Biochimica et Biophysica Acta 818 (1985) 13–22
Elsevier

BBA 72660

Phospholipid vesicle fusion and drug loading: temperature, solute and cholesterol effects, and, a rapid preparation for solute-loaded vesicles

Daniel S. McConnell and Stephen E. Schullery *

Chemistry Department, Eastern Michigan University, Ypsilanti, MI 48197 (U.S.A.)

(Received January 22nd, 1985)

Key words: Phospholipid vesicle; Membrane fusion; Temperature effect; Cholesterol; Solute concentration; Trapping efficiency

The fusion of sonicated dipalmitoylphosphatidylcholine (DPPC) vesicles was studied by gel-exclusion chromatography as a function of temperature, permeable and impermeable solute concentration, and cholesterol content of the bilayer membrane. Fusion is faster at lower temperatures: there is no fusion at or above 35.5°C (0.10 M DPPC/0.1 M K_2SO_4/0.01 M Hepes buffer (pH 7.4)/0.02% NaN_3). There is about 10% fusion after 1 week at 30°C, and about 60% fusion after 2 days at 13–25°C. Between 13 and 8°C, the fusion product changes from 700-Å-diameter vesicles to the 950-Å vesicles previously reported by Wong et al. (Wong, M., Anthony, F.W., Tillack, T.W. and Thompson, T.E. (1982) Biochemistry 21, 4126–4132). At 1°C, fusion is about 90% complete after 1 day. Membrane-impermeable solutes (NaCl, trehalose and glucose) inhibit fusion in a manner reflecting the total particle concentration. There is no detectable fusion after 3 days (22°C) in either 1.0 M NaCl or 2.0 M sugar, the highest concentrations studied. A suggested explanation is that impermeable solutes osmotically inhibit the influx of solution that accompanies the fusion of vesicles to form a larger vesicle, and, could conceivably thereby inhibit the fusion reaction. By contrast, membrane-permeable solutes (glycerol, ethylene glycol, propylene glycol and ethanol) dramatically increase the fusion rate. 1.0 M ethanol causes 100% fusion in 15–30 min at 22°C. The simultaneous presence of 0.15 M NaCl entirely negates the fusion-promoting effect of 1.0 M ethanol. 1 mol% cholesterol completely inhibits fusion in 0.1 M KCl (20°C), and greatly slows it down either in 1.0 M ethanol at 20°C or in 0.1 M KCl at 4°C. A suggested mechanism is that cholesterol might concentrate in and stabilize bilayer lattice defect sites that are critical for the fusion reaction. The trapping efficiency of vesicles formed by the fast ethanol fusion conditions in the presence of the water-soluble markers, chromate and arsenazo III, ranged from 9.0 to 12.7% of the marker captured in the vesicles, corresponding to trapped volumes of 1.8 to 2.5 l/mol DPPC. Bromophenol blue gave anomalously high values of 67% and 13 l/mol DPPC, which presumably reflect binding, in addition to encapsulation.

* To whom correspondence should be addressed.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FUV, fusion vesicle; SUV, small unilamellar vesicle; MLV, multilamellar vesicle; Pipes, 1,4-piperazinediethanesulfonic acid.

Introduction

Small unilamellar vesicles (SUVs), formed by sonication of saturated phosphatidylcholines, spontaneously fuse when incubated below their main liquid-crystal phase-transition temperature,

T_m [1–5]. These fusion vesicles (FUVs) have several advantages as a model membrane system. Because the vesicles contain only a single purified lipid, and no fusogens are involved, the SUV/FUV system appears to be a very elementary example of bilayer membrane fusion. Furthermore, the dipalmitoylphosphatidylcholine (DPPC) FUVs originally reported by Schullery et al. [2] are unilamellar and remarkably homogeneous and stable; FUVs prepared at room temperature have a 700 Å diameter with a standard deviation of only 105 Å, and have been kept at room temperature with no evidence of change for at least 2 years (Felgner, P., personal communication). The purity, stability and homogeneity of the FUVs suggest their potential value as drug-delivery vesicles. For example, the sharp 41.5°C phase transition of DPPC FUVs should permit a form of targeted drug delivery by using local hyperthermia to cause circulating FUVs to release their contents while passing through the target tissue [6]. Unilamellar vesicles of comparable size prepared by other methods have a broader phase transition, presumably due to impurities introduced by the other procedures [7].

The properties of FUVs depend somewhat on the lipid and the fusion conditions. The DPPC FUVs produced at room temperature [2] are of 700 Å diameter, homogeneous, stable and free of detectable contamination by multilamellar (MLV) or oligolamellar forms. By contrast, Wong et al. [8] observed that DPPC SUVs produce a bimodal FUV population when fusion is carried out at 4°C – complete fusion to 700-Å FUVs in 7 days was followed by further fusion to 950-Å FUVs in 3–4 weeks. Unlike the 700-Å FUVs, the 950-Å FUVs aggregated and settled-out upon standing. FUVs produced by distearoylphosphatidylcholine (DSPC) [1] or by dimyristoylphosphatidylcholine (DMPC) [7] are also heterogeneous, with DSPC FUVs exhibiting a bimodal distribution of 600-Å and 1000-Å-diameter vesicles.

