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Bile salt damage of egg phosphatidylcholine liposomes *

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Physiochemical damage of egg phosphatidylcholine liposomes, caused by the salts of three bile acids, chenodeoxycholic acid, ursodeoxycholic acid, and cholic acid, has been investigated. Of the three bile salts, that of chenodeoxycholic acid was the most destructive, and the effect of the damage was examined by monitoring the induced 6-carboxyfluorescein release from the liposomes. For all three of the bile salts and under the experimental conditions, the minimum (effective) concentrations causing the 6-carboxyfluorescein release were below their critical micelle concentrations. In the case of the salt of chenodeoxycholic acid, the presence of cholesterol in the liposomal bilayers did not show any significant effect on the induced 6-carboxyfluorescein release, while, for the salts of ursodeoxycholic acid and cholic acid, the presence of cholesterol tended to depress the release. Permeation of bile salts into the membranes of liposomal bilayers made these membranes more fluid, and this fluidity was monitored by measuring the change in fluorescence polarization using 1,6-diphenylhexatriene entrapped in the liposomes. Coating the liposomes with polysaccharides, to make them more hydrophobic, led to their easier lysis by the bile salts.

Introduction

The majority of gallstones are comprised, for the most part or completely, of cholesterol. Cholesterol is itself insoluble in water but it can be cosolubilized in bile upon the formation of mixed micelles or bilayer disks comprising cholesterol, phospholipids, and bile salts [1–5]. A dependable method for dissolving such gallstones is the oral administration of certain bile salts which concentrate therein and influence the secretory rates of other biliary lipids [6]. However, the in vitro study using artificial bile showed that the cholesterol solubilizing ability of ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid) was far less than that of chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid) [7]. The salt of the primary bile acid, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), does not dissolve gallstones [8,9].

On the other hand, liposomes have gained wide acceptance in chemotherapy as potential drug carriers [10,12]. However, there still remain several problems to be overcome. One of the disadvantages of using liposomes as drug carriers is their instability in blood [13] and in the gastrointestinal tract [14]. The physicochemical stability of liposomes in the gastrointestinal milieu is largely affected by the surface active bile salts [14,15]. Hence, it is hoped that a knowledge of the ability

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of bile salts to interact with liposomes will assist in the design of an improved liposomal drug carrier for oral administration.

Materials and Methods

Materials. Egg yolk phosphatidylcholine was isolated and purified from egg yolk as described in the literature [16,17] and found to be pure by thin-layer chromatography (using a precoated silica gel plate (Spotfilm Tokyo Kasei, Tokyo) and development with chloroform/ methanol/ water (65 : 25 : 4, v/v)). Ursodeoxycholic and chenodeoxycholic acids were a kind gift from Professor H. Igimi, School of Medicine, Fukuoka University. Cholic acid was commercially available from Nakarai Chemicals, Kyoto, Japan, and was recrystallized from ethanol/hexane before use. 6-Carboxyfluorescein, the marker entrapped in the liposomes, was purchased from Eastman Kodak, Rochester, NY, and 1.6-diphenyl-1,3,5-hexatriene was from the Aldrich Chemical Co., Milwaukee, WI. *O*-Palmitoylpullulan, whose weight average molecular weight is 50000 and whose primary alcohol moieties are substituted by 1.8 palmitoyl residues per 100 glucose units, was the same as that used in previous works [18,19]. This polysaccharide, chemically modified in part, was used to toughen the liposomes against external stimuli such as the changes in temperature, pH, ionic strength, and/or polarity or the interaction with lipases and to make them behave more like the cell walls of plant and bacterial cells [18,19].

Preparation of liposomes. Small, unilamellar vesicles, encapsulating 6-carboxyfluorescein in the interior water phase, were prepared by the method described elsewhere [20–22].

Polysaccharide-coated liposomes were prepared as follows [18,19]. A sonicated small, unilamellar vesicle suspension (4.0 ml prepared from 2.5 mg of egg phosphatidylcholine) containing 200 mM 6-carboxyfluorescein in the interior water phase of the liposomes, in 20 mM Tris-HCl buffer (pH 8.6), was mixed with 20 mM aqueous Tris-HCl buffered solution (1.0 ml) containing 10 mg of *O*-palmitoylpullulan and 200 mM sodium chloride and kept for 30 min at 20.0°C, under stirring. After gel filtration on a Sepharose 4B column (11.6 cm × 40 cm), the polysaccharide-coated liposomes, loaded with 6-carboxyfluorescein, were isolated [18,19]. The liposome concentration was determined as inorganic phosphate according to Allen's procedure [23].

