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From mixed micelles to liposomes: Critical steps during detergent removal by membrane dialysis

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Abstract

To elucidate the critical steps during liposome preparation by detergent removal from mixed micelles, kinetics of bile salt removal were measured by monitoring radioactively labelled cholate, desoxycholate (DC) or chenodeoxycholate (CDC) in the dialysis buffer. Three different phases of bile salt interaction with lipids were found. In a first phase, a rapid removal of cholate from mixed micelles was estimated. At the end of phase I, liposome formation was completed after 4 h when using pure egg lecithin and after 3–5 h, when 30 mol% cholesterol or sphingomyelin was mixed with egg lecithin. Phases II and III showed a pronounced lower bile salt release from liposomes and were correlated with desorption from outer liposome surface and flip–flop from the inner monolayer to the outside. DC and CDC are only slowly removable from mixed micelles. Liposome formation is therefore complete only after ≈ 20 h. When using cholate and high lipid concentrations, formation of oligolamellar structures is due to fusion of liposomes containing a high amount of bile salt. Therefore, detergent removal has to be very fast to result in exclusively unilamellar vesicles.

Keywords: Liposome preparation; Detergent removal; Membrane dialysis; Bile salts; Mixed micelles; Kinetics

1. Introduction

Liposomes can be prepared by different methods. After disperging suitable membrane lipids in an aqueous phase and spontaneous formation of multilamellar large vesicles (MLV), mechanical procedures such as ultrasonication (Huang, 1969), homogenization by a french press (Barenholz et al., 1979) or by other high pressure devices (Mayhew et al., 1984; Brandl et al., 1990), or extrusion through polycarbonate membranes with defined pore sizes (Olson et al., 1979) lead to a reduction in size and number of lamellae of the vesicles. A second group of procedures starts with dissolving the lipids in an organic solvent and mixing it with

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an aqueous phase. The concentration of the organic solvent is then reduced by suitable procedures. Ether can be evaporated from an excess of warm water (Deamer and Bangham, 1976), ethanol can be diluted (Batzri and Korn, 1973) or the organic solvent is evaporated until the coherent outer organic phase is removed and a phase reversion occurs (reverse phase evaporation, Szoka and Papahadjopoulos, 1978). A third group of preparation procedures uses suitable detergents, e.g. bile salts or alkylglycosides. Far above their critical micellar concentration (c.m.c.), they dissolve membrane lipids in an aqueous phase to form mixed micelles, in which the detergents are in equilibrium with free detergent monomers. Upon reducing the monomer concentration, mixed micelles loose their bound detergent molecules and are forced to fuse to larger disc-like aggregates and finally vesiculate and form liposomes when the free detergent concentration is around the c.m.c. Common procedures of detergent removal from the mixed micelles are dilution (Schurtenberger et al., 1984), gel chromatography (Brunner et al., 1976), dialysis through hollow fibres (Rhoden and Goldin, 1979) or through thin membranes (Milsmann et al., 1978). The major advantage of detergent removal techniques is the possibility to tailor the size of the liposomes and to yield almost exclusively large unilamellar vesicles (LUV). However, when preparing liposomes with this detergent removal method, this high quality of the product is only obtained when low lipid concentrations are used. Above $\approx 2\%$ lipid (w/w), size distribution of liposomes as well as their number of lamellae increase. To elucidate the critical steps of liposome formation, in this contribution detergent removal kinetics during controlled dialysis are measured and the influence of residual detergent on newly formed liposomes is shown.

2. Material and methods

2.1. Preparation of mixed micelles

Sodium salts of cholic acid (C), deoxycholic acid (DC) and chenodeoxycholic acid (CDC) were purchased from Serva, egg lecithin prepared from fresh egg yolk and cholesterol were from Sigma Chemicals. A total of 136 μmol of lecithin or a lecithin/cholesterol mixture (7:3 mol/mol) were mixed with 227 μmol of the trihydroxy bile salt sodium cholate or 60 μmol of the dihydroxy bile salts DC or CDC. Bile salts were labelled with 1.85 MBq of the corresponding radioactive bile salt (^{3}H-labelled C, ^{14}C-labelled DC or CDC; NEN, Dreieich, FRG). Lipid and bile salt were dissolved in methanol and solvent was removed completely under reduced pressure. The dry and clear lipid/detergent film was dissolved in 8 ml of phosphate buffer (10 mmol/l phosphate, 150 mmol/l NaCl, pH adjusted to 7.35) to form a clear mixed micelle solution with a final lipid concentration of 17 mmol/l and 28.3 mmol/l of cholate or 7.5 mmol/l of DC or CDC.

