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Phospholipid vesicles as drug delivery systems. Part II. A study on kinetic fusion between vesicles containing CDP-choline and dipalmitoylphosphatidylcholine vesicles

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Abstract

We have studied the phenomenon of vesicle fusion by differential scanning calorimetric (DSC) and scanning dilatometric (SD) measurements of pure and mixed phospholipids containing CDP-choline. In our study on mixed phospholipid systems, two preparation methods for multilamellar vesicles were used: homogeneous and heterogeneous. The fusion, characteristic of the charged system, is inhibited in the presence of the drug, but occurs when dipalmitoylphosphatidylserine (DPPS) was "diluted" in dipalmitoylphosphatidylcholine (DPPC). In addition, we have evaluated the inclusion efficiency in pure and mixed phospholipids, finding that DPPC/DPPS (3:1 molar ratio) liposomes are the best "host–guest" system, considering the fusion properties and the inclusion efficiency.

INTRODUCTION

Phospholipidic vesicles are known to form good "host–guest" systems, because they can incorporate into their structure different kinds of guest molecules which are either hydrosoluble (in the spaces of the bilayer) or lipophilic (between the hydrophobic chains of the bilayer).

The use of liposomes as "carriers" to enhance incorporation of nucleotides, enzymes, sugars and anti-tumour drugs into cultured cells has already been demonstrated [1–3].

Pharmaceutical compounds loaded with liposomes are incorporated into cells both by endocytotic and non-endocytotic mechanisms which have been proposed to involve fusion of vesicles with the plasma membrane. The

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non-endocytotic pathway predominates in the uptake of negatively charged vesicles.

The convenience of these systems lies in their easy preparation, perfect biocompatibility and absolute absence of toxicity.

For this reason it is necessary to study not only the drug release phenomenon but also the fusion between the vesicles, which is one of the determinant steps in drug absorption, when lipid vesicles containing a hydrophilic drug, such as CDP-choline are used.

Rather than attempt to review the roles of all membrane components, we have focussed our attention on pure or simple mixtures of lipid membrane systems, which mimic most aspects of natural fusion phenomena without compositional complexity.

Many experimental techniques (fluorescence, NMR, light-scattering, DSC) have been used to detect the fusion phenomenon [4–9]. Recently, some of us have designed a new scanning dilatometer (SD) developing a non-perturbing thermodynamic technique capable of detecting isothermal fusion [10] between lipid vesicles by the volumetric properties of the sample under scanning temperature conditions [11].

The aim of this work was to study the possible fusion phenomenon among liposomes containing CDP-choline and target vesicles of dipalmitoylphosphatidylcholine.

EXPERIMENTAL

Liposomes preparation

The neutral liposomes consisted of dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylethanolamine (DPPE) (all purchased from Fluka Chemicals Co., Buchs, Switzerland).

For negative liposomes, dipalmitoylphosphatidic acid (DPPA) (Fluka Chemicals Co., Buchs, Switzerland) or dipalmitoylphosphatidylserine (DPPS) (Sigma Chemical Co., St. Louis, USA), or a mixture of DPPC and DPPS at various molar ratios were used.

The phospholipid purity was judged by two-dimensional thin-layer chromatography [12].

The required amount of phospholipid was weighed into a Quickfit round-bottomed flask and dissolved in a small volume of chloroform. The organic solvent was slowly removed at reduced pressure on a rotary evaporator at 35°C, such that a thin film of dry lipid was deposited on the inner wall of the flask, followed by overnight high-vacuum storage. Vesicles containing the drug were prepared by adding an aqueous solution of CDP-choline (Cyanamid-Italia) in 0.1 M NaCl (analytical grade) to the lyophilized phospholipid film.

This procedure was carried out at a temperature higher than that of the phospholipid gel–liquid-crystal phase transition to allow full hydration of the samples: DPPC and DMPC were heated at 55°C, whereas DPPE, DPPS and DPPA were heated at 75°C. Then the flask was shaken twice on a mechanical agitator for 2 min to produce multilamellar vesicles (MLVs).

