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PREPARATION OF MONODISPERSE VESICLES WITH VARIABLE SIZE BY DILUTION OF MIXED MICELLAR SOLUTIONS OF BILE SALT AND PHOSPHATIDYLCHOLINE

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We have investigated by means of quasi-elastic light scattering the aggregative behavior of aqueous mixed micellar solutions of glycocholate and phosphatidylcholine. Upon dilution with buffer the micellar size and the polydispersity increases dramatically, and, as the system is diluted beyond the mixed micellar phase boundary, a spontaneous transition from polydisperse micelles to monodisperse vesicles occurs. The radius of the vesicles formed upon dilution depends strongly upon the final composition of the solution and can be varied between 120 and 550 Å. In contrast to the thermodynamically stable mixed micelles these vesicle solutions can be brought into a metastable state in which it is possible to remove by dialysis the bile salt molecules from the mixed vesicles without changing their radius by more then 10%. The combination of dilution and dialysis thus represents a method for the preparation of unilamellar, monodisperse and detergent-free vesicles with a desired radius that can be chosen between 120 and 500 Å.

Vesicles have become of considerable importance as model membranes and drug delivery systems [1–4]. A number of methods for the preparation and characterization of vesicles have been published (for recent reviews see Refs. 5 and 6). In particular by detergent removal from mixed micellar solution by means of gel filtration [7] or dialysis [8] one can produce very homogeneous small unilamellar vesicles. However, for a variety of applications it could be useful to have a method for reproducible preparation of monodisperse vesicles of a chosen size [9,10]. Recent quasi-elastic light scattering studies of the aggregation behavior of mixed micellar solutions of bile salt and phosphatidylcholine brought across the mixed micellar phase boundary by means of dilution with aqueous buffer have indicated that a micelle-to-vesicle transition occurs spontaneously as the phase boundary is crossed [11] and that the vesicle radius can be varied between 120 and 550 Å by choosing the final solution composition [12]. In the present work we will show that it is possible to prepare small unilamellar and detergent-free versicles of a chosen size by dilution of mixed micellar solutions and subsequent detergent removal by dialysis.

Egg yolk phosphatidylcholine was obtained from Lipid Products (South Nutfield, Surrey, U.K. (grade I)) and the sodium salt of glycocholic acid from Calbiochem. Glycocholate was dissolved in ethanol, filtered and recrystallized. The radiolabelled compounds di[1-^{14}C]palmitoylphosphatidylcholine (100 mCi/mmol) and [^{3}H(G)]glycocholic acid (1.7 Ci/mmol) were obtained from

Abbreviations: $\bar{R}_H$, apparent mean hydrodynamic radius; V, polydispersity index.

New England Nuclear (Boston, MA) and phosphatidyl[*N-methyl*-^{14}C]choline from Amersham International (Buckinghamshire, U.K.). All other reagents used were of analytical grade.

Mixed micellar bile salt-phosphatidylcholine solutions with different phosphatidylcholine to bile salt molar ratios were prepared by the method of coprecipitation [13]. After dissolving an appropriate amount of each lipid in ethanol the mixture was dried in vacuo until the dry weight was constant. Buffer (0.15 M NaCl/Tris, pH 8) was then added to obtain stock solutions with a total lipid concentration of 50 mg/ml. The final concentrations were then prepared from the stock solutions by a number of rapid dilution steps. Each sample was flushed with purified N_2, sealed and incubated at 20 °C until the light scattering properties were time independent. Whereas the micellar systems reached equilibrium within minutes, the incubation time needed for solutions containing vesicles was usually of the order of 48 h.

Bile salt was removed from the incubated vesicle solutions by dialysis. These solutions (1–5 ml) were dialyzed for 24 h against different volumes of 0.15 M NaCl/Tris buffer (usually 4 litre) using a rotating teflon dialysis cell (Diachema Ltd., Rüschlikon/Zürich) and a cellulose disk membrane with 10000 molecular weight cut off (Ref. No. 10.16, Diachema Ltd.). The concentration of glycocholate and lecithin was determined with a $^3H/^{14}C$ dual label counting program on a Beckman LS 7800 liquid scintillation counter.