In order to determine the optimum conditions for formation and drug-loading of FUVs, and to help clarify the mechanism of vesicle fusion, we have carried out the following studies on the fusion of DPPC SUVs: fusion was studied at eight temperatures, from 1 to 35.5°C. The effects of both permeable and impermeable solute concentration and the effect of incorporation of cholesterol into the SUV membrane were determined. The trapping efficiency and trapped volume for incorporation of three water-soluble markers into FUVs under the optimum fusion conditions was measured. Vesicle fusion was monitored by gel-exclusion chromatography, and the vesicle size of some of the preparations was checked by laser light scattering.

We were surprised at how sensitive the fusion process was to all three of the parameters investigated. Contrary to our expectation, fusion does not readily occur until DPPC SUVs are about 10°C below T_m, and the dependence of the FUV size distribution on temperature differed somewhat from that reported by Wong et al. [8]. Impermeable solutes, whether ionic or nonionic, inhibit fusion, whereas permeable solutes dramatically increase the fusion rate. Cholesterol at only 1 mol% (relative to total lipid) blocks the fusion.

Our results are discussed in terms of the aggregation/fusion model presented by Wong and Thompson [9]. In addition, we suggest that ease of osmotic flow into the new FUVs immediately following fusion is an important determinant of the fusion rate, and that cholesterol inhibits fusion by preferentially locating in the lattice-defect regions of the gel-state vesicles.

Experimental section

DPPC, lots C160-45 and C160-70, supplied as a dry powder, was purchased from Avanti Polar Lipids, Birmingham, AL. Cholesterol from Eastman Organic Chemicals (Rochester, NY) was purified by recrystallization three times from ethanol; a second sample of cholesterol, lot CH-11, was purchased from Avanti. Other chemicals used were of highest commercially available purity.

SUVs were prepared at a concentration of 0.10 M by adding 0.615 ml aqueous buffer to 0.050 g dry DPPC, followed by sonication at 50°C using a MSE, Ltd. 100-W sonicator with 1 cm tip-diameter probe. The sample tube was sealed to the probe by wrapping with Teflon tape. The dispersion was sonicated to very near optical clarity using about ten alternate 30-s on/off bursts. No residual MLVs were detectable by gel-exclusion chromatography. All preparations were buffered with 0.01 M Hepes

at pH 7.4, and preserved with 0.02% NaN_3. Samples containing mixtures of cholesterol and DPPC were codissolved in a minimum of chloroform, then vacuum-dried at 50°C overnight, followed by sonication as described above.

Gel-exclusion chromatography was done using jacketed, upward-flow, Sepharose CL-2B columns (Pharmacia) with flow adapters. Flow adapters with polyethylene netting (Bio-Rad) gave better lipid recovery than those with nylon netting (Reynolds, J., personal communication). The temperature dependence studies used a column of 1 cm diameter × 37.3 cm length. The solute and cholesterol studies used a 1 cm × 26.2 cm column. A few samples were also run on a 1 cm × 39.5 cm column of Bio-Gel A-150M (Bio-Rad), which has a larger exclusion limit than Sepharose Cl-2B.

The sample was incubated in a 2-ml glass syringe connected by polyethylene tubing to a Pharmacia LV-3 valve and then to the lower flow adapter of the column; the entire assembly including the flow adapter was immersed in a thermostatted water-bath. Typically, a 1–2-drop aliquot (0.04–0.08 ml) of the sample was loaded onto the column by observing the simultaneous release of 1–2 drops of effluent. Samples were generally eluted with the same buffer as they were prepared in, and care was taken to maintain isoosmotic conditions across the vesicle membranes. The buffer was degassed by storage at 45°C. Column effluent was continuously monitored at 260 nm using a flow cell in a Hitachi model 100-30 spectrophotometer connected to strip-chart recorder. 1-ml fractions were collected by a LKB model 2112 RediRac fraction collector, and were analyzed for phosphorus by the method of McClare [10].

Elution profiles were constructed by drawing smooth curves through plots of the phosphorus in 1-ml fractions. All curves were normalized to a constant area by adjusting the total phosphorus to a constant value.

FUVs were loaded with water-soluble markers (as model drug compounds) by including the marker along with 1.0 M ethanol in the standard Hepes/NaN_3 buffer solution, before sonication and fusion. Successful fusion in the presence of the marker was verified by running an aliquot on the Sepharose column.

Trapping efficiencies of water-soluble markers were determined using the method of Fry et al. [11] to separate loaded vesicles from external marker solute. Sephadex G-50 (Pharmacia) columns were prepared in 6-ml Monoject plastic syringes using porous polyethylene disc (No. 13650, Bel-Art Products, Pequannock, NJ) bed supports. 0.5 ml of FUVs loaded with marker were diluted to 1.0 ml, loaded on a precentrifuged column, allowed to equilibrate for 10 min, then very gently centrifuged (setting 5 in International model K) for 10 min, followed by a brief faster centrifugation (setting 10) for 30 s. Trapped marker was determined spectrophotometrically after addition of 25% Triton X-100 detergent in 1.0 M Tris (base form) to disrupt the vesicles and control pH. Control studies performed with each marker permitted correction for the small amount (1–4%) of free marker that eluted with the vesicles. Greater than 90% of the lipid was recovered in the 1-ml centrifugate. The marker could be quantitatively recovered by subsequent washing of the column.