For DSC experiments, 120 μl of each sample, containing about 5 mg of lipid, were sealed in an aluminium pan and submitted to analysis. For SD experiments, 600 μl of each sample, containing about 25 mg of lipid, were degassed for 10 min at 40°C with a water-pump and then sealed in a quartz cylinder and analysed. In these experiments, the scanning procedure was started 1 h after the sample preparation in order to obtain a better stabilization of volumes.

In the fusion experiments the samples were prepared in two ways. (a) Heterogeneous mode: pure phospholipid or phospholipid mixture vesicles were obtained separately and then mixed at temperatures lower than the gel–liquid-crystal transition of the two components. (b) Homogeneous mode: pure phospholipid or phospholipid mixture vesicles were dissolved in the same organic solution and then the standard procedure for MLV preparation was followed.

After each experiment, the total inorganic phosphorus was determined by spectrophotometry [13].

Apparatus

Scanning dilatometry

A Mettler TC 10A processor equipped with a TMA 40 thermomechanical analyser, previously calibrated for temperature and length, was used in order to obtain measurements of length as a function of temperature at a constant pressure [14]. The fusion kinetics experiment was carried out using isothermal dilatometry (ID) technique at 70°C.

Differential scanning calorimetry

A Mettler TC 10A processor equipped with a DSC 20 measuring cell was used for calorimetric analysis, after calibration for temperature and energy [14].

Density measurements

The sample densities were calculated to obtain precise values for the starting volumes in the ID experiments. Densities were measured with an Anton Paar "vibrating tube" digital densitometer (DMA 602/60) [14].

Analysis of CDP-choline-loaded liposomes

The separation of the fraction of CDP-choline that was not encapsulated was performed by centrifugation for 30 min at 8000 rpm; the apparatus was

a Beckman J2-21 centrifuge equipped with a Beckman JA-20 fixed-angle rotor.

For determination of encapsulated CDP-choline, 5% sodium dodecyl sulphate (SDS) was added to the vesicles; the sample was then vortexed for 2 min.

After brief heating (5 min, 60°C), the vesicles were dissolved and the CDP-choline content was estimated by HPLC (Hewlett-Packard, Model HP 1090 M) using a variable wavelength UV-detector (D.A.D. HP). The separation was performed by a Bondapak C_{18} (10 μm, i.d. 3.9 mm, length 300 mm, Waters Associates Milford, MA) with a flow-rate of 1.5 ml min^{-1} and a pressure of 27.58 psi. The mobile phase consisted of 0.1% monobasic ammonium phosphate (w/v)/methanol/methylcyanide/10% (w/v) tetrabutylammonium hydroxide (89.11/9.9/0.49/0.49, v/v). The detection was performed at 280 nm.

RESULTS AND DISCUSSION

The study of the fusion phenomenon among vesicles is of immense importance in the understanding of the mechanisms which govern the absorption of this pharmaceutical formulation.

CDP-choline encapsulation yields in neutral and charged phospholipid vesicles are shown in Table 1. The best yield was obtained from DPPA and DPPA–DPPS (1:1 molar ratio). This could be due to the larger aqueous spaces between the bilayers of DPPA and DPPA–DPPS (1:1 molar ratio)

TABLE 1

Molar fraction of drug entrapped per mole of lipid (molar concentration of CDP-choline/molar concentration of phospholipid) $\times 10^2$ and fusion behaviour in the presence of CDP-choline for pure and mixed phospholipid vesicles [a]

Liposome composition	Molar fraction of drug entrapped	Fusion behaviour
DPPC	0.59	No fusion
DMPC	0.58	No fusion
DPPA	2.04	No fusion
DPPS	0.00	No fusion
DPPE	0.00	No fusion
DPPC–DPPS (1:1)	1.99	No fusion
DPPC–DPPS (3:1)	0.98	Fusion
DPPC–DPPA (1:1)	1.20	No fusion
DPPA–DPPS (1:1)	2.32	No fusion

[a] We have investigated all possible combinations of the four pure phospholipids (data not reported in the table).

