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Liposomes prepared dynamically by interactions between bile salt and phospholipid molecules

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Quasi-elastic light scattering (QELS) studies showed that vesicles can spontaneously form from sodium glycocholate-egg phosphatidylcholine mixed micelles upon dilution with physiological saline buffer (pH 7.5). A water-soluble drug, cytosine arabinoside, did not affect the transition pattern. Transitions were also obtained when dilutions were performed using diluted serum solutions (5% and 10% serum in buffer). However, if intermicellar bile salt monomer concentration (imc) was kept constant in diluent media, the transition was completely suppressed. Approximately 10% of the cytosine arabinoside was trapped inside vesicles which were formed in buffer, although this value decreased to approx. 2.5% when diluted serum was used. These results suggest that the mixed micelle to vesicle transition can be an alternative means to sonication in loading drugs into in vitro vesicles. A good correlation was found between the transmission electron microscopy (TEM) diameter and the mean hydrodynamic diameter obtained by QELS.

Introduction

Bile salts are biological detergents that have amphiphilic properties and form membrane-toxic simple micelles in aqueous media above the critical micelle concentration (cmc). In the presence of phospholipids, bile salts form thermodynamically stable mixed micelles which are less toxic [1,2]. Mazer et al. [3] proposed the 'mixed disk' model for the structure of bile salt-egg phosphatidylcholine mixed micelles, based on a quasi-elastic light scattering study of the aggregative behavior of native biliary lipids. Phospholipid vesicles (liposomes) recently became important as drug delivery systems and models for biological membranes. It was previously shown by quasi-elastic light scattering that bile salt and egg phosphatidylcholine mixed micelles spontaneously form vesicles upon direct aqueous dilution [4,5] or equilibrium dialysis [4].

We are currently investigating the potential of bile salt-egg phosphatidylcholine mixed micelles as drug delivery vehicles. We have shown that liposomes containing the drug can be produced in vitro by direct dilution of mixed micelles with buffer [6]. It has been well known that conventional liposomes immediately bind plasma proteins in the blood stream upon injection and then these proteins signal phagocytic cells of reticuloendothelial system (RES) to remove the liposomes from the circulatory system [7]. It is our intention to pursue our new model as an alternative drug delivery device to sonication since the final value of drug trapped inside vesicles is higher in our system and the detection and uptake of the liposomes, prepared in the presence of blood components, by RES may be minimized. There are two main purposes for this study: (1) To provide more evidence for the formation of vesicles by two approaches independent of QELS; (i) determining the trapping efficiency from the amount of cytosine arabinoside trapped inside the vesicles, (ii) examining the morphology of the particles formed during transition by transmission electron microscopy. (2) To study the micelle to vesicle transition by means of QELS in the presence of blood components by diluting the micellar system with serum solution.

Experimental procedures

Materials

Sodium glycocholate and egg-yolk phosphatidylcholine were obtained from Sigma. Both bile salt and phospholipid gave one spot on thin-layer chromatogra-

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phy in chloroform/isopropanol/acetic acid/water (30 : 30 : 4 : 1, v/v) and chloroform/methanol/ammonium hydroxide/water (60 : 35 : 5 : 2.5, v/v), respectively. Human serum was purchased from Hazelton Research products (Lenexa, Kansas). Methanol and acetonitrile were HPLC grade and the other reagents used were reagent grade. All studies were carried out at room temperature unless otherwise noted.

Methods

(a) Solutions. Aqueous glycocholate-egg phosphatidylcholine (GC/egg PC) mixed micellar solutions were prepared by co-precipitation [8]. After dissolving the appropriate amounts of each lipid (egg PC/GC, molar ratio = 0.8) in ethanol, the mixture was thoroughly dried under a stream of purified nitrogen and then dried in vacuo for 2–3 days until the dry weight was constant. 0.01 M Tris buffer (pH 7.5) containing 0.150 M NaCl and 0.001 M NaN_3, or serum was then added to obtain stock solutions with a total lipid concentration of 50 mg/ml. The solutions were flushed with nitrogen, sealed and equilibrated for 2 days at room temperature. The final solutions were prepared by rapid dilutions from the stock solution. Diluents were: (i) Buffer, (ii) Buffer containing glycocholate or cytosine arabinoside, (iii) Serum solutions (5% and 10%) in buffer. Each solution was likewise flushed with nitrogen, sealed, and left for 2 days at room temperature to reach equilibrium.