Diffusion coefficient D, apparent mean hydrodynamic radius $\bar{R}_H$ and polydispersity index V were determined by quasi-elastic light scattering. Our light scattering apparatus for both static and dynamic measurements consists of an argon ion laser (Spectra Physics, model 171, $\lambda_0 = 5145$ Å), a temperature controlled scattering cell holder, a digital autocorrelator (Malvern K 7023, 96 channels) and an 'on-line' data analysis performed by a Nova 3 computer. Details are given elsewhere [14].

Sample purification and data analysis are described in Ref. 15. A cumulant analysis [16] was used to determine the mean diffusion coefficient $\bar{D}$ and the polydispersity V (as defined in Ref. 15). From $\bar{D}$ an apparent mean hydrodynamic radius $\bar{R}_H$ as defined by Equation 1 was calculated [15,17]

$$\bar{R}_H = \frac{kT}{6\pi\eta\bar{D}} \quad (1)$$

where k is Boltzmann's constant, T the absolute temperature and η the viscosity of the solvent.

Dilution of a mixed micellar solution of bile salt and phosphatidylcholine causes a marked increase in micellar size and polydispersity (see Fig. 1A, B). At the approach of the mixed micellar phase limit $\bar{R}_H$ appears to diverge.

If the mixed micellar solution is diluted beyond the phase limit one observes a spontaneous transition to almost monodisperse vesicles [11]. The size of the vesicles decreases with increasing dilution starting at approx. 500 Å close to the phase boundary and levelling off at 120 Å [12]. The increase of micellar size with dilution and the existence of a micellar phase boundary can be explained in terms of the mixed disk model proposed by Mazer et al. [18] by taking into account the partition equilibrium between bile salt molecules in the mixed micelles and bile salt monomers in the intermicellar solution [12,18].

The dependence of the vesicle size upon the final solution composition has been investigated

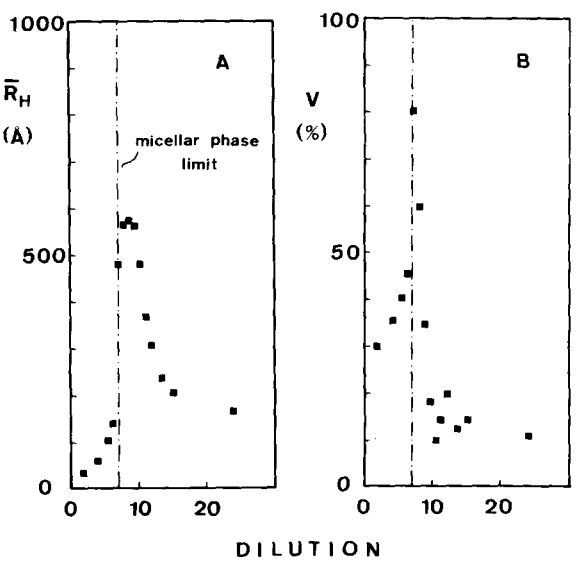

Fig. 1. Effect of dilution on the mean apparent hydrodynamic radius $\bar{R}_H$ (A) and polydispersity index V (B) of a mixed micellar solution of glycocholate/egg phosphatidylcholine (PC/bile salt = 0.75, total lipid concentration = 50 mg/ml, $t = 20$ °C). The micellar phase limit is calculated using the mixed disk model (Refs. 18, 19).

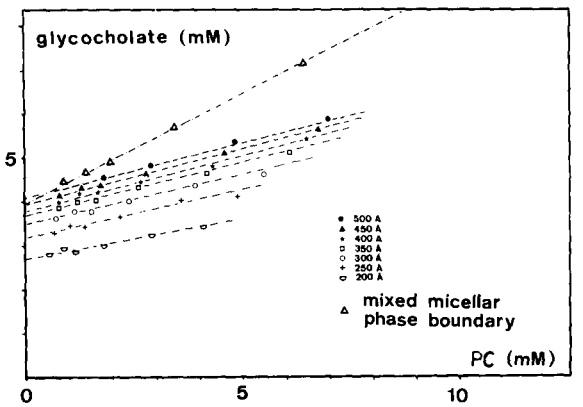

Fig. 2. Dependence of the hydrodynamic radius of glycocholate/egg phosphatidylcholine vesicles upon the solution composition in 0.15 M NaCl/Tris buffer at 20 °C. Dilution of mixed micellar stock solutions produces vesicles of radii. (●) 500 Å, (▲) 450 Å, (★) 400 Å, (□) 350 Å, (○) 300 Å, (+) 250 Å, (⌓) 200 Å, depending upon the concentrations of glycocholate and phosphatidylcholine.