Light-scattering measurements were made using a Langley Ford LSA-2 instrument with a model 1096 correlator which analyzed the data by Keppel's method of cumulants [12]. For this purpose, the original 0.10 M DPPC samples were diluted by adding 10 μl of vesicles to 2.0 ml of matching buffer.

Results

Temperature dependence

Elution profiles for DPPC SUV/FUV incubated at various temperatures between 1 and 30°C are shown in Fig 1A–E. All curves are normalized to the same area. The elution volumes of the 700-Å FUVs and the SUVs were 21 and 29 ml, respectively. The free volume (accessible to the smallest solutes) was 31.5 ml, and the void volume, where the 950-Å FUVs appear, was 11 ml.

The elution profile of SUVs incubated at 35.5°C (not shown) remained unchanged for 1 week, indicating that no fusion occurs at that temperature.

Fig. 1A shows that fusion is very slow at 30°C; essentially no fusion was seen after 1 day, and only about 10% fusion after 7 days.

The elution profiles for fusion at 25°C are shown in Fig. 1B, and are essentially the same as those observed at 22 and 13°C (not shown). In this

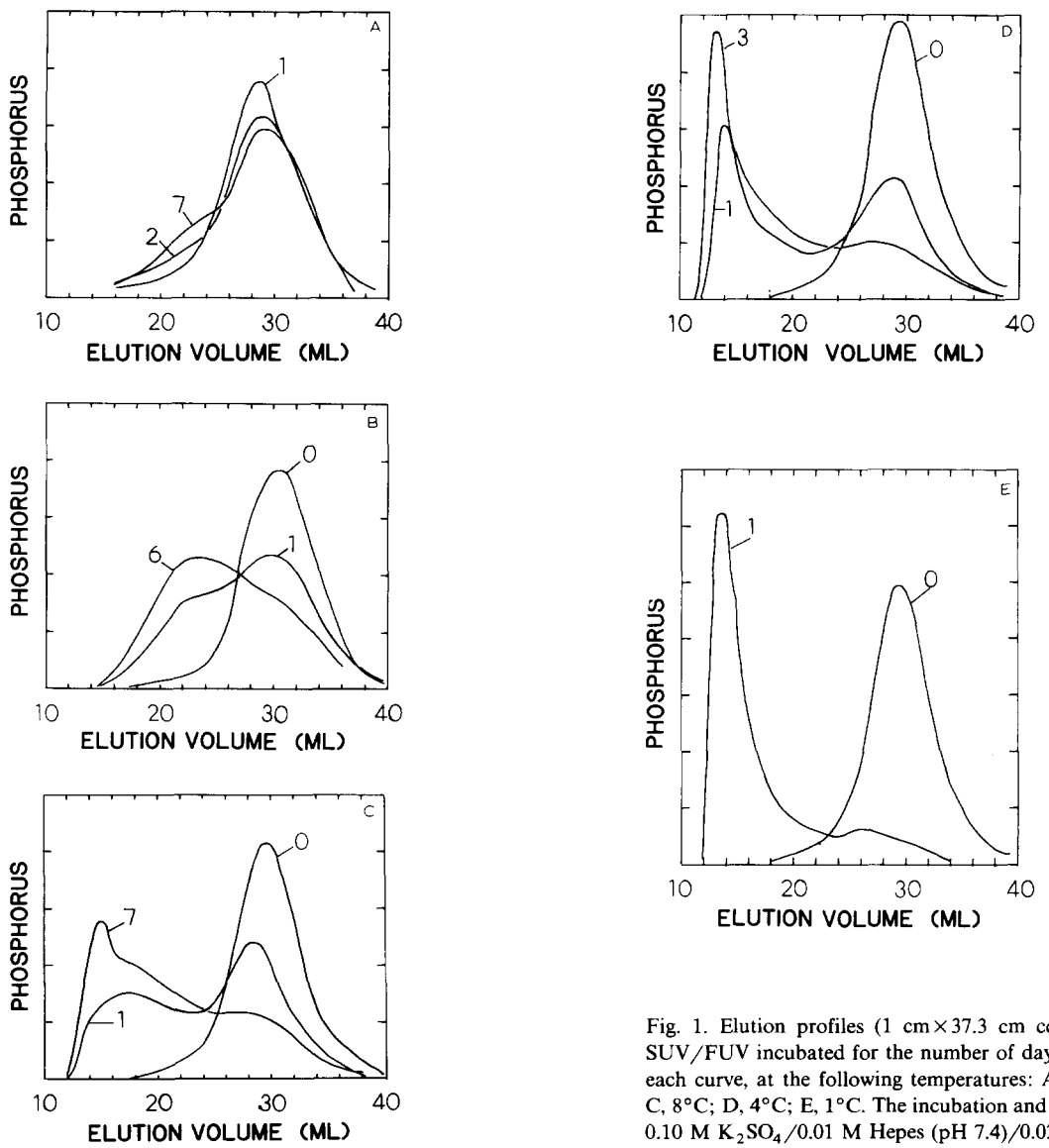

Fig. 1. Elution profiles (1 cm × 37.3 cm column) for DPPC SUV/FUV incubated for the number of days indicated beside each curve, at the following temperatures: A, 30°C; B, 25°C; C, 8°C; D, 4°C; E, 1°C. The incubation and elution buffer was 0.10 M K_2SO_4/0.01 M Hepes (pH 7.4)/0.02% NaN_3.

temperature range, as previously reported at 21°C [2], about 2/3 of the lipid appears in FUVs after 2 days, and fusion of the remaining SUVs is very slow. Normalization of the curves to a constant area results in a well-defined isobestic point, indicating that there are only two species, the SUVs and the 700-Å FUVs, without vesicles of intermediate size.