(b) Quasi-elastic light scattering (QELS) measurements. These were performed utilizing a Nicomp Model 270 submicron particle sizer (Pacific Scientific, Menlo Park, CA) equipped with a 5 mW Helium-Neon Laser at an exciting wavelength of 632.8 nm and with a 64-channel autocorrelation function, a temperature-controlled scattering cell holder and an ADM 11 video display terminal computer (Lear Siegler Inc., Anaheim, CA) for analyzing the fluctuations in scattered light intensity generated by the diffusion of particles in solution. The mean hydrodynamic particle diameter, $\bar{d}_h$, was obtained from the Stokes-Einstein relation using the measured diffusion coefficient obtained from the fit. Each reported experimental result is the average of three $\bar{d}_h$ values obtained from analysis of autocorrelation functions accumulated for 30 min.

(c) Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This was performed according to the method of Laemmli [9].

(d) Trapped volumes and trapping efficiencies. Vesicles were prepared in the presence of cytosine arabinoside (5 mg/ml), water-soluble drug, in the diluent media. Untrapped drug was removed by the extensive dialysis against drug-free buffer (0.01 M Tris (pH 7.5) containing 0.150 M NaCl and 0.001 M NaN_3) at room temperature using Spectrapor dialysis membrane tubing with a molecular weight cutoff of 12000 (Spectrum Medical Industries, Inc.). The concentrations of cytosine arabinoside trapped within vesicles were determined by high-pressure liquid chromatography (HPLC) after breaking down the liposomal membrane with methanol Reverse-phase HPLC was performed, using a system consisting of a Waters 600 pump fitted with a U6k manual injector, RCM-100, 490 programmable multiwave length detector and 745 data module (Millipore). A Novapak C_{18} Radial-Pak column (particle size 4 μm, 10 cm length × 8 mm internal diameter) was used. The mobile phase was methanol and water (95 : 5, v/v) which were degassed with a high-purity grade of helium gas (Union Carbide, Linde Division). The flow rate was 1.0 ml/min and detection was made at 254 nm and 0.500 AUFS.

Trapped volumes were calculated based on the diameter of the vesicles assuming the bilayer thickness of 4 nm and the area per phospholipid molecule of 0.7 nm^2 [10,11]. Trapping efficiencies were calculated from the ratio of the drug concentrations after and before removal of untrapped drug.

(e) Transmission Electron Microscopy (TEM). Diameters of mixed micelles and mixed vesicles were determined by TEM following staining. A small drop of the sample in Tris buffer (pH = 7.5) was placed onto carbon-coated copper grid. Excess sample was gently drained off with filter paper, leaving a thin film of the samples on the grid. One drop of 1% phosphotungstic acid buffer was then added to the grid and allowed to stand for 1 min. The excess solution was withdrawn with filter paper and allowed to air dry for 2–3 min. Samples were viewed on a Joel Jem 100S transmission electron microscopy and photographed at 160000 × magnification. At least 700 particles were measured on several electron micrographs covering different areas on a grid.

Results

Micelle to vesicle transition in buffer

Fig. 1 shows the change in the mean hydrodynamic diameter, $\bar{d}_h$, of the particles in glycocholate-egg phosphatidylcholine mixed micellar solution (initial total lipid concentration 50 mg/ml, egg PC/GC molar ratio = 0.8) upon dilution. When the mixed micellar system was diluted around the micellar phase limit (dilution factor of 10), micellar $\bar{d}_h$ values increased strongly from 22 nm to 140 nm. With further dilutions, $\bar{d}_h$ values decreased toward an asymptotic value of approx. 34 nm, consistent with the vesicle sizes obtained by others [4,5]. These results demonstrate that bile salt-egg phosphatidylcholine mixed micelles are spontaneously transformed into bile salt-egg phosphatidylcholine vesicles [5,6,12].

