ± 4.0
	Dermis	<1[a]	<1[a]	<1[a]	12.0 ± 4.0	14.0 ± 4.0

Note: In the calculations a three-compartment model was used: thread (300 μm), epidermis (125 μm) and dermis (to 2.5 mm). The rate constant of ASL reduction $k_1 = 1l\ mol^{-1} \times s^{-1}$ is taken to be the same in the epidermis and in the dermis, while the concentration of the reducing agent in the epidermis is $3 \times 10^{-2}\ mol\ l^{-1}$ and in the dermis $9\times10^{-4}\ mol\ l^{-1}$, as calculated from the best correlation with the reduction kinetic curve for an ASL solution.

[a] Calculated curves are not sensitive to the variation of the parameter.

(thread and skin). The reduction kinetics and evolution of concentration profiles, were calculated separately for liposome-entrapped and released spin probe by the model in which the Fick equation was used to describe the transport of ASL into the skin (10). Different diffusion coefficient and liposome decay rates were taken into account in the thread, epidermis, and dermis. The lineshape of 1D-EPRI spectra was calculated from the concentration profiles ($C(z_i,t)$) calculated from the reduction kinetic imaging experiment. The calculated spectra should fit the spectra measured by 1D-EPRI. Only the combination of two independent experiments measured simultaneously on the same sample provided relevant information on the transport characteristics of liposome-entrapped and released substances.

The transport measurements of DPPC:Ch liposomes were performed at room temperature, while the measurements of DMPC:Ch liposomes were performed at 15°C and 30°C.

Bilayer Fluidity Measurements

Liposome bilayer fluidity was measured on Bruker ESP 300 EPR spectrometer using the lipophilic spin probe MeFASL(10,3) as a marker that reflects the ordering and dynamics of phospholipid alkyl chains in its surroundings. The desired amount of water dispersion of unlabelled liposomes was put into a glass tube with MeFASL(10,3), uniformly distributed on the wall in the molecular ratio phospholipid:spin probe = 500:1, and incubated for 20

Table III. The influence of cholesterol on entrapped volume (V_i), transport parameter (Δl), and on the parameters used in calculating the kinetics of the EPR spectra amplitudes decrease in the DMPC liposomes at 15°C (A) and 30°C (B)

		Preparation/ compartment	ASL in water	ASL in DMPC MLV	ASL in MLV DMPC:Ch=9:1 (mol:mol)	ASL in MLV DMPC:Ch=7:3 (mol:mol)	ASL in MLV DMPC:Ch=1:1 (mol:mol)
	V_i (%)	—	—	1.1 ± 0.2	5.0 ± 0.3	9.2 ± 0.7	16.0 ± 2.0
	Δl	—	0.00	0.00	0.00	0.04 ± 0.02	0.08 ± 0.02
A		Thread	—	0.0	0.0	0.0	0.0
	$k_2(10^{-3}\ s^{-1})$	Epidermis	—	14.0 ± 4.0	11.0 ± 3.0	10.0 ± 1.0	10.0 ± 1.0
		Dermis	—	<0.1[a]	< 0.1[a]	< 0.1[a]	< 0.1[a]
		Thread	10.0 ± 3.0	1.5 ± 0.2	1.4 ± 0.2	1.6 ± 0.2	1.3 ± 0.2
	$D(10^{-7}\ cm^2\ s^{-1})$	Epidermis	0.3 ± 0.05	0.7 ± 0.2	0.8 ± 0.2	10.0 ± 4.0	11.0 ± 4.0
		Dermis	<1[a]	<1[a]	<1[a]	3.5 ± 1.0	5.1 ± 1.0
	V_i(%)	—	—	1.1 ± 0.2	5.0 ± 0.3	9.2 ± 0.7	16.0 ± 2.0
	Δl	—	0.00	0.01 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	0.12 ± 0.03
B		Thread	—	0.0	0.0	0.0	0.0
	$k_2(10^{-3}\ s^{-1})$	Epidermis	—	11.0 ± 4.0	10.0 ± 4.0	12.0 ± 1.0	11.0 ± 1.0
		Dermis	—	<0.1[a]	<0.1[a]	<0.1[a]	<0.1[a]
		Thread	10.0 ± 3.0	1.6 ± 0.3	1.4 ± 0.3	1.6 ± 0.2	1.6 ± 0.2
	$D(10^{-7}\ cm^2\ s^{-1})$	Epidermis	0.3 ± 0.06	0.9 ± 0.2	1.1 ± 0.2	15.0 ± 4.0	16.0 ± 4.0
		Dermis	<1[a]	<1[a]	<1[a]	5.3 ± 0.6	12.0 ± 4.0