Monitoring the release of 6-carboxyfluorescein. A 3 ml sample of 0.1 mM 6-carboxyfluorescein-loaded liposomal suspension in 20 mM Tris-HCl buffer (pH 8.6) containing 200 mM NaCl was placed in a thermoregulated cuvette cell and preincubated for 10 min at 37.0°C. During the preincubation, we found no spontaneous release of the marker, 6-carboxyfluorescein. The carboxyfluorescein-release, induced by adding an appropriate amount of bile salt solution (1–100 μl) was followed by monitoring an increase in the fluorescence intensity, I, at 520 nm, by excitation at 470 nm on a Hitachi 650-10S fluorescence spectrometer. The intensity of the total amount of the liposome-encapsulated marker, I_∞, was determined after complete destruction of the liposomes with 100 μl of Triton X-100 (10% v/v). The percentage of 6-carboxyfluorescein released was obtained by the following equation: % 6-carboxyfluorescein release = $((I_t - I_0)/(I_\infty - I_0))100$, where t and 0 refer to the times of sampling and to the instant when the bile salt solution was added to the liposomal suspension. All the measurements were duplicated or triplicated if necessary.

Measurement of surface tension. The equilibrium surface tension values of the bile salt solutions were measured on a White Electrical Co. torsion balance type 'OS' with a 13-mm platinum ring. A dual-walled vessel was maintained at 37.0°C by water circulation from a Grant SC10 circulator. Values of γ were averaged from at least ten readings after waiting for them to reach a steady value. All measurements were duplicated.

Steady-state fluorescence polarization measurement. Steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene intercalated in liposomal bilayers was measured as a function of bile salt concentration on a Union Giken fluorescence polarization spectrophotometer FS-501S, which was connected to a Sord Microcomputer M200 Mark II system to control the measurement conditions and collect all the data. Diphenylhexatriene was excited at 360 nm and its fluorescence was detected using a sharp cut-off filter L-39 (Hoya Glass Works, Tokyo) to shut out the light of

wavelengths less than 370 nm.

Liposomes containing 1,6-diphenyl-1,3,5-hexatriene were formed and isolated by the same method as that described previously [24,25]. To prepare a thin film, a chloroform solution of phosphatidylcholine was mixed with 1,6-diphenyl-1,3,5-hexatriene dissolved in tetrahydrofurane. The molar ratio of 1,6-diphenyl-1,3,5-hexatriene to the lipid was maintained at less than or equal to 130 [26] throughout all the runs. Such a ratio of the fluorescent probe to lipid in the lipid bilayer membranes is enough to affect the surrounding lipids and to give information about the membrane fluidity but has no effect on the fluorescence polarization results [27]. For the measurement, the resulting liposome suspension was further diluted to give a lipid concentration of $5.4 \cdot 10^{-5}$ M, at which concentration the effect of light scattering on the fluorescence polarization measurements was negligible [28,29].

The following relationship was employed to obtain the steady-state fluorescence polarization (P) of the probe:

$$P = \frac{I_{VV} - C_f \cdot I_{VH}}{I_{VV} + 2C_f \cdot I_{VH}}$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of the excitation (first letter) and analyzer (second letter) polarizers, respectively. $C_f (= I_{VH}/I_{HH})$ is the grating correction factor [24,25]. It then follows that the membrane fluidity is proportional to the reciprocal of the P value.

Results

The effect of concentration on the surface tension properties of cholic acid, ursodeoxycholic acid and chenodeoxycholic acid was examined in 20 mM Tris-HCl solution (pH 8.6) containing 200 mM NaCl at 37.0°C.

The sharp breaks in the curves of surface tension against log[bile salt], shown in Fig. 1, determine the values of the critical micelle concentration, under these conditions, and decrease in the order of cholic acid (5.9 mM) > ursodeoxycholic acid (2.8 mM) > chenodeoxycholic acid (1.7 mM) (absolute data are not shown). It should also be noted that the equilibrium surface tension in the plateau region, when aggregation is complete, is higher for ursodeoxycholic acid than for the values of cholic acid or chenodeoxycholic acid, which are equal.