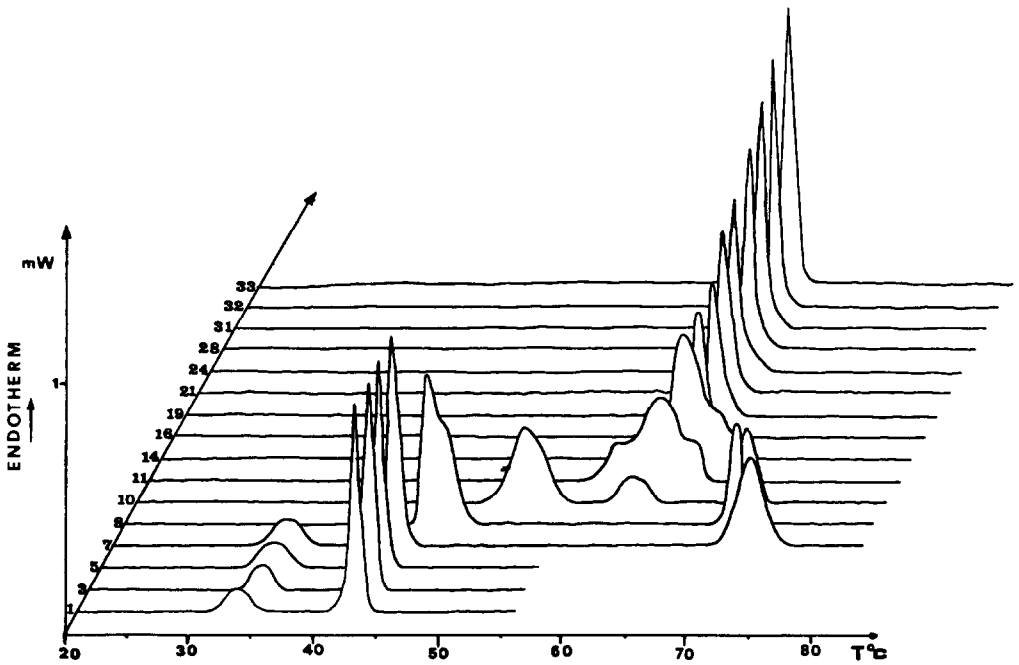

Fig. 1. Differential scanning calorimetric traces in heating mode for DPPC–DPPS (1:1 molar ratio) in 0.1 M NaCl. This sample was prepared in the heterogeneous mode.

liposomes, caused by the inter-bilayer electrostatic repulsions of the phospholipid polar heads.

DPPS vesicles, at the high CDP-choline molar fraction of 0.5 used to prepare liposomes, do not form MLVs: a solid aggregate is produced which shows a broad, low peak in the DSC analysis [14]. A similar observation indicates that the surface area of the DPPS polar head is greately increased by its strong interaction with the drug molecules. For this reason we note the formation of vesicles with a high radius of curvature that, according to the theory of Israelachvili et al. [15], cause a drastic reduction in the aqueous space between the bilayers of the MLVs into which the guest molecule ought to be received. This hypothesis is confirmed by the fusion experiments reported below.

Our group is, at present, carrying out some "in vivo" bioavailability tests using CDP-choline-loaded liposomes. For this purpose, by considering that a reduction of the carbon atoms lowers the phase transition temperature and that the fusion phenomenon occurs only when vesicles exist in their liquid-crystal state (Fig. 1), we have checked the encapsulation ability of liposomes prepared from DMPC which, for every hydrophobic chain, has two methylene groups fewer than the other phospholipids employed.

Table 1 shows that there is no difference in the load capacity and fusion behaviour among phospholipids containing the same polar head with hydrophobic tails of different length.

The amount of drug enclosed in the liposomes was determined as reported under the experimental section.

In the fusion experiments, samples were prepared in two ways, the heterogeneous and homogeneous modes, as reported above.

It was necessary to use the two methods to make mixed vesicles. The heterogeneous method was used to simulate the "in vivo" fusion process using DPPC as the target vesicles, given that the cellular external layer consists essentially of phosphatidylcoline polar head. The homogeneous method was used to study the vesicles interaction [14]. It is known that DPPS gives rise to the fusion phenomenon; thus, this system was prepared to provide an effective "host–guest" system containing CDP-choline in order to improve the absorption of this drug.