[a] Calculation data and abbreviations are shown in Table II.

min. by continuous stirring above Tc to label the liposome bilayer. Typical parameters by which the phospholipid bilayer dynamics and ordering are characterized are correlation time (τ), which reflects the dynamics of lipid alkyl chains, and order parameter (S) (an average deviation of alkyl chains from the normal to the bilayer surface). The order parameter can vary from 1 for completely ordered to 0 for isotropic systems.

For a rough estimate of relative changes in liposome bilayer fluidity with temperature and Ch concentration, maximum hyperfine splitting $2A_{II}$ was determined from the EPR spectra. It is directly related to the average order parameter of the entire liposome bilayer (11).

However, after the addition of Ch lateral domains, with different fluidity characteristics, are formed in liposome bilayer (7,12). To obtain more precise information about this heterogeneous structure, the lineshape of the experimental EPR spectra should be compared with the spectra calculated by the model, which takes into account the existence of several coexisting domains with different ordering and dynamics of alkyl chains. In the model, an isotropic motion of the lipophilic probe around the long molecular axis and the restricted motion in the direction perpendicular to the bilayer surface was taken into account (13). From the best fit to the experimental spectra order parameter, correlation time, and relative portion of distinct domain (W) in the bilayer, were estimated. In the calculation S, τ inside each domain and W of distinct domains were varied until the best fit of calculated to the experimental spectra were obtained.

Statistics

Data are presented as mean ± standard deviation (AM ± SD). The significance was determined by the Student t-test; levels of 0.05 were taken as indicative of significant differences.

RESULTS

Entrapped Volume

The influence of extrusion on entrapped volume V_i and liposome size is shown in Table I. After extrusion, the liposome size as well as polidispersity index decreases, but V_i does not change significantly. It seems that extrusion influences the lamelarity of liposomes and that the aqueous space between the bilayers does not contribute significantly to V_i. The influence of lipid composition on V_i is presented in Tables II and III. A significant increase is observed after the addition of 30 or 50 mol% of Ch.

Transport Measurements

In Table I, the influence of vesicle size and polidispersity on Δl after the application of liposomes to the skin is shown. It can be seen that in a size region from 1100-300 nm the average diameter of liposomes only slightly influences the transport into the skin, which corresponds with some other already published data (14,15). Therefore, we concentrated our further investigations only on MLV.

In Tables II and III, the transport parameters are shown for DPPC and DMPC liposomes at different concentrations of Ch at selected temperatures. Typical parameters obtained by the EPR kinetic imaging method were the rate constant of ASL reduction (k_1), rate constant for liposome decay (k_2), and diffusion coefficients (D) of free ASL and ASL, which penetrates into the skin protected from reduction (liposome entrapped). They are different in the epidermis and dermis.

Transport parameters $\Delta 1$ and D show that a significant delivery of ASL into the skin occurs only when 30 or 50 mol% of Ch is present in the liposome bilayer. When ASL is applied to the skin entrapped in liposomes of pure phospholipids (DPPC or DMPC), or in a presence of 10 mol % of Ch, only a negligible increase of D in the epidermis and dermis is observed, compared to the aqueous solution of ASL. No transport of ASL into the deeper skin layers is observed when ASL is applied to the skin in the solution, as is evident from Tables II and III, which is not surprising with respect to the positive charge of this molecule. From a comparison of the results below and above Tc (Tables III.A. and III.B,), it is evident that after the transition of liposome bilayer from the solid ordered (gel) to the liquid disordered (liqid crystal) phase[4] only a minor increase in D was observed, while a pronounced increase was observed in both phases when 30 or 50 mol% of Ch is present in the bilayer