It is of interest that the transition phenomenon was not affected at all by a hydrophilic compound (cytosine arabinoside) present in the diluent buffer (Fig. 1). Fur-

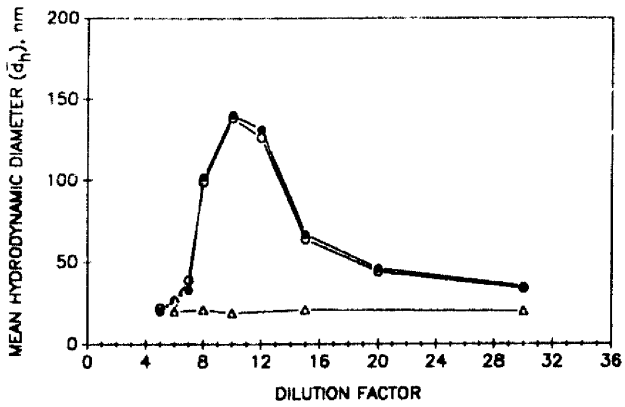

Fig. 1. Effect of dilution on mean hydrodynamic diameter, $\bar{d}_h$, of glycocholate-egg phosphatidylcholine mixed micellar solution (initial total lipid concentration = 50 mg/ml, egg PC/GC molar ratio = 0.8) at 23°C in buffer (0.020 M Tris, 0.15 M NaCl, (pH 7.5), 0.001 M NaN_3); The upper curve, diluent buffer alone (○), diluent buffer with cytosine arabinoside (5 mg/ml) and (●) and diluent buffer containing glycocholate (2.1 mg/ml, 4.3 mM) (△).

thermore, when the mixed micellar solution was diluted with buffer containing the intermicellar concentration (imc) of glycocholate (2.1 mg/ml, 4.3 mM), the micellar sizes did not change and no transition was observed. This is shown by the lower horizontal line in Fig. 1.

Micelle to vesicle transition in serum

In an attempt to investigate the possibility that the micelle to vesicle transition (Fig. 1) can also occur in the presence of blood components, we examined the effect of dilution with four different concentrations of human serum (5%, 10%, 50% and 100%) on the mean hydrodynamic diameter, $\bar{d}_h$, values of glycocholate-egg phosphatidylcholine mixed micellar colloids. (Fig. 2A and B). The effect of HDL particles present in serum on the size analysis was assumed to be insignificant since it only represented approx. 4% of serum components.

The transition patterns in 5% and 10% serum (Fig. 2A and B) were similar to that observed with buffer alone (Fig. 1). However, the growth of micelles was faster and the transition occurred at a lesser dilution (dilution factor of 7) in the presence of serum. By comparison to pure buffer as a diluent, the vesicle formation was slower and the final vesicle sizes were bigger when serum was included in the dilution media. As observed in the pure buffer system, the presence of a hydrophilic drug in serum did not affect the transition pattern and the micellar size remained constant in the presence of glycocholate at imc in diluent serum, as shown in the horizontal lower lines. Dilution of glycocholate-egg phosphatidylcholine mixed micelles with 50% and 100% serum strongly interfered with the size divergence, so that $\bar{d}_h$ seemed to be unchanged over the entire range of total lipid concentrations (not shown). Furthermore, it was observed that the turbidity of the solutions did not significantly change with dilution.

The turbidity of serum itself interfered the light-scattering properties of bile salt-egg phosphatidylcholine aqueous solutions. Based on the reports [13,14] which showed that the filtration process did not affect the particle morphology, all samples in serum were filtered through 1.0 μm disposable membrane filters (Gelmanscience, AcroDisc CR) before the QELS measurements were performed. However, to test the possibility that the filtration process might remove or change serum proteins, and so interfere with a possibly important factor in plasma for the transition, some samples taken before and after filtration were analyzed on SDS PAGE. The results are shown in Fig. 3. The intensities and the separation patterns of proteins including those with M_r 66000 and 28000 (serum albumin and apolipoprotein A-I, respectively) were almost identical throughout the lanes. Therefore it is clear that there was no gross change in protein composition and protein concentrations upon filtration, indicating that most serum proteins were preserved after filtration.