Figure 1 shows the change in the gel–liquid-crystal transition temperature in various DSC experiments for the DPPC–DPPS (1:1 molar ratio) system in 0.1 M NaCl, prepared in heterogeneous mode.

The first six scans were run until 55°C was reached, i.e. lower than the gel–liquid-crystal transition point of DPPS MLVs. No variation in the gel–ripple ($L_\beta \rightarrow P_{\beta'}$) at 34°C, and the ripple–liquid-crystal ($P_{\beta'} \rightarrow L_\alpha$) at 42°C, transition peaks was observed in these tests: this indicates the absence of any interaction between DPPC and DPPS vesicles.

The 7th scan was carried out up to 80°C, three peaks being obtained: one at 34°C, showing the $L_\beta \rightarrow P_{\beta'}$ transition of DPPC; one at 42°C, due to the $P_{\beta'} \rightarrow L_\alpha$ transition of DPPC; and one at 65°C, showing the gel–liquid-crystal transition ($L_\beta \rightarrow L_\alpha$) of DPPS. This indicates the absence of any interaction between the DPPS bilayers and DPPC.

The subsequent cycles show the disappearance of the DPPC transition peaks and the increase of two unresolved peaks at 48°C, while the DPPS transition peak shifts to a lower temperature.

In further thermal cycles, the two signals shift toward a single peak centred at 61.5°C.

The DSC scans indicate that the two compounds are mixing together to form a stable phase with a transition at 61.5°C. Moreover, fusion occurs if, and only if, both DPPC and DPPS vesicles are in the liquid–crystal state. The same values were detected when samples were prepared in the homogeneous mode (Fig. 2). However, the evolution of the DSC curves is clearly different for samples prepared in the homogeneous or in the heterogeneous mode. In fact, the first cycle the samples prepared in the homogeneous mode show a broad, low peak at 60.2°C, which becomes a high, sharp peak centred at 61.5°C during the following scans.

Such results lead to the conclusion that the two products follow different mechanisms in their fusion, although reaching the same phase when they attain a thermodynamic equilibrium state.

The analysis of the width at half-height $\Delta T(1/2)$ of the gel–liquid-crystal transition peak confirms this hypothesis: $\Delta T(1/2)$ is 1.00°C in the DSC

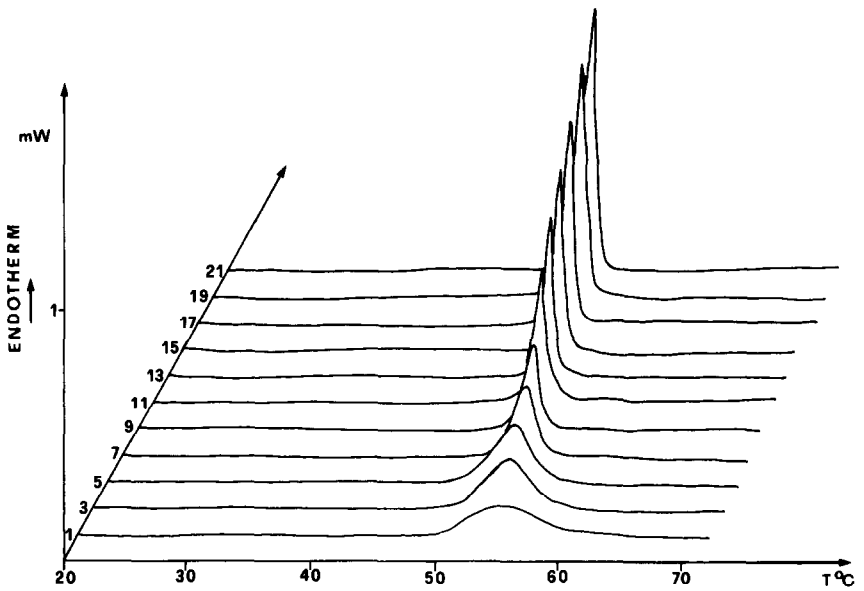

Fig. 2. Differential scanning calorimetric traces in heating mode for DPPC–DPPS (1:1 molar ratio) in 0.1 M NaCl. This sample was prepared in the homogeneous mode.

peak and 2.00°C in the SD peak for samples obtained in both a homogeneous and a heterogeneous way. A similar trend was observed for the DPPC–DPPS (3:1 molar ratio) system.