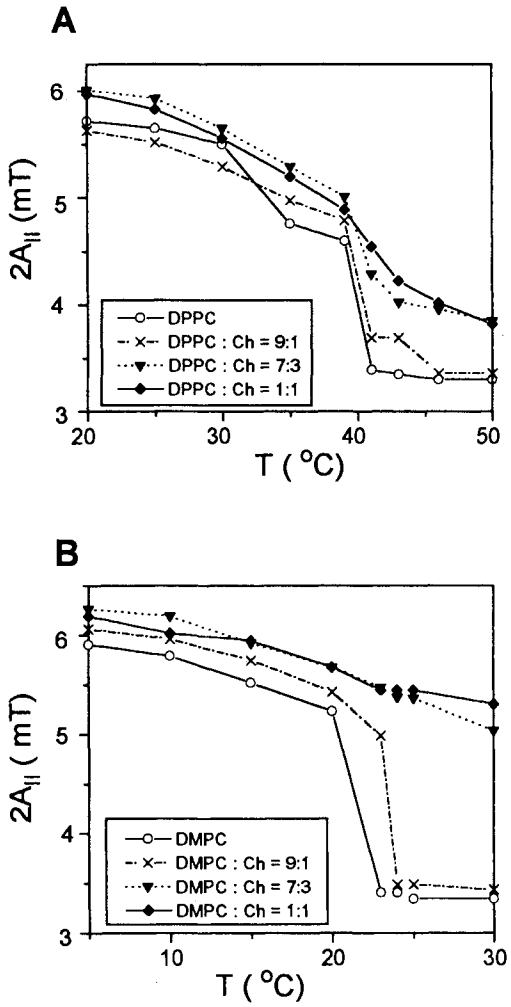

Fig. 3. Temperature dependence of the maximum hyperfine splitting $2A_{II}$ of MeFASL(10,3) in the bilayer of: A. DPPC:Ch liposomes; B. DMPC:Ch liposomes.

[4] The nomenclature of Bloom for the description of different phases of lipids (16) is used.

of DMPC liposomes. The difference in transport characteristics of liposomes with 0 or 10 mol% of Ch with respect to those with 30 or 50 mol% of Ch is well visible from the concentration distribution profiles of ASL ($C(z)/C_0$) in pig ear skin 25 min. after the application of different DPPC:Ch liposomes to the skin (Fig. 1). Similar concentration distribution profiles were also obtained for DMPC liposomes. ASL from liposomes with a higher concentration of Ch can penetrate deeper into the skin. On the other hand, ASL from liposomes with a low Ch remain primarily on the surface of the skin.

Bilayer Fluidity

An EPR spectrum of MeFASL(10,3) in DPPC:Ch liposomes is shown in Fig. 2. The determination of maximum hyperfine splitting $2A_{II}$ is also indicated. Temperature dependence of $2A_{II}$ is presented in Fig. 3. The results obtained by EPR method are in accordance with differential scanning calorimetry studies on phosphatidylcholine:cholesterol systems (7,12) and with the DPPC:Ch phase diagram (16). As shown in Figs. 3A and 3B, for a liposome bilayer composed of pure phospholipids, a drastic decrease of $2A_{II}$ is observed at the temperatures that correspond to the main Tc from the solid ordered to the liquid disordered phase. For pure DPPC liposomes a pretransition temperature at 32°C is also observable, which disappears at 10 mol% of Ch. At a higher Ch content, the main phase transition disappears, as expected with respect to the other phase transition studies of phospholipids:Ch mixtures (7,16). According to the phase diagram (16), above 30 mol% of Ch a fluid phase with orientationally ordered acyl chains is formed (liquid ordered phase).