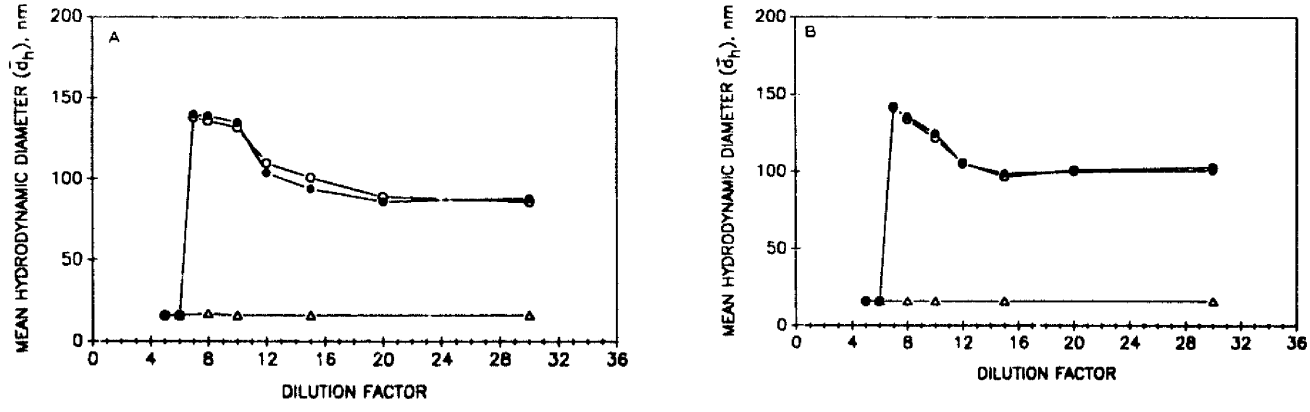

Fig. 2. Effect of dilution on mean hydrodynamic diameter, $\bar{d}_h$, of glycocholate-egg phosphatidylcholine mixed micellar solution (initial total lipid concentration = 50 mg/ml, egg PC/GC molar ratio = 0.8) at 23°C in 5% (A), and 10% (B) human serum: diluent serum alone (○), diluent serum with cytosine arabinoside (●) and diluent serum containing glycocholate (2.1 mg/ml, 4.3 mM) (△).

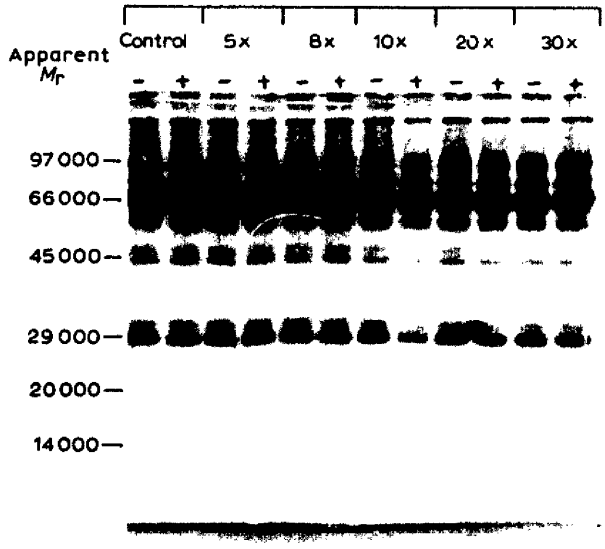

Fig. 3. SDS-PAGE analysis of samples diluted with human serum before (−) and after (+) filtration. Control represents serum alone and 5×, 8×, 10×, 20× and 30× are the dilution factors.

Trapped volume and trapping efficiency

Cytosine arabinoside was chosen as a model drug to calculate the trapping capacity and to compare it with the value reported in the literature. The amount of cytosine arabinoside experimentally entrapped inside vesicles can be determined from the calculated internal volume. The theoretical values of the trapped volume were determined from the diameters of the vesicles that were known from QELS measurements, simply using the relationship between vesicle diameter and internal volume [10,11]. We assumed that vesicles (dilution factor of 20 and 30) consisted of purely phospholipid bilayer since bile salts were diluted far below the critical micellar concentration.