systematically for dilution series at different PC/bile salt ratios (see Fig. 2) [12,19]. This study has led to the hypothesis that the vesicle size is primarily determined by the amount of bile salt incorporated in the bilayer. On this basis it was possible to develop a simple phenomenological equilibrium model that accounts for the light scattering results shown in Fig. 2 [12]. Therefore dilution of mixed micellar bile salt-phosphatidylcholine solutions is a production method for almost monodisperse vesicles with radii that can be chosen between 120 and 500 Å using Fig. 2 *. However, the large amount of bile salt in the bilayer and in the aqueous phase may prohibit certain applications. This limitation can be overcome by removing bile salts with dialysis, taking advantage of the fact that previously equilibrated mixed vesicles can stay in a metastable state, in which the vesicle radius remains close to its original value, even if bile salt is removed. [12,19]. This means that the vesicle size is not only determined by the values of the physical and chemical parameters, and that it can also depend upon the particular sequence of steps leading to the final state of the system. The data in Table I as seen in the context of Fig. 2 can serve as an example for this 'path dependence'. From Fig. 2, and considering that the minimum size for detergent-free vesicles produced by detergent removal or sonication is approx. 120 Å [5], one would expect that complete removal of bile salt by means of dialysis from solutions containing mixed micelles or vesicles should cause the formation of small vesicles with a radius of 120 Å. As shown in Table I, dialysis of a mixed micellar solution results in vesicles with $\bar{R}_H$ equal to 140 Å, in satisfactory agreement with this expectation. However, when previously equilibrated solutions of mixed vesicles are dialyzed, the bile salt molecules are almost completely removed while the vesicle radius decreases only slightly (approx. 10%).

This particular property of mixed vesicle solutions offers a method for the preparation of unilamellar, monodisperse and detergent free vesicles with radii that can be chosen between 120 and 500 Å. One simply has to combine mixed vesicle preparation by dilution of a mixed micellar solution with subsequent detergent removal through dialysis. The final concentration of bile salt (glycocholate) is less than 0.5% of the initial concentra-

TABLE I

APPARENT MEAN HYDRODYNAMIC RADIUS $\bar{R}_H$ AND BILE SALT AND PHOSPHATIDYLCHOLINE CONCENTRATION (C_{BS}, C_{PC}) OF VESICLE SOLUTIONS BEFORE AND AFTER DIALYSIS

The mixed vesicles were prepared through rapid dilution of mixed micellar solutions. (PC/BS = 0.3 and 0.75, total lipid concentration C_{tot} = 50 mg/ml, t = 20 °C)

Before dialysis				After 24 h dialysis			
C_{BS} (mM)	C_{PC} (mM)	$\bar{R}_H$ (Å)	V (%)	C_{BS} (mM)	C_{PC} (mM)	$\bar{R}_H$ (Å)	V (%)
17.54 [a]	5.26	24	60	0.20	4.99	140	30
4.05	1.20	525	20	0.03	1.13	470	25
4.03	1.18	516	17	0.04	1.11	460	20
3.76	1.09	430	17	0.04	1.07	390	15
3.70	1.07	370	15	0.01	0.99	340	15
1.48	1.12	135	–	0.02	1.0	140	15

[a] This composition corresponds to a micellar solution.

* The phase diagram in Fig. 2 for glycocholate/egg phosphatidylcholine mixed vesicles at 20 °C had to be modified slightly when different lots of egg phosphatidylcholine were used, owing to small changes in the phosphatidylcholine composition [19]. For a practical use of the relation between vesicle size and solution composition it is therefore necessary to first 'calibrate' the system by measuring the hydrodynamic radius as a function of dilution for at least two PC/bile salt ratios.

tion and the polydispersity index V is below 20%. An examination of the stability of these solutions by means of PC/bile salt showed no changes in the light scattering parameters over a period of two months. It is possible to increase the low vesicle concentration (after the dialysis) by ultrafiltration. Using an ultrafiltration system (Amicon model 8MC) and a filter (Diaflo XM 50) with a molecular wight cut off of 50000 the concentration could be increased by a factor of four without changing the vesicle size. Further systematic investigation of the time dependence of these concentrated solutions remains to be done.

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