The fusion kinetics of DPPC and DPPS vesicles with and without CDP-choline have been investigated using ID analysis. By this technique we can follow the kinetics of the vesicle fusion phenomenon without introduction of any foreign molecule into the lipid system such as fluorescent probes [4]. Moreover, ID is very reliable in the quantitative investigation of the fusion kinetics; DSC, however, can only provide qualitative information from the variations in the shape of the transition peak, which sharpens with increasing curvature of the vesicles.

When two vesicles fuse the resulting volume is slightly smaller than the sum of the single volumes, because in small vesicles the packing of the lipid molecules is strained owing to the curvature effect. This strain decreases with increasing radius of curvature, which allows a more efficient packing of the lipid molecules; as a consequence the total volume of the vesicles becomes smaller.

It is implicit that the curvature effect is greater the smaller the radius of curvature of the interacting vesicles. In our experiments, we found that DPPC–DPPS (1:1 molar ratio) samples prepared in the homogeneous or heterogeneous way with CDP-choline, do not give fusion. Similar behaviour can be observed for the mixed systems obtained from all possible combinations of the four phospholipids. The only system containing CDP-choline

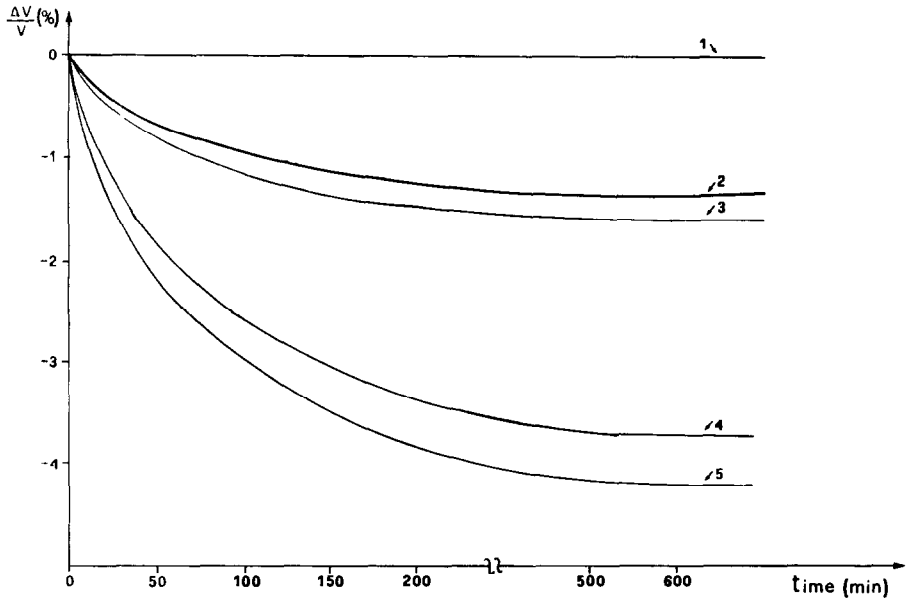

Fig. 3. ΔV value change in isothermal conditions (70°C) for various MLVs with and without CDP-choline. Curve 1, DPPC–DPPS (1:1 molar ratio) MLVs, prepared in heterogeneous mode in 0.1 M NaCl with CDP-choline. Curve 2, DPPC–DPPS (3:1 molar ratio) MLVs, prepared in homogeneous mode in 0.1 M NaCl with CDP-choline. Curve 3, DPPC–DPPS (3:1 molar ratio) MLVs, prepared in homogeneous mode in 0.1 M NaCl without CDP-choline. Curve 4, DPPC–DPPS (3:1 molar ratio) MLVs, prepared in heterogeneous mode in 0.1 M NaCl with CPD-choline. Curve 5, DPPC–DPPS (3:1 molar ratio) MLVs, prepared in heterogeneous mode in 0.1 M NaCl without CDP-choline.

which shows fusion is that prepared with DPPC and DPPS in a molar ratio of 3:1.