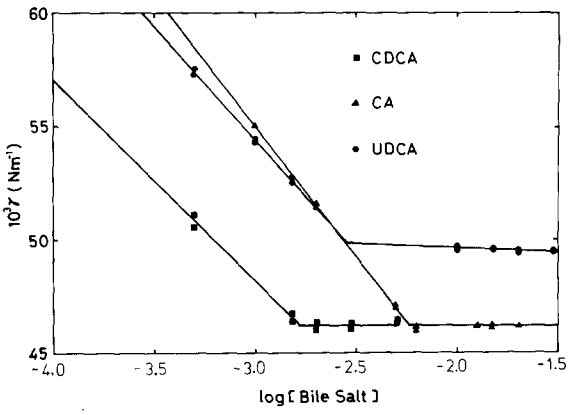

Fig. 1. Equilibrium surface tension values of chenodeoxycholic acid (CDCA), cholic acid (CA), and ursodeoxycholic acid (UDCA) plotted against the logarithmic concentration of the steroids in 20.0 mM Tris-HCl buffer (pH 8.6) containing 200 mM NaCl at 37.0°C.

Fig. 2 shows the percentage 6-carboxyfluorescein-release five minutes after the addition of the bile salts to a 0.1 mM small, unilamellar vesicle suspension, both in the absence and presence of cholesterol, after preincubation for 10 min at 37.0°C in 20 mM Tris-HCl buffer (pH 8.6) containing 200 mM NaCl. From the data it is seen that the order of effectiveness of the bile salts to effect lysis of the liposomal bilayers is chenodeoxycholic acid ≫ ursodeoxycholic acid > cholic acid. This is the reverse sequence to their critical micelle concentration. At first glance the effect of adding cholesterol into the liposomal bilayer seems to be drastic only in the case of cholic acid. However, then we determined the minimum effective concentration of cholesterol needed for the bile salts to perturb the membranes. The values were estimated graphically, using Fig. 2, from the intersection of a tangent line, passing through the middle point of the sigmoidal curve, with the abscissa. The concentrations needed in solutions of 0, 25 and 50 mol% cholesterol content, respectively, were: chenodeoxycholic acid, 0.20, 0.26 and 0.26 mM; ursodeoxycholic acid, 1.25, 1.60 and 0.78 mM; cholic acid, 1.00, 2.00 and 1.40 mM. We

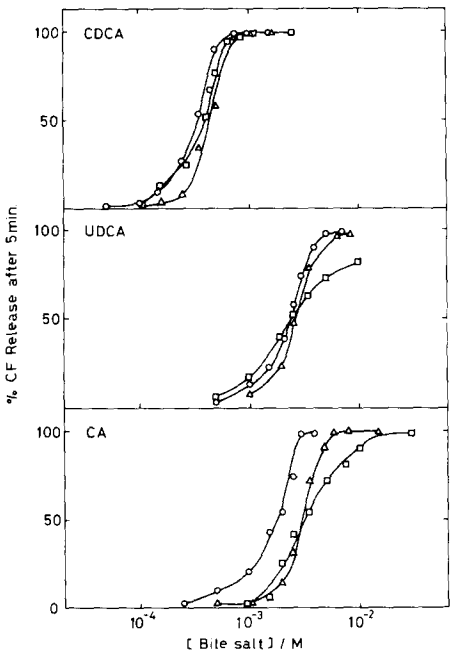

Fig. 2. Effect of cholesterol content on the bile salt-induced leakage, after 5 min, of 6-carboxyfluorescein (CF) from a 0.1 mM egg phosphatidylcholine small, unilamellar vesicle suspension in 200 mM NaCl at 37.0°C: ○———○, without cholesterol; △———△, containing 25 mol% of cholesterol and 75 mol% phosphatidylcholine; and □———□, containing 50 mol% of cholesterol.

now find a different phenomenon, as follows. In the case of chenodeoxycholic acid, even if the cholesterol content were increased up to 50 mol% of that of phosphatidylcholine, the efficiency of perturbation of the membranes was not significantly altered. On the other hand, in the case of ursodeoxycholic acid and cholic acid, addition of cholesterol into the liposomes tended to depress the 6-carboxyfluorescein-release.