At temperatures below 13°C, a third and larger vesicle which elutes near the void volume of Sepharose CL-2B is produced. Fig. 1C, D and E, corresponding to 8, 4 and 1°C incubations, show that these larger FUVs form more rapidly at lower temperatures.

Wong et al. [8] showed that the larger vesicles produced at 4°C are homogeneous, unilamellar, stable, osmotically active vesicles of 950 Å diameter. They postulated that the 950-Å vesicles are formed by fusion of two 700-Å FUVs. Evidence of the 700-Å FUV intermediate can be seen in the elution profiles for 8°C fusion (Fig. 1C), but the 1 and 4°C results (Fig. 1D and E) are ambiguous in

this respect. However, a low level of intermediate size vesicles could easily be lost in the tails of the SUV and 950-Å FUV peaks.

Because Wong et al. [8] reported nearly complete conversion of 4°C SUVs to 700-Å FUVs before the 950-Å FUVs formed, in disagreement with our findings, we investigated the 4°C fusion case in more detail. They used a different buffer system, at the same pH but of lower ionic and osmotic strength: 20 mM Pipes/0.010 M NaCl/0.02% NaN_3 (pH 7.4). Therefore, we carried out the 4°C fusion in a very low ionic strength buffer: 0.0050 M Hepes/0.02% NaN_3 (pH 7.4). Also, aliquots were chromatographed after shorter incubation times, in an effort to detect the intermediate form. Fig. 2 shows that under these low ionic strength conditions, the 700 Å form is clearly seen. However, in contrast to the results of Wong et al. [8], the larger 950-Å FUV has appeared after only 5 h, when approximately half of the lipid is still in the SUV form. Elution profiles for 4°C fusion in 0.15 M KCl buffer (not shown) are essentially the same as in our original 0.1 M K_2SO_4 buffer, thereby eliminating the possibility of a specific sulfate ion effect.

A composite of 1-day-fusion elution profiles for various incubation temperatures is shown in Fig. 3. The increase in fusion rate at lower temperatures, and the shift to the larger size FUVs are clearly seen.

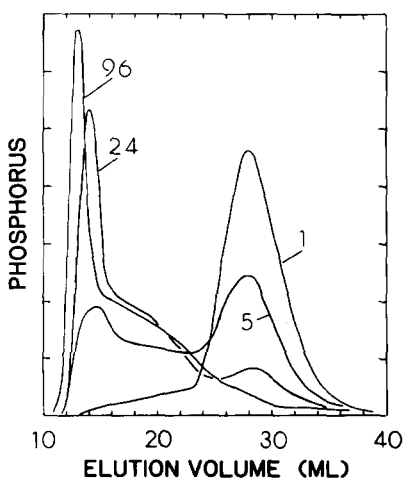

Fig. 2. Elution profiles (1 cm×37.3 cm column) for DPPC SUV/FUV incubated at 4°C for the number of hours indicated beside each curve, in 0.005 M Hepes (pH 7.4)/0.02% NaN_3.

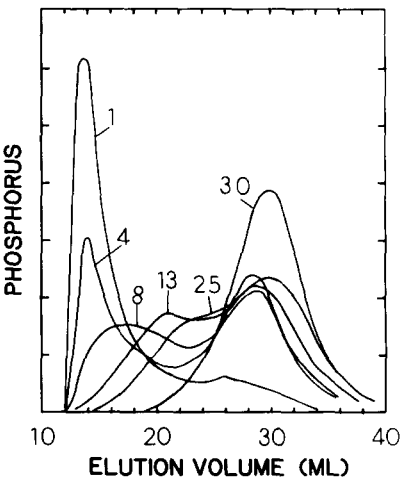

Fig. 3. Elution profiles (1 cm×37.3 cm column) for DPPC SUV/FUV incubated for 1 day at the temperatures indicated beside each curve. The incubation and elution buffer was 0.10 M K_2SO_4/0.01 M Hepes (pH 7.4)/0.02% NaN_3.

Light scattering

An attempt was made, using quasi-elastic light scattering, to verify the size distribution and stability of some of the FUV preparations used in the temperature study. In as much as the light-scattering method is tricked by small amounts of large particles or aggregates [9], values obtained must be interpreted as upper limits on the average vesicle size. Our results are presented in Table I. The incubation time at initial fusion temperature, and subsequent storage time at room temperature are given for five different fusion temperatures. The average diameter, $\bar{d}$, was measured first at 25°C, then after a 5–10 min equilibration at 45°C.