Figure 3 shows the isothermal volume change as a function of time related to the fusion of DPPC–DPPS (3:1 molar ratio) vesicles, prepared in both the homogeneous and heterogeneous way, in the presence or absence of the drug.

We found that for the DPPC–DPPS (3:1 molar ratio) system prepared in the heterogeneous way, the volume reduction linked to the fusion phenomenon is less than 4.0% without CDP-choline, whereas it was less than 1.4% for samples prepared in the homogeneous mode without CDP-choline. Such a difference can be explained by considering that, in this latter case, fusion takes place among vesicles of various sizes but with the same composition; whereas in samples obtained heterogeneously, both size and composition are different. In fact, in the liquid-crystal state, DPPS liposomes are less well packed than DPPC [11], owing to the electrostatic repulsions between DPPS charged polar heads; instead, when mixed with DPPC, the repulsive forces of these vesicles are lowered by the "dilution" effect due to zwitterionic DPPC polar heads.

None of the samples containing CDP-choline and prepared in either mode showed fusion, as confirmed by the DSC and SD data. In addition, the DPPC–DPPS (1:1 molar ratio) system did not display the fusion process, because the CDP-choline adsorbed on the DPPS surface drastically reduced the reticular defects required for the fusion to take place. Only the DPPC–DPPS (3:1 molar ratio) system showed this phenomenon; in fact, the DPPC zwitterionic "dilution" effect inhibits the strong reduction of the reticular defects.

It is interesting to note that the time necessary for the fusion of DPPC–DPPS (3:1 molar ratio) vesicles, obtained in the heterogeneous mode, with or without the drug, is almost equal, the only difference being the volume variation. Such apparently anomalous behaviour can be ascribed to the strong interactions between the drug and DPPS, in agreement with the DSC and SD results reported above, when they come into contact inside the vesicles and after their fusion.

Thus, the time required to reach thermodynamic equilibrium is shorter in the absence of the drug that might be adsorbed on the DPPS surface by means of hydrogen bonds [14]. This causes a decrease in the reticular defects necessary for DPPS fusion.

The fusion times have been calculated from a series of graphs (see Fig. 3); in particular, the fusion-end time has been established as the point at which the volume variation becomes indistinguishable from the instrument noise. With increasing CDP-choline concentration, the fusion of the DPPC and DPPS vesicles is inhibited as a consequence of a "saturation" effect.

CONCLUSIONS

For DPPS and DPPC liposomes prepared in the heterogeneous mode, the fusion phenomenon occurs only when both vesicles are in the liquid-crystal state. This behaviour is confirmed by isothermal volumetric data, which show no volume variation at temperatures lower than that of the phase transition. These results are in good agreement with literature data [10,16–18].

From the kinetic data we argue that the system DPPC–DPPS (3:1 molar ratio) is a good CDP-choline delivery device; in fact, it could represent an efficient host–guest system, resulting in the fusion phenomenon. Moreover, at temperatures lower than that of the liquid–crystal state, no decrease in entrapped drug concentration was observed.

The fusion phenomenon was absent in systems where CDP-choline was encapsulated in DPPC, DPPA, or mixtures of the four phospholipids employed.

On the basis of the results discussed above, we hypothesize that mixtures of DPPS do not give the fusion phenomenon in the presence of CDP-

choline, owing to the reduction of the reticular defects due to the strong hydrogen interactions among the DPPS polar heads and the drug.

Therefore, we have chosen a DPPC–DPPS (3 : 1 molar ratio) mixture as a "host–guest" system for "in vivo" experiments; this "dilutes" the DPPS, which is responsible for the fusion phenomenon essential to the drug's release into the tissues. In addition, it should be mentioned that this system can encapsulate a considerable amount of drug.

The DPPC–DPPS (3 : 1 molar ratio) mixture does have a problematic property: a transition temperature higher than that of the human body. This problem can be removed by substituting DMPC for DPPC.

These latter results will be reported shortly.

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