Fig. 3 shows the effect of the addition of bile salts on the steady-state fluorescence polarization of a 0.1 mM small, unilamellar vesicle suspension. It can be seen that the fluidity of the membranes increases upon the permeation of the bile salts into the liposomal bilayers. The change in fluidity was measured by monitoring the change in P value, $P_0 - P$, before and after the injection of the bile salt into the egg phosphatidylcholine small, unilamellar vesicle suspension containing 1,6-diphenyl-1,3,5-hexatriene in the bilayers. The fluidity increased as a function of bile salt concentration, in a manner very similar to that of the bile salt-induced 6-carboxyfluorescein-release (Fig. 2).

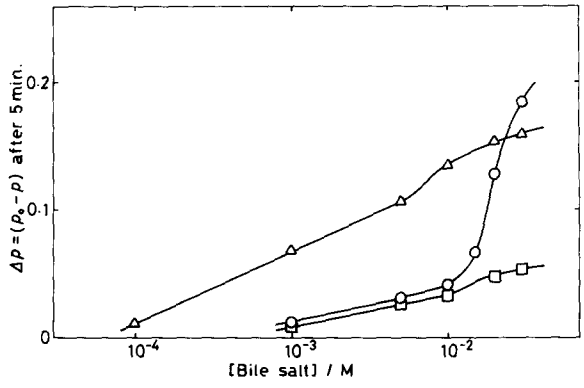

Fig. 3. Change in fluorescence polarization ($P_0 - P$), or apparent membrane fluidity, induced by the addition of bile salts, plotted against the bile salt concentration. Small, unilamellar vesicle suspensions (0.1 mM) were prepared from egg phosphatidylcholine containing 20 mM Tris-HCl (pH 8.6) with 200 mM NaCl at 37.0°C; △———△, chenodeoxycholic acid; □———□, ursodeoxycholic acid; and ○———○, cholic acid.

In Fig. 4 is shown the effect of coating the liposomes with *O*-palmitoylpullulan. The percentage 6-carboxyfluorescein-release, 5 minutes after the addition of bile salts to a 0.1 mM small, unilamellar vesicle suspension of hardened liposomes is compared with that in the presence of

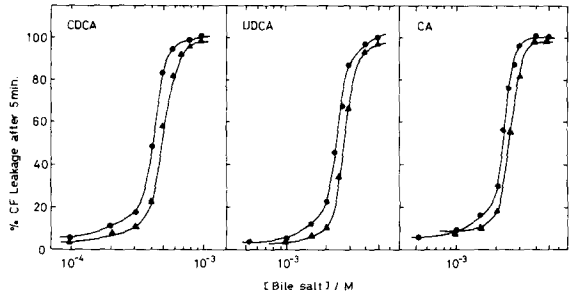

Fig. 4. Effect of coating the liposomes with *O*-palmitoylpullulan on the induced leakage of 6-carboxyfluorescein (CF). The percentage leakage of 6-carboxyfluorescein from 0.1 mM egg phosphatidylcholine small, unilamellar vesicles (4.0 ml) coated with 10 mg of *O*-palmitoylpullulan (see Materials and Methods) was monitored 5 min after injection of the aqueous bile salt solution in 20.0 mM Tris-HCl (pH 8.6) containing 200 mM NaCl at 37.0°C; ●———●, *O*-palmitoylpullulan-coated liposomes and ▲———▲, conventional liposomes without polysaccharide coating.

uncoated liposomes. The experimental conditions for the data in Figs. 3 and 4 were the same as those given in Fig. 2.

Discussion

Although many investigations have been made on the physicochemical properties of bile salts and these have been extensively reviewed [30,31], such studies have not normally included the salt of ursodeoxycholic acid. Moreover, Carey and Small [30] have clearly documented evidence that any physicochemical measurement, such as the determination of the critical micelle concentration, is very sensitive to the effect of pH, temperature, and increasing counterion concentration. Thus, any attempt to compare or correlate the effect of bile salt structure with micellar or other properties must be made using data obtained under identical environmental conditions. We have therefore used a standard set of conditions, i.e. a 20 mM Tris-HCl solution (pH 8.6) containing 200 mM NaCl at 37.0°C, for all kinetic and physical measurements described herein.

