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A Two-Step Model of Disintegration Kinetics of Liposomes in Bile Salts

MARIKO NAGATA, TOSHIHISA YOTSUYANAGI* and KEN IKEDA

Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan

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The disintegration kinetics of egg phosphatidylcholine small unilamellar liposomes (SUV) in sodium deoxycholate (SDOC) and sodium cholate (SC) was investigated as a mimic of the disintegration behavior of orally administered liposomes in the intestine. The disintegrating action of the bile salts was followed by measuring turbidity changes with a stopped-flow apparatus, from which the pseudo-first-order disintegration rate constant (k_{obs}) was calculated as a function of bile salt concentration (up to 25 mM). The disintegration rate of the SUV in SDOC was considerably higher than that in SC, and the k_{obs} increased with a tendency to reach a plateau in SDOC but not in SC. At 20 mM bile salts, the disintegration half-life of the SUV (1.5 mM phosphorus) was 0.003 s in SDOC and was 0.69 s in SC. A model in which the penetration–saturation step of bile salt molecules and the subsequent lamellar–micellar transition step are involved was applied to analyses of the disintegration kinetics. The results showed that SDOC molecules penetrate into the bilayer structure faster than SC molecules by a factor of 5×10^2. This is probably due to the difference of molecular surface available for hydrophobic interaction.

Keywords——liposome; bile salt; disintegration; kinetics; model; egg phosphatidylcholine; sodium deoxycholate; sodium cholate

Introduction

In studies to explore the advantages of liposomally-associated drugs, liposomes have been mainly administered by parenteral routes. However, the use of liposomes as drug vehicles has also been extended to oral administration,[1-5] though the absorption mechanism of liposomal drugs remains a subject of controversy.

In the intestinal tract, it is unlikely that the physical integrity of liposomes is maintained because of the disintegrating or solubilizing ability of physiological surfactants, *i.e.* bile salts.[6-9] Bile salts form mixed micelles with phosphatidylcholine under normal physiological conditions, contributing to the solubilization of phosphatidylcholine as well as cholesterol and fat ingested. Naturally, drug-loaded liposomes are also likely to be disintegrated by bile salts. It is therefore important to assess the disintegration rate of liposomal vehicles in bile salts, in order to obtain some insight into the fate of orally administered drug-loaded liposomes.

In the present work, the disintegration kinetics was followed by measuring turbidity changes of egg phosphatidylcholine small unilamellar liposomes in the presence of sodium deoxycholate and sodium cholate as model systems.

Experimental

Materials——Phosphatidylcholine (PC) was extracted from egg yolk and purified by column chromatography on silicic acid (Mallinkrodt, St. Louis, MO).[10] Sodium deoxycholate (SDOC) and sodium cholate (SC) (Sigma) were chromatographically pure and were used without purification. All other chemicals were of reagent grade.

Preparation of Liposomes——PC was dispersed in Tris buffer (pH 7.4) as described elsewhere.[11] Multilamellar liposomers were then sonicated for 60 min by a probe-type sonifier under a nitrogen stream. Titanium fragments from the probe were removed by centrifugation at 10000 × *g* for 10 min. The resulting small unilamellar suspension (SUV) was allowed to equilibrate at room temperature for several hours and filtered (0.1 μm, cellulose nitrate, Toyo Roshi Co., Tokyo). The PC content was assayed based on phosphorus.[12]

Kinetic Measurement——The disintegration kinetics of liposomes in bile salt was followed at 25 °C by measuring the turbidity change at 400 nm with a stopped-flow apparatus (Model RA-401, Union Giken, Osaka) equipped with a UV-VIS detector and a microcomputer (M223 MarkIII, Sord Computer Systems). It was assumed that the turbidity changes directly reflect the disappearance rate of the vesicles since the sizes of the resulting mixed micelles are markedly reduced in the presence of an excess of bile salts.[13] The reaction was carried out in an excess of bile salt so that pseudo-first-order kinetics hold. An equal volume of the liposome suspension containing 3.0 mM phosphorus was quickly mixed with bile salt solutions at various concentrations up to 50 mM (Tris buffer, pH 7.4). The concentrations of lipid and bile salt are diluted to half in a cell of the apparatus. Slight increases (2—3%) relative to the initial turbidity caused by bile salts were observed when the concentration of the surfactant was low. The point at which the turbidity returned to the original level was assumed to be $t=0$ for kinetic calculations. Computer-generated curve fitting gave pseudo-first-order rate constants. It was confirmed that PC and bile salts have no absorption at 400 nm and the final turbidity becomes zero after complete disintegration.