Table I shows the trapped volumes and the trapping efficiencies of the vesicles prepared in three dilution media (buffer, 5% and 10% serum containing cytosine arabinoside) at two different lipid concentrations. Final vesicles (dilution factor of 30) formed in buffer had approx. 10% of trapping efficiency which is significantly higher when compared to that obtained by sonication of aqueous suspension of phospholipids (1–2% efficiency) [11]. Interestingly, the vesicles produced in 5% and 10% serum have approx. 2.5% of trapping efficiency. This may be due to binding and/or interaction of cytosine arabinoside and phospholipid bilayer with serum components. The trapping efficiency of the vesicles formed in buffer at the dilution factor of 20 was decreased to approx. 7%, indicating that the vesicles were not completely sealed and some leakage occurred.

TABLE I

Trapped volumes and trapping efficiencies of vesicles formed by dilution at two different lipid concentrations

The experimental procedure for calculating trapped volume (μl/μmol lipid) and trapping efficiency for cytosine arabinoside were described under Methods.

Sample		Lipid concn. (mg/ml)	Trapped-volume (μl/μmol lipid)	Trapping [a] efficiency (%)
dilution medium	dilution factor			
buffer	20	1.40	1.38	7.32 ± 0.22
	30	0.93	1.08	9.72 ± 0.28
5% serum	20	1.40	2.64	2.4 ± 0.10
	30	0.93	2.64	2.51 ± 0.12
10% serum	20	1.40	3.0	2.46 ± 0.09
	30	0.93	3.0	2.43 ± 0.11

[a] Numbers represent the results of three experiments (mean ± S.E.).

Electron microscopy of glycocholate-egg PC aqueous solutions

Transmission electron microscopy studies were conducted in order to examine the morphological changes in the particles produced upon dilution during the transition from mixed micelles to mixed vesicles. Fig. 4 shows the electron micrographs of three diluted samples, dilution factor of 5 (mixed micelles, A), 8 (mixed micelles, B) and 10 (mixed vesicles, C) with corresponding $\bar{d}_h$ values measured by QELS of 22, 99 and 140 nm, respectively.

As shown in Table II, there is close correlation between the mean hydrodynamic diameter, $\bar{d}_h$, measured by QELS (Fig. 1) and the TEM diameter (Fig. 4). We have also found that there was no morphological difference for the same three colloidal systems in the presence of cytosine arabinoside (not shown).

Discussion

We have shown that the micelle to vesicle transition of glycocholate-egg phosphatidylcholine particles spontaneously occurs upon dilution in physiological saline buffer (pH 7.5). The dilution of a mixed micellar solution up to the macroscopic mixed micellar phase limit results in an abrupt increase in the micellar size (Fig. 1). This micellar growth is also reflected in the development of turbidity in the transition region. This happens because upon dilution bile salts dissociate from mixed

TABLE II

The comparison of the particle diameter from quasi-elastic light scattering (QELS) and transmission electron microscopy (TEM)

Dilution factor	QELS diameter ($\bar{d}_h$, nm)	TEM diameter (nm)
5	22	10–40 (18) [a]
8	99	50–150 (91)
10	140	80–200 (134)

[a] Numbers in parenthesis represent the mean of 700–800 particles.