Although bile salts do not exhibit the distinct end-to-end polarity which characterizes many aqueous surfactants they do exhibit planar polarity. Space-filling molecular models (Carey-Pauling-Koltun) suggest that the protruding methyl groups lie above the plane of the steroidal skeleton, while the hydrophilic groups lie below this plane. The curvature of the skeleton brings the hydroxyl groups at the 3α and 7α positions quite close together and the mobility of the side chain allows its polar group to lie in the same plane as the hydroxyl groups. While chenodeoxycholic acid still has two OH groups lying in the hydrophilic plane, ursodeoxycholic acid, with its 7β-OH group, no longer exhibits such distinct planar polarity. Carey et al. [32] have demonstrated that, while it requires 12 molecules of chenodeoxycholic acid to solubilize one molecule of cholesterol, it requires 298 molecules of ursodeoxycholic acid. The values given for solubilization of one molecule of cholesterol by cholic acid are somewhat variable, but generally lie within the range 35–50 [33–35], although Ekwall [36] reported an approximate value of 16. However, comparison of these solubilization values with those of the corresponding critical micelle concentrations reveals that the efficiency of these three bile acids to solubilize cholesterol bears no direct relationship to their ability to form micelles, although it does seem to be related to the polarity of the molecules. Moreover, this efficiency is not proportional to their usefulness as therapeutic agents in gallstone dissolution. We therefore studied the ability of these salts to perturb the egg phosphatidylcholine liposomal bilayers. If we limit the discussion to consideration of the present experimental conditions, then the effective concentrations are always lower than the respective critical micelle concentration values and it is the monomer of the bile salt itself, not the micelles or the aggregates, which permeates into and perturbs the egg phosphatidylcholine liposomal bilayers, Fig. 2 and the text. Small [30,37] has noted that the critical micelle concentration of bile salt solutions is markedly decreased by the addition of phosphatidylcholine, but the consequences of this observation are not applicable in this present situation. When we discuss the effect of phosphatidylcholine on the critical micelle concentration of the bile salts, the most important point to be considered is the methodology used in mixing the phosphatidylcholine and bile salt. If one were firstly to prepare a mixture of lipids and bile salts or, alernatively, firstly to add the lipids into an aqueous solution of bile salt, and secondly to measure the critical micelle concentration, then this value should certainly be lowered by the presence of the lipid. On the other hand, if one were firstly to prepare the suspension and secondly add the bile salts into the suspension (as we have done), the critical micelle concentration should be little affected by the presence of the lipid which is present almost exclusively in the form of a rather stable lipid (liposome) assembly and only very rarely as a dispersion of monomer. If the concentration of lipid monomers extracted from the liposomes by the bile salt micelles were so high as to affect the critical micelle concentration of the bile salt, then the liposomes would be almost completely destructed and all the 6-carboxyfluorescein would be released. Since this situation is not observed, we believe we have justification for the statement made above, about the relative magnitude of the minimum, effective concentration of bile salt necessary to damage the

liposomal membranes and the critical micelle concentration. It should be stressed, however, that the concentration of bile salt which causes lysis will be affected by the surface area of the liposomes, i.e. by the concentration of phosphatidylcholine, and so the conclusion can be related only to the present experimental conditions.

The most interesting finding of this work is that for all three of the bile salts, even for that of chenodeoxycholic acid, the 6-carboxyfluorescein leakage (namely, lysis of the liposomal membranes) starts at a molar ratio of bile salt to lecithin higher than, or close to, 3 : 1, which corresponds to the mean physiological ratio for human gallbladder bile. Previous works show that, at a bile salt/phosphatidylcholine molar ratio exceeding 2 : 1, mixed micelles or mixed-disk aggregates are formed [2,3]. Hence it seems that the lysis of the liposomes is accompanied by the morphological change of the aggregates from a lipid bilayer to a mixed micellar structure.

Fig. 2 also shows the effect of the cholesterol content of the liposomal bilayers on the bile salt damage of the liposomes. The curves for 50 mol% cholesterol seem to be different from the other two curves and in the case of cholic acid, the intercept is clearly affected. A plausible explanation would seem to be that when bile salts, cholesterol and lipids interact altogether and form a specific molecular assembly, it is to be expected from the corresponding phase diagram of the three components that, in our present system, the co-interaction among the three components should be more affected by the concentration of one of these than by the others. The effect of the bile salts in the perturbation of the lipid membranes may become rather small at 50 mol% cholesterol content. In other words, 50 mol% cholesterol may be enough to act like a buffer for the absorbed bile salts which permeate and causes the lysis. This effect is also closely related to the well-known one of cholesterol, when added into lipid bilayer membranes, altering the fluidity of the membranes and abolishing their distinct phase transition.