A large decrease in $\bar{d}$ upon heating to 45°C was seen for the 1°C and 4°C FUVs, indicating extensive aggregation of these vesicles. Furthermore, consecutive measurements at 45°C showed a continuing slow disaggregation in most of the samples. The $\bar{d}$ was about 850 Å for 13°C and 21°C FUVs, and about 1100 Å for the 8°C FUVs. The discrepancy between these values and the expected $\bar{d}$ of 700 and 950 Å presumably corresponds to the large-particle bias of the light-scattering method [9]. In contrast to the 1°C and 4°C samples, the relatively small change upon heating the 8°C, 13°C

TABLE I

AVERAGE DIAMETER $\bar{d}$ OF FUVs MEASURED BY QUASI-ELASTIC LIGHT SCATTERING

Salt concentration, incubation time and temperature, subsequent storage time and temperature, and sample temperature during light-scattering measurement are indicated.

Sample	$\bar{d}$ (Å)	
	25°C	45°C
1°C for 6 days; 2 months at room temp.; 0.1 M K_2SO_4	18000	7600
4°C for 5 days; 5 weeks at room temp.; 0.15 M KCl	5300	2100
8°C for 8 days; 2.5 months at room temp.; 0.1 M K_2SO_4	1200	1100
13°C for 6 days; 7 weeks at room temp.; 0.1 M K_2SO_4	870	850
21°C for 2 weeks; 0.1 M NaCl	860	–

and 21°C samples to 45°C indicates little aggregation, at least after the 200-fold dilution for sample preparation.

Effect of impermeable solute concentration

The effects of glucose and trehalose concentration up to 2.0 M and of NaCl up to 1.0 M were studied. A composite of 3-day-fusion (22°C) elution profiles for various NaCl concentrations is shown in Fig. 4. Increasing concentrations progressively inhibit fusion, up to 1.0 M NaCl, which completely inhibited fusion on this time scale. Comparable osmolarities of glucose and trehalose (not shown) gave essentially identical fusion inhibition as NaCl, thus ruling out an ionic mechanism, and suggesting an osmotic mechanism for this effect.

Effect of permeable solute concentration

The effect of permeable solutes, as compared to the impermeable solutes, was studied in order to discriminate between a mechanism dependent simply on solute particle concentration and a true osmotic effect requiring different relative permeabilities of solute and solvent. The bilayer-permeable solutes [13] ethylene glycol, propylene glycol, glycerol and ethanol were studied. In contrast to

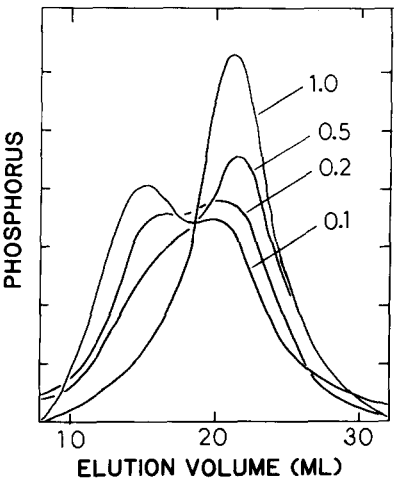

Fig. 4. Elution profiles (1 cm×26.2 cm column) for DPPC SUV/FUV incubated for 3 days in the various molarities of NaCl indicated beside each curve, and in 0.01 M Hepes (pH 7.4) and 0.02% NaN_3.

the impermeable solutes, the permeable solutes gave dramatic (and comparable) increases in fusion rate. Fig. 5 shows that fusion in 1.0 M ethanol (22°C) is complete in between 15 and 30 min. Elution profiles at longer times (not shown) were identical to the 30-min profile.

In order to run samples at such short incubation times, it was necessary to remove aliquots

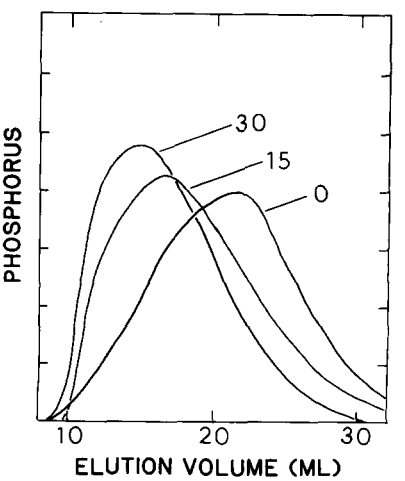

Fig. 5. Elution profiles (1 cm×26.2 cm column) for DPPC SUV/FUV incubated for the number of minutes indicated beside each curve, in 1.0 M ethanol/0.01 M Hepes (pH 7.4)/ 0.02% NaN_3 at 22°C.

from the incubation sample, and quench the fusion reaction. Fusion quenching was accomplished by simply storing the aliquot at 45°C until the column was available. Appropriate controls verified that fusion was indeed quenched for at least several days by this procedure. Also, in order to prevent early fusion, and to obtain accurate zero-time elution profiles of the SUVs in the presence of permeable solutes, it was necessary to maintain the sample reservoir, the connecting tubing, valve and flow adapter, and the column above T_m.