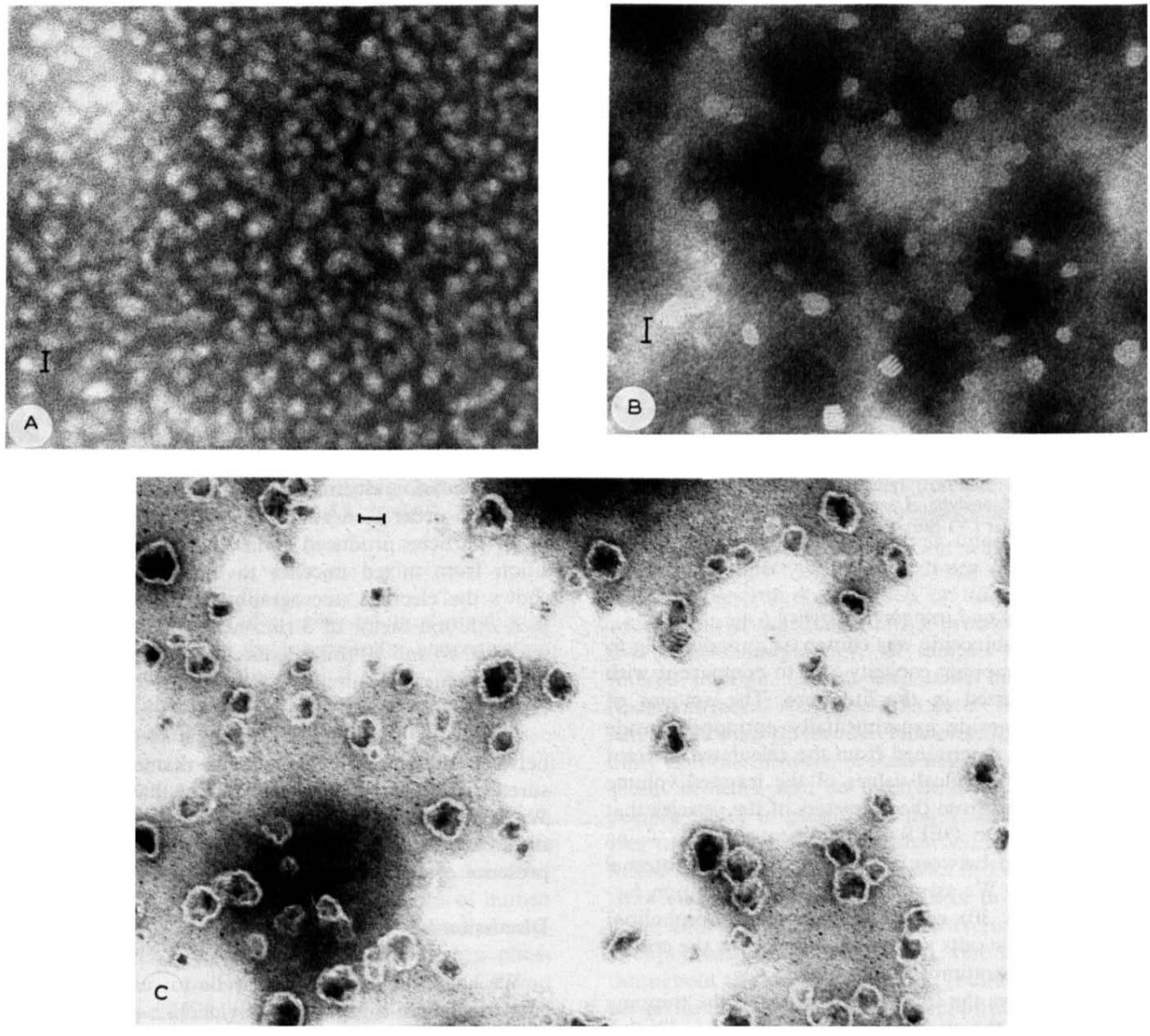

Fig. 4. Transmission electron micrographs of glycocholate-egg phosphatidylcholine mixed micelles and vesicles (egg PC/GC, molar ratio = 0.8) formed in isotonic buffer (pH 7.5), negatively stained with 1% phosphotungstic acid and photographed at 160000 × magnification. Bar equals 100 nm. (A) Mixed micelles (dilution factor of 5), (B) mixed micelles around the phase limit (dilution factor of 8) and (C) mixed vesicles (dilution factor of 10).

micelles in order to reestablish the imc and so maintain the equilibrium between the bile salt monomers in mixed micelles and those present in the intermicellar solution (imc) [3,16]. Thus, the relative amount of bile salts available for solubilizing egg phosphatidylcholine will decrease during the transition, resulting in micellar growth [4]. Over the transition there is a change in the shape of the colloidal particles from roughly spherical to rodlike, as demonstrated by our recent findings on similar systems using small-angle neutron scattering analysis [17]. In order to confirm that micellar growth is due to dissociation of bile salt molecules, we examined the effect of the intermicellar bile salt monomer concentration (imc) on the micellar size by keeping it constant in diluent media. The imc (2.1 mg/ml, 4.3 mM) of glycocholate-egg phosphatidylcholine mixed micelles at 23°C was deduced by a trial and error method [18]. This value is comparable to those reported by Schurtenberger et al. [5] and Mazer et al. [3]. The presence of imc in the diluent media (buffer or serum) completely suppressed the transition, indicating that bile salt-egg phosphatidylcholine mixed micellar size is mainly determined by the remaining amount of bile salts incorporated in the phospholipid bilayer.