It is reasonable to expect that the extent and nature of the membrane perturbation should be affected by both the structure of the bile salts and the physicochemical properties of the membrane, such as the cholesterol content, the structure of the lipid, the temperature, the ionic strength, the pH of the buffer phase, and so forth. Therefore, it is necessary to make comparisons between both the slope parameters (see Fig. 2) and the intercept parameters obtained in the extrapolation method for a sigmoidal phenomenon (see Results).

In order to understand the relationship between the membrane fluidity and the bile salt damage of liposomes [14,15], the membrane fluidity, as estimated from the steady-state fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene in bilayers [24,25], was investigated as a function of the bile salt concentration (Fig. 3). The membrane fluidity increased as a function of bile salt concentration in a manner very similar to that of the bile salt-induced release of 6-carboxyfluorescein from the liposomes (Fig. 2). This means that the damage of the liposomes caused by the bile salts is closely related to the membrane fluidity brought about by permeation of bile salts into the liposomal bilayers. The present finding on membrane fluidity is exactly identical to the result reported by Low and Coleman [15], who found by using erythrocytes from different species differing in their lipid composition that membranes of high fluidity were lysed more readily than those of low fluidity. Another interesting point is the correlation among the chemical structure of the three bile salts, the induced 6-carboxyfluorescein-release and the induced membrane fluidity. The hydroxyl group at C-7 of chenodeoxycholic acid has an α configuration, while that of ursodeoxycholic acid has a β one, and this difference is the only one between the structure of the two bile acids. Ursodeoxycholic acid, with its marked difference in polarity, is not expected to perturb the surface structure as much as the other two species and this is the result that we find in its higher surface tension values (Fig. 1). Vochten and Joos have demonstrated a similar phenomenon for the surface tension properties of the salts of cholanoic acid compared with cholic acid [38]. Even though the configurational difference between chenodeoxycholic acid and ursodeoxycholic acid is small, the differences in the induced fluidity of the membranes and the 6-carboxyfluorescein-release, upon the interaction of the bile salts with the bilayers, are relatively large. Moreover, the presence of a further hydroxyl group at C-12 in cholic acid also has a significant effect

on perturbation of the lipid bilayer membranes, a fact which is evident from the large difference in both the induced 6-carboxyfluorescein release and fluidity between the salts of chenodeoxycholic acid and cholic acid. In other words, sodium cholate, bearing the additional hydroxyl group, may form different aggregates by itself and/or interact with lipid molecules in a different manner in the liposomal bilayer membranes, compared with the behavior of the other two bile salts.

Two of the present authors (J.S. and K.I.) have reported recently that coating the outermost surface of liposomes with chemically modified polysaccharides, such as *O*-palmitoylpullulan and *O*-palmitoylamylopectin, brings about an increase of the stability in the chemical, physicochemical, and enzymic destruction of liposomes [18,19]. We therefore expected that we would be able to show that the stability of such coated liposomes in the gastrointestinal milieu, when the liposomes were orally administered, would be increased. We therefore coated the surface of the liposomes with *O*-palmitoylpullulan (see Materials and Method) and investigated their resistance to bile salt damage. Unfortunately, however, and contrary to what should be expected, coating the surface of liposomes with polysaccharide resulted in an easier 6-carboxyfluorescein-release for all of the bile salts (Fig. 4). When the liposomes were coated with a more branched polysaccharide, *O*-palmitoylamylopectin (with molecular weight 112000 as substituted by 4.9 palmitoyl residues per 100 glucose units) [18,19], the percentage 6-carboxyfluorescein release at the same 6-carboxyfluorescein concentration was even more enhanced, compared with the case of liposomes coated by the more linear polysaccharide, *O*-palmitoylpullulan (though data are not shown). These results suggest that an increase in the hydrophobicity of the liposomal surface, by coating it with polysaccharides, leads to more favorable interaction of the bile salts with the liposomes and consequently to easier lysis of the liposomes. This interaction becomes stronger when the liposomes are coated with a more branched polysaccharide such as *O*-palmitoylamylopectin. The disappointing conclusion which arises from these present results is that coating liposomes with polysaccharide derivatives will fail to make the liposomes tough, in the gastrointestinal milieu, when they are employed in oral administration.

Acknowledgements

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