Fusion in the presence of 1.0 M ethanol along with either 0.15 M NaCl or 0.30 M glucose was studied to see whether the fusion promoter or inhibitor would dominate. As seen in Fig. 6, the elution profiles for the ethanol/NaCl and ethanol/glucose combinations have the same shape as for FUVs prepared in 0.15 M NaCl alone. Thus, the presence of even a moderate amount of impermeable solute negates the fusion-promoting effect of the permeable solute.

The elution volumes of both SUVs and FUVs prepared in and eluted with 1.0 M ethanol were increased by about 4 ml (Fig. 6). This was shown to be due to an ethanol effect on the gel, rather than due to an actual difference in vesicle sizes in the presence or absence of ethanol. The same increase in elution volume was seen for the chromate ion when it was eluted with the ethanol-containing buffer.

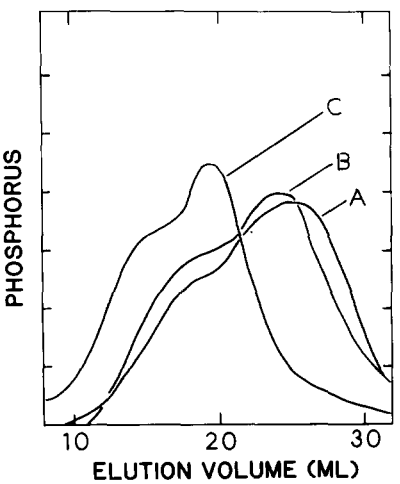

Fig. 6. Elution profiles (1 cm×26.2 cm column) for DPPC SUV/FUV formed after 3 days at 22°C in the following buffers: A, 1.0 M ethanol/0.15 M NaCl; B, 1.0 M ethanol/0.30 M glucose; C, 0.15 M NaCl. All buffers contained 0.01 M Hepes (pH 7.4) and 0.02% NaN_3

Effect of cholesterol

DPPC SUVs were prepared with 1.0 and 15 mol% cholesterol (relative to total lipid). At both cholesterol levels, there was no evidence of fusion after 5 days incubation in 0.10 M KCl at 22°C. Identical results were obtained with cholesterol from two different suppliers. The control experiment in which DPPC without cholesterol was carried through the chloroform-dissolution and vacuum-drying steps gave the expected degree of fusion based on the earlier studies.

Fusion of vesicles containing 1.0 mol% cholesterol in the presence of 1.0 M ethanol did occur, but at a greatly reduced rate compared to vesicles not containing cholesterol. Approx. 40% of the lipid was in FUVs after 6 days at 20°C in 1.0 M ethanol. Similarly, 1.0 mol% cholesterol greatly reduced but did not stop fusion at 4°C (in 0.1 M KCl).

Solute trapping

The compatibility of the fast ethanol fusion procedure with simultaneous incorporation of water-soluble markers was investigated for three different compounds: chromate, arsenazo III and

TABLE II

TRAPPING EFFICIENCY AND TRAPPED VOLUME OF FUV's PREPARED IN 1.0 M ETHANOL (22°C) WITH TWO DIFFERENT WATER-SOLUBLE MARKERS

Trapping efficiency is calculated as percent of marker present in the original SUV preparation that remains with the FUVs after separation from external marker on the Sephadex column. The trapped volume (expressed per mol of DPPC) is the volume necessary to contain the measured amount of trapped solute, assuming the solute concentration inside the vesicles equals the initial, bulk solute concentration. The original molarity of the marker, and the results of replicate determinations are given.

Marker	Concn. (M)	Trapping efficiency (%)	Trapped volume (l/mol)
Arsenazo III	$2.0 \cdot 10^{-3}$	9.0	1.86
	$4.0 \cdot 10^{-4}$	9.9, 12.0	1.98, 2.48
Sodium chromate	$1.0 \cdot 10^{-2}$	9.4, 12.7	1.84, 2.50

Bromophenol blue. DPPC SUVs were both sonicated and allowed to fuse in the presence of 1.0 M ethanol and the marker. In all cases, formation of normal-size SUVs and subsequent fusion to 700-Å-diameter vesicles was verified by Sepharose chromatography.

The trapping efficiency, defined as percent of marker incorporated into vesicles, ranged from 9.0 to 12.7% for chromate and arsenazo (Table II). These trapping efficiencies corresponded to trapped volumes ranging from 1.8 to 2.5 l/mol DPPC, as expected for vesicles of this size [2].

The trapping efficiency for Bromophenol blue was anomalously high, 61–70%, which is close to the theoretical limit for close-packed spheres with very thin walls; we assume this corresponds to binding, in addition to encapsulation.

Discussion

Temperature dependence

The temperature dependence of DPPC vesicle fusion can be summarized as follows: above about 35°C, SUVs are apparently stable indefinitely. Note that 35°C is below the T_m of DPPC MLVs, FUVs and SUVs. Around 30°C, fusion does occur, but it is very slow, i.e., insignificant after 1 day, but a few percent after a few days. In the region of 13–25°C, fusion to 700-Å FUVs occurs as previously reported at 21°C [2], i.e., a rapid initial phase for the first day, such that approx. 2/3 of the lipid is in FUVs after a few days, followed by prolonged slower fusion. In the range from 1 to about 8°C, fusion occurs to the 950-Å FUVs reported by Wong et al. [8], with fusion rate increasing with decreasing temperature, so that, at 1°C, fusion is about 90% complete in 1 day.