2.2. Kinetics of detergent removal during dialysis

Detergent removal was performed by using the method of fast and controlled dialysis of mixed detergent/lipid micelles (Milsmann et al., 1978). A total of 7.5 ml of the clear mixed micelle solution was filled into a suitable dialysis apparatus (LIPOPREP, Diachema, Langnau, Switzerland), the dialysis chamber of which contains a magnetic stirrer and is covered on both sides with a highly permeable dialysis membrane with a cutoff of 10 kDa and a dialysis area of each 9.6 cm^2. Dialysis was performed with a continuous flow of 35 ml/h of buffer on each side. For the measurement of the detergent removal kinetics during liposome formation from mixed micelles, dialysates of both sides were combined and pumped with a HPLC pump (2150, LKB Instruments) with the same flow rate through a radioactivity monitor (RAMONA, Raytest, Straubenhart, FRG). The containing bile salt concentration was measured by splitting 10% of the combined dialysate and mixing it with scintillation cocktail (Rialuma, Baker Chemicals, Deventer, Holland) in a ratio of 1:2.5. Chemiluminescence, which correlates to radioactivity in the dialysate, was monitored for 21 h. Different stages of bile salt removal could be detected from the logarithm of the bile salt decrease in the dialysate. Residual detergent at dif-

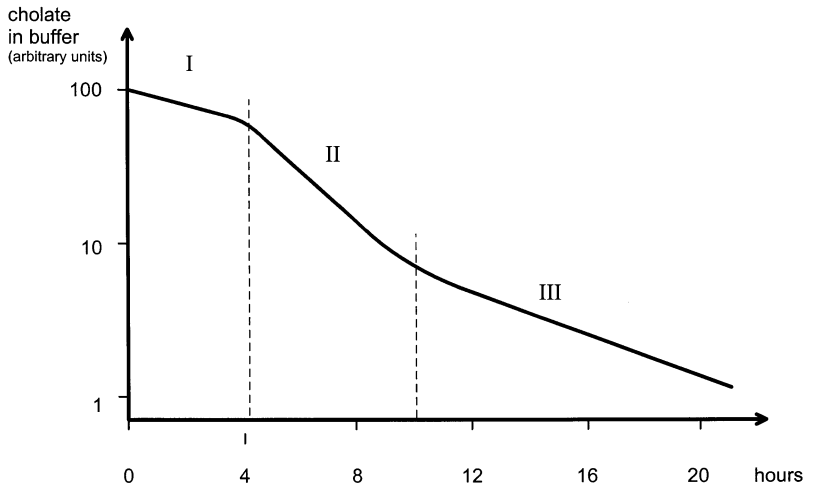

Fig. 1. Sodium cholate in dialysis buffer during preparation of EPC liposomes by controlled membrane dialysis of mixed micelles. Mixed micelles were prepared from 17 mM EPC and 28 mM sodium cholate, labelled with ^{3}H-cholate. Cholate concentration was followed for 21 h by radioactivity in dialysis buffer, which is indicated in arbitrary units. I, release of bile salt molecules, which are desorbed from mixed micelles; non-linear transition between phases I and II correlates with liposome formation, which is indicated by a strong increase of turbidity of the dispersion in the dialysis chamber; II, III, release of bile salt molecules from vesicles; different removal kinetics in phases II and III may indicate a faster decrease of bile salts adsorbed to the outer LUV monolayer in II compared to the rate-limiting flip–flop from the inner to the outer monolayer in III.

ferent time points was measured directly in the retentate. All experiments were performed in triplicate.

2.3. Liposome characterization

Liposome size was characterized by the z-average measured by dynamic laser light scattering (NANOSIZER, Coulter Electronics, FRG).

3. Results and discussion

For the measurement of the kinetics of detergent removal for liposome preparation from mixed micelles, we followed the bile salt concentration in the dialysis buffer, which turned out to be more sensitive for the detection of different phases than the concentration changes in the dialysis chamber. In Fig. 1, the typical concentration profile of cholate (C) in the dialysis buffer during controlled dialysis, starting with mixed micelles, is shown. In this case, cholate was removed from mixed micelles containing egg lecithin (EPC). The profile is similar, when 30 mol% cholesterol or sphingomyelin are mixed with EPC (Table 1). A high and nearly constant concentration in phase I is measured before an increase of turbidity in the dialysis chamber indicates the formation of liposomes from mixed micelles. Therefore, phase I correlates with the removal of bile salt (BS) monomers, which are in equilibrium to BS involved in mixed micellar aggregates (Schubert and Schmidt, 1988). In diluted BS/lipid mixed micelles, BS are supposed to be predominantly adsorbed at the edge of the disk-like structures and, to a smaller extent also to the bilayer-like surfaces. (Mazer et al., 1976). Upon BS removal from the edges, disks fuse laterally. Edges disappear during vesicle formation and the decrease of the BS removal rate in phase II therefore reflects the BS monomer concentration, which is desorbing from the vesicle membrane surface. This is substantiated by an experiment in which cholate was added in a sublytic concentration of 7 mM to preformed LUV from EPC (Table 1). After an incubation time of 30 min, removal of cholate from liposomes was started. The profile of cholate

Table 1
Content of different bile salts in the retentate after different phases of removal by membrane dialysis for liposome preparation