For some representative EPR spectra the lineshape was calculated by computer simulation of the experimental spectra. As an example, in Fig. 2 the best fit of the calculated spectrum (Fig. 2A, dotted line) to the experimental spectrum (Fig. 2A, full line) of MeFASL(10,3) in the bilayer of DPPC:Ch (9:1) liposomes is shown. The spectrum is a superimposition of the spectra of three types of domain with a different ordering and dynamics of acyl chains (Fig. 2B: a,b,c).

The dependence of order parameter and relative portion of distinct domains in the bilayer of DMPC liposomes on the concentration of Ch at 15°C (below Tc of pure DMPC) are shown in Fig. 4A. One can see that the bilayer composed only of pure DMPC is homogeneous and of one type of domain

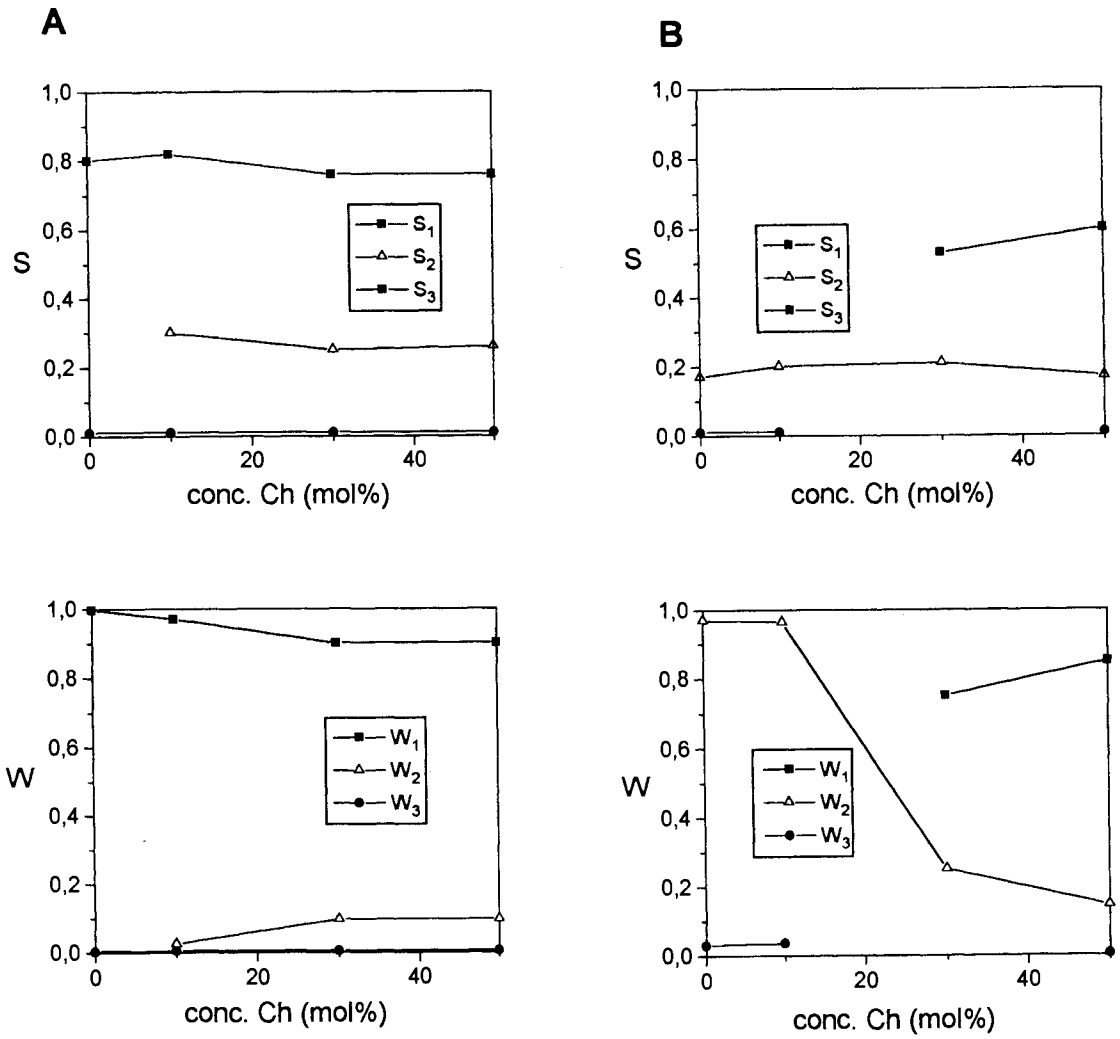

Fig. 4. Influence of cholesterol on the formation and order parameter of coexisting domains in the bilayer of DMPC liposomes: S = order parameter, W = portion of a specific domain in the bilayer: A. at 15°C; B. at 30°C.

with S ≅ 0.8. According to phase diagram (16), it corresponds to the bilayer in the solid ordered phase. The small, almost isotropic domain (S = 0.01, W = 0.02) most probably belongs to MeFASL(10,3) in micelles. After the addition of Ch, the bilayer becomes heterogeneous with two coexisting domains and a new domain appears with S ≅ 0.3. Similar dependence was obtained for DPPC liposomes at 20°C.

Fig. 4B shows the bilayer fluidity characteristics of DMPC:Ch liposomes at 30°C (above Tc of pure DMPC). The liposome bilayer from DMPC or DMPC:Ch (9:1) is homogeneous, with one type of domain (S ≅ 0.17, W ≅ 0.97) which corresponds to the bilayer in the solid disordered phase. At increased Ch content (30 mol%) the portion of this domain decreases and a new type of domain with a higher order parameter (S = 0.53; W = 0.75) appears. According to the phase diagram at this Ch concentration, bilayers are in the liquid ordered phase through the whole invesigated temperature range.

DISCUSSION

Our results show that for the chosen lipid systems (DPPC:Ch and DMPC:Ch) the significant transport of a liposome entrapped substance into the skin was observed only for liposomes with the addition of 30 mol% or more of Ch. The aggregate (solid ordered or liquid disordered) state of phospholipids in the liposome bilayer is less important. It seems that the concentration of Ch in liposomes is of prime importance for the delivery of ASL into the skin. It should be increased to the range where liposomes are in a liquid ordered phase over the whole temperature range measured where the phase transition is abolished (Fig.3).