Wong and Thompson [9] have proposed a model for the aggregation and fusion of SUVs which qualitatively accounts for the curious temperature dependence. They propose a reversible aggregation of SUVs to 18-mers, which are then triggered to irreversibly fuse to 700-Å FUVs by collision either with other 18-mers or other 700-Å FUVs. Similarly, 700-Å FUVs reversibly aggregate to dimers, which, upon collision with other dimers, irreversibly fuse to form two 950-Å FUVs. The 950-Å FUVs can aggregate, but do not fuse further. This mechanism is qualitatively consistent with both the aggregation and fusion kinetics reported by them and others [14], as well as the apparent negative activation energy for fusion, suggested by their results and elaborated by our data. As they have pointed out, slower fusion at higher temperatures is expected if the disaggregation rate constants increase faster with temperature than do the aggregation and fusion rate constants. Thus, our results are at least qualitatively consistent with the Wong-Thompson model. Quantitative evaluation of the rate constants and their activation energies remains to be done. Also, it remains to be shown that such a set of rate constants, with their respective activation energies, can account for the formation of 18-mers, rather than some other size aggregate, and for the total absence of 950-Å FUVs above 13°C, even after many months.

It is not clear why, at 4°C, we do not see complete conversion to 700-Å FUVs prior to formation of 950-Å FUVs, as reported by Wong et al. [8]. We have ruled out an ionic or osmotic strength effect as the cause (Fig. 2). The remaining differences we know of in our procedures are that we used Hepes buffer rather than Pipes, and we did not employ a high-speed centrifugation step after sonication. We have chosen to study the non-centrifuged system because we are interested in developing the quickest possible procedure (with minimum sample loss) for FUV preparation and drug loading. Furthermore, centrifugation appears unnecessary for our purposes because, following our sonication procedure, we have never observed either an elution profile peak attributable to residual MLVs, or a visible settling-out of titanium particles (as happens, for example, when an unpolished sonication probe is used).

Wong has suggested that the fusion reaction may be critically sensitive to the size of the initial SUV, which in turn may be dependent on the sonication buffer. If this is true, the buffer difference, along with our not using prolonged high-speed centrifugation to obtain a maximally homogeneous SUV preparation [15], could conceivably have caused our different results. Although Wong saw some evidence of fusion-rate irreproducibility in the absence of the centrifugation step, our results were quite reproducible (Wong, M. and Thompson, T.E., personal communications). Workers using the below-room-temperature fusion

conditions are cautioned to verify the size of their vesicle preparations, and to do so by a method that will not be tricked by the extensive aggregation that occurs under these conditions (Table I).

Solute effects

The dramatic solute effects on fusion rate were completely unexpected. Early studies [2] had indicated that fusion was relatively insensitive to the presence or the concentration (less than about 0.15 M) of various salts. Also, our initial hypothesis was that trehalose, which had been reported to be unique among sugars in affecting membrane stability [16,17], might also be a unique inhibitor of SUV fusion. However, we now believe the following generalizations can be made: any membrane-impermeable solute, be it ionic or nonionic, at concentrations approaching 1.0 M will inhibit the low-temperature-induced fusion of DPPC SUVs. Furthermore, any permeable solute will promote the fusion of DPPC SUVs. When both permeable and impermeable solutes are present, the inhibition effect of the impermeable solute will dominate.

The solute effects appear to involve neither formation of abnormal FUVs nor gross perturbation of the bilayer membrane. The elution profiles indicate that normal-size SUVs and FUVs are obtained in the presence of all solutes tried. The ethanol-FUVs were checked on the Bio-Gel A-150M column, which has a larger exclusion limit than Sepharose 2B-CL, and they eluted where expected for 700-Å FUVs; there was no evidence of a larger form mixed in with the FUVs that might have been unresolved on the Sepharose column. The experiments with fusion in the presence of both permeable and impermeable solutes, in which the impermeable-solute effect dominates, indicate that the permeable solutes do not grossly affect the permeability properties of the bilayer. The identical effect of salts and sugars, and the opposing effects of large sugars and small polyalcohols, also argue against perturbation of the bilayer lipid or its hydration layer being a significant factor in the effects observed.

A possible explanation for the impermeable-solute effect is as follows. When two or more vesicles fuse to form a larger vesicle, there must be some mechanism for net influx of solvent, unless the fused vesicle remains in a highly collapsed state. The facts that the trapped volume of 700-Å FUVs is smaller than expected for spheres [2], and that fluorescence changes can be produced by osmotically swelling and shrinking FUVs loaded with the self-quenching fluorophore 6-carboxyfluorescein (Felgner, P., personal communication), indicate that the 700 Å FUVs are partly-collapsed spheres. However, the increased trapped volume originally used by Schullery et al. [2] as proof of fusion, shows that there is at least some increase in the internal volume following SUV fusion. If this solvent influx occurs to a significant extent by diffusion (rather than bulk flow through membrane-rupture sites), the presence of impermeable solutes would create an osmotic pressure gradient which could conceivably stop solvent influx, and thereby inhibit the fusion reaction. Although permeable solutes would flow in along with the solvent, and not cause an osmotic problem, we have no explanation for how they increase the fusion rate.