Bile salt	Lipid	End (h) of		Residual detergent (mM) after			Liposome diameter (nm)
		Phase I	Phase II	Phase I	Phase II	Phase III (21 h)	
C (28 mM)	EPC	4.2 ± 0.7	10.3 ± 1.6	10.5 ± 1.3	2.6 ± 0.3	0.4 ± 0.05	69 ± 3
C (28 mM)	EPC/Cho 7:3 mol/mol	3.2 ± 0.6	7.7 ± 1.2	8.5 ± 1.1	1.8 ± 0.5	0.2 ± 0.04	77 ± 3
C (28 mM)	EPC/Sph 7:3 mol/mol	4.7 ± 0.2	9.7 ± 0.6	8.2 ± 0.5	2.0 ± 0.7	0.3 ± 0.05	71 ± 8
C (7 mM, sublytic)	EPC liposomes		7.4 ± 0.2			0.07 ± 0.04	71 ± 3 (unchanged)
DC (8 mM)	EPC	20				1.3 ± 0.3	150 ± 10
CDC (8 mM)	EPC	20				1.0 ± 0.12	158 ± 13

EPC, egg phosphatidylcholine; Cho, cholesterol; Sph, egg sphingomyelin; C, cholate; DC, deoxycholate; CDC, chenodeoxycholate; total lipid concentration, 17 mM. A concentration of 28 mM cholate or 8 mM DC or CDC was used to prepare a clear solution of mixed bile salt/lipid micelles. At 7 mM cholate no solubilization of EPC liposomes was found.

in the dialysis buffer corresponds to that in Fig. 1 after phase I, when cholate is removed from closed vesicles. Incubation of egg lecithin liposomes with sublytic cholate concentration has no effect on liposome size or lamellarity, when the lipid concentration is low, as shown in this case for 17 mM EPC (Table 1). Also when using higher lipid concentrations of ≈ 50 mM, structural changes could not be observed, when cholate was added at half the concentration leading to the onset of membrane solubilization (Schubert et al., 1991). However, at this high lipid concentration, liposomal membranes tend to fuse to large oligolamellar structures, when BS concentration is close to the limit of the solubilization onset. At this maximal subsolubilizing concentration, bile salts induce permanent membrane defects, as shown earlier by freeze fracture electron microscopy (Schubert et al., 1986). As a consequence, it is obvious that the high cholate content immediately after vesicle formation upon detergent removal is the critical point for the preparation of large unilamellar vesicles (LUV). Even when using the fast and controlled dialysis through thin membranes, the removal of detergent from the dialysis chamber is relatively slow. When dialysing only sodium cholate without any lipid, its half-life time in the retentate is ≈ 1.5 h (data not shown). This is in the order of the removal of cholate from mixed micelles with different lipid, which is between 2 and 3 h, as estimated from the data in Table 1 for phase I. Due to this relatively slow removal of detergent during dialysis, the probability of collision and fusion of cholate-rich vesicles is high and, in addition, is correlated to lipid concentration. This problem can be circumvented by maximal increase of the detergent removal rate without dilution, which is possible by an active removal process like cross-flow filtration (Peschka et al., 1997).

The more lipophilc dihydroxy bile salts DC and CDC show a stronger binding to membrane lipids in mixed micelles and vesicles. Association constants are higher ($12\,200\ M^{-1}$) compared to cholate ($4800\ M^{-1}$) (Schubert and Schmidt, 1988). This obviously results in a prolonged time required for vesicle formation, which is ≈ 20 h. Furthermore, it also has an effect on the vesicle size and the mechanism of liposome formation. The prolonged time for lateral fusion of disk-like mixed micelles favours larger aggregates, which finally vesiculate. After 20 h liposomes are therefore larger and still contain the maximal subsolubilizing concentration of DC or CDC.

For cholate-containing mixtures, vesicle formation is complete after 3–5 h, depending on the lipid composition. Also in this case, formation

time of liposomes can be correlated with the binding strength. Due to the lower association constants to EPC membranes containing cholesterol (2800 M^{-1}), cholate is more easily released form mixed micelles, leading to faster vesiculation.

After liposome formation, cholate concentration in buffer rapidly decreases in phase II, as shown in the logarithmic scale in Fig. 1. This points to a sudden decrease of cholate monomers in the lipid dispersion after vesicle closure. Most of the residual detergent is therefore adsorbed to the membranes or encapsulated into the liposomes. Two distinct phases II and III of detergent removal probably are due to the desorption of cholate from the outer liposome monolayer and a rate-limiting flip–flop from the inner monolayer to the outside, respectively. This suggestion is substantiated by comparing the residual amount of cholate at the end of dialysis after 21 h, when starting with mixed micelles or with intact liposomes exposed to a sublytic cholate concentration. In the latter case, less cholate is then expected to be found in the inner part of the liposomes, when flip–flop (in this case from outer to inner monolayer) is slow. As a consequence, the residual bile salt after dialysis is then lower, as shown in Table 1.

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