In view of liposome bilayer fluidity measurements, we assume that the heterogeneous structure of the liposome bilayers with several coexisting domains is of prime importance for the transport. Neither for a highly ordered homogeneous bilayer with S = 0.8 (DPPC or DMPC below Tc), nor for a less ordered bilayer with S ≅ 0.2 (for DMPC above Tc), no significant transport of ASL into deeper skin layers was observed so long as Ch is below 30 mol%. At 30 mol% or more of Ch when the domain structure of both formulations, DPPC and DMPC (at 15°C and at 30°C), becomes very similar, significant transport is observed. In this region, both formulations have two domains with S ≅ 0.8 and 0.2, and the portion of the less ordered domain is 10%. Above the Tc of pure DMPC, order parameter of the more ordered phase decreases to S = 0.6 and the fraction of the less ordered phase increases to W = 0.20, which does not influence significantly the transport characteristics (Table III). We assume that the appearance of the second domain in the right proportion enables better contact between the liposomes and the skin and some budding of the liposome bilayer could occur, which may cause a spilling of the liposome interior into the epidermis. A very similar domain structure was also observed for the mixture of hydrogenated soya lecithin:Ch mixture (7:3), which also revealed enhanced transport into the skin (6).

There are also some other options which could influence our observation, which should be considered. One is the increased entrapped volume of liposomes with a higher Ch content (Tables II and III). However, DMPC:Ch (9:1) liposomes have the same V_i (5%) as DPPC:Ch (7:3) liposomes, but l changes with time ($\Delta 1 > 0$) and D is for an order of magnitude larger in DPPC:Ch (7:3) than in DMPC (9:1) liposomes. Thus, an increased V_i alone could not explain our results. This hypothesis also corresponds with our previous measurements (6,14) where no direct correlation between V_i and $\Delta 1$ was observed but with an appropriate domain structure of liposome bilayer.

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