Cholesterol effect

A possible explanation for the effectiveness of only 1% cholesterol in stopping the fusion reaction is that cholesterol might preferentially locate in gel-phase bilayer-defect sites. This could interfere with a fusion-initiation step which requires strained defect sites being jostled or pressed against each other. In contrast to the cholesterol effect, neutral glycosphingolipid has little effect on DPPC SUV fusion, while incorporation of 5 mol% or less ganglioside inhibits fusion (presumably by charge repulsion) (Ref. 18, and unpublished results of Friere, M., cited therein). It is also notable that Bromophenol blue, which apparently binds to the bilayer, does not affect the fusion.

The stabilization of DPPC SUVs by only 1 mol% cholesterol, would seem to offer a convenient preparation of stable DPPC SUVs, suitable for physical studies on both sides of the transition.

Implications for drug loading

Our results indicate that a preparation of stable unilamellar vesicles, of about 700 Å diameter, loaded with a water-soluble drug can be prepared in 2–3 h time. The recommended protocol would be as follows: sonicate 0.10 M DPPC in an aqueous solution (at 50°C) of 1.0 M ethanol/0.01 M

Hepes, up to a few millimolar concentration of the drug, and less than about 0.01 M salts or other impermeable solutes. Permit fusion to occur by incubating the sample at room temperature for 30–60 min. Separate the trapped drug from the external drug using the Sephadex centrifugation method of Fry et al. [11]. Store at room temperature.

Approx. 10% of the drug can be expected to be trapped in the vesicles. The untrapped drug can be easily and quantitatively recovered. It is possible that concentrations of drug higher than a few millimolar can be loaded; however, a drastic slowing-down of the fusion rate is expected at concentrations of impermeable drugs between 0.1 and 1.0 M. The anomalously high apparent trapping of Bromophenol blue, presumably due to dye binding to or in the bilayer, is under further investigation. It is conceivable that such binding might be useful for delivery of certain drugs. It is interesting that this dye-bilayer interaction did not affect the size of the SUV or FUV, or the fusion rate.

The long-term stability of the loaded vesicles and their leak rate for the trapped drug must be determined for each case. If leakage is a problem, perhaps long-term stability could be achieved by storage in the presence of the external drug, and performing the Sephadex centrifugation shortly prior to use. Further experiments are underway to determine the leakiness of FUVs to a variety of solutes.

The reviewers called our attention to the instability in blood of vesicles without cholesterol. Since we have shown that even 1 mol% cholesterol greatly slows down fusion, this may present a serious obstacle to the ultimate application of DPPC FUVs for drug delivery through the circulatory system, although Weinstein et al. [6] successfully used sonicated DPPC SUVs, which, based on their stated procedure, we presume was, in fact, a mixture of FUVs and SUVs.

Acknowledgement

We gratefully acknowledge the assistance of Dr. Norman Ho and Dr. Walter Jetzer at the University of Michigan College of Pharmacy in the use of their light-scattering apparatus.

References

1 Larrabee, A.L. (1979) Biochemistry 18, 3321–3326
2 Schullery, S.E., Schmidt, D.F., Felgner, P., Tillack, T.W. and Thompson, T.E. (1980) Biochemistry 19, 3919–3923
3 Gaber, B.P. and Sheridan, J.P. (1982) Biochim. Biophys. Acta 685, 87–93
4 Nicolussi, A., Massari, S. and Colonna, R. (1982) Biochemistry 21, 2134–2140
5 Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry 15, 1393–1401
6 Weinstein, J.N., Magin, R.L., Yatvin, M.B. and Zaharko, D.S. (1979) Science 204, 188–191
7 Parenta, R.A. and Lentz, B.R. (1984) Biochemistry 23, 2353–2362
8 Wong, M., Anthony, F.W., Tillack, T.W. and Thompson, T.E. (1982) Biochemistry 21, 4126–4132
9 Wong, M. and Thompson, T.E. (1982) Biochemistry 21, 4133–4139
10 McClare, C.W.F. (1971) Anal. Biochem. 39, 527–530
11 Fry, D.W., White, C. and Goldman, I.D. (1978) Anal. Biochem. 90, 809–815
12 Koppel, D.E. (1972) J. Chem. Phys. 57, 4814–4820
13 Bangham, A.D., De Gier, J. and Greville, G.D. (1967) Chem. Phys. Lipids 1, 225–246
14 Schmidt, C.F., Lichtenberg, D. and Thompson, T.E. (1981) Biochemistry 20, 4792–4797
15 Barenholz, Y., Gibbes, D., Litman, B.J., Thompson, T.E. and Carlson, F.D. (1977) Biochemistry 16, 2806
16 Crowe, J.H., Crowe, L. and Chapman, D. (1984) Science 223, 701–703
17 Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S. and Womersley, C. (1984) Biochim. Biophys. Acta 769, 141–150
18 Felgner, P.L., Friere, E., Barenholz, Y. and Thompson, T.E. (1981) Biochemistry 20, 2168–2172