A novel feature of using mixed micelles as a drug delivery device is to increase drug-loading into vesicles

which spontaneously form in vitro in a natural environment and so become more stable. Although the aggregation behavior of bile salt-egg phosphatidylcholine mixed micellar solutions has been studied physically and chemically in buffer, there is a lack of evidence about the vesicle formation in plasma. Quasi-elastic light scattering studies of glycocholate-egg phosphatidylcholine mixed micelles in the presence of 5% and 10% serum (Fig. 2A, B) suggest that there is a possibility to produce in vitro vesicles in the presence of blood components which may mimic self-antigens and thus induce immunologic tolerance [19]. It is however, obvious that there are some differences in the transition pattern between that in buffer and in the serum system. First, the macroscopic mixed micellar boundary limit is shifted to a lower dilution factor, and the particle growth within the mixed micellar region is very steep in serum. This may be due to significant binding of bile salt to serum components such as albumin [20,21] and lipoproteins [20,22] with a concomitant increase in egg PC/GC molar ratio in the micelles. Secondly, in diluted serum the final vesicle size was increased. This suggests that some serum components, presumably lipoproteins, may be distributed between the bilayers of vesicles and intervesicular aqueous space, thus altering the kinetics and the final sizes of vesicles formed by dilution of mixed glycocholate-egg phosphatidylcholine micellar solutions [5].

At two higher serum concentrations (50% and 100% serum) the micelle to vesicle transition was not observed. This must be due to the strong influence of serum components during the transition. It is not known at this stage which serum component(s) has a major interaction with the colloidal particles. However, it is reasonable to speculate that a possible mechanism for the suppressing role of serum components could be either to solubilize the phosphatidylcholine molecules or to prevent the dissociation of bile salt molecules from mixed micelles. This suppression of the transition formed here is consistent with the result of a recent study [5], in which QELS analysis of taurocholate-egg phosphatidylcholine colloids in buffer solution containing 0.5 mg/ml of apolipoprotein A-I showed the complete abolishment of the transition.

It is clear that cytosine arabinoside does not interfere with the transition in aqueous media, suggesting that the supramolecular rearrangement within micellar and vesicular zone is not affected by hydrophilic compounds.

To confirm our QELS results we prepared phospholipid vesicles in the presence of cytosine arabinoside. Approx. 10% of the drug was trapped inside the aqueous compartment of the vesicles formed in buffer. It is of interest that the trapping efficiency decreased to approx. 2.5% in the presence of low concentrations of serum, suggesting that cytosine arabinoside may bind to serum albumin [15] and/or that other serum components may be involved in drug-encapsulation by interaction with phospholipid bilayer during the transition [7].

We also found that the drug concentrations trapped in the particles formed both in 50% and in 100% serum were insignificant, which was comparable to the QELS results. This new observation can be answered by further studies with pure individual serum proteins.

Further evidence that the micelle to vesicle transition is associated with the change in the size and shape of the colloidal particles is provided by the electron microscopy. It is evident that the morphology of colloid particles changed significantly upon dilution and that vesicles form above the phase limit (Fig. 4). It is also seen in Fig. 4C that particles are interacting with each other at the transition region, presumably by lipid exchange. It is reported that low concentrations of bile salts bind to membrane and increase the spontaneous rate of intervesicular phospholipid transfer [23]. It is likely that the equilibrium vesicle sizes can be reached by a net lipid transfer from smaller to larger vesicles in the presence of bile salts [24]. This would occur either by molecular rearrangement of mixed vesicles or by significant dynamic alterations to the phospholipid components [25].

We conclude that the quasi-elastic light scattering results obtained in this study, along with trapped drug determinations and the electron micrographs of the colloidal samples, support our model that vesicles are spontaneously formed in vitro from bile salt-egg phosphatidylcholine mixed micelles upon aqueous dilution and that can be an alternative means to sonication in loading drugs.

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