Results and Discussion

Disintegration Kinetics of Liposomes

Figure 1 shows typical examples of the disintegration behavior of the SUV liposomes in SDOC followed by measuring the turbidity changes. The apparent disintegration rate of liposomes is generally dependent on the vesicle size when the liposomes are multilamellar, since the bilayers are disintegrated by being stripped off one by one from the outermost shell to the next inner one.[14] The liposomes employed in this study were small unilamellar ones throughout. Therefore, the observed rate can be regarded as an ultimate disintegration rate for a single bilayer and serve as a parameter of the disintegration process of the bilayer in different surfactants. The rate of the reaction of liposomes and bile salt is expressed in terms of turbidity changes as follows:

$$-\frac{d[T]}{dt}=k_b[BS][T] \qquad (1)$$

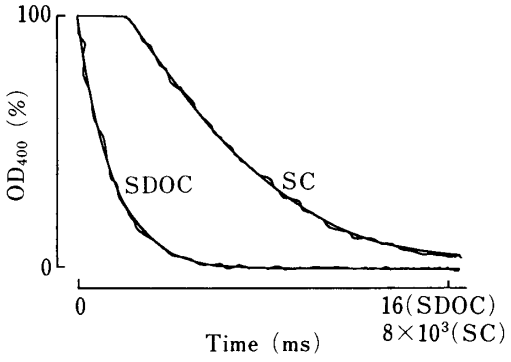

Fig. 1. Typical Examples of the Disappearance of Turbidity of Small Unilamellar Liposomes in SDOC and SC

Smooth lines indicate the computer-generated fitting curves for the experimental curves where first-order kinetics was assumed. Egg phosphatidylcholine (1.5 mM phosphorus) was mixed with bile salt (25 mM) at pH 7.4 and 25 °C.

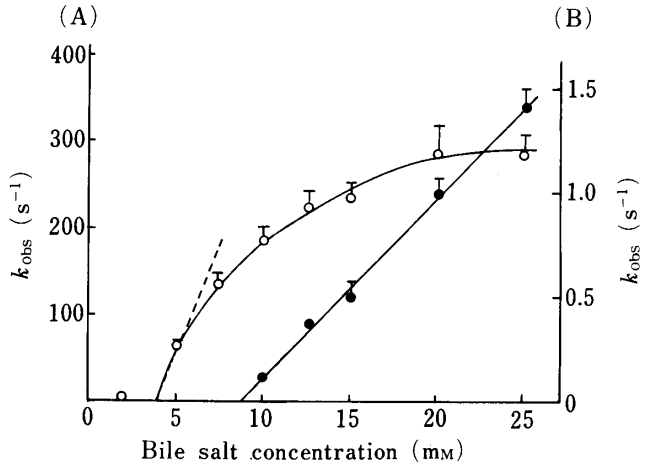

Fig. 2. Plot of the Pseudo-First-Order Disintegration Rate Constant (k_{obs}) as a Function of Bile Salt at pH 7.4 and 25 °C

(A), SDOC (○); (B), SC (●). Egg phosphatidylcholine: 1.5 mM (phosphorus). Each point represents the mean ± S.D. ($n=3$—6).

where $[T]$ is the turbidity and replaces the liposome concentration, and $[BS]$ is the concentration of bile salt. k_b is the second-order rate constant.[7,14] If the concentration of $[BS]$ is in excess of the concentration of lipid and remains almost unchanged during the disintegration process, we have

$$-\frac{d[T]}{dt}=k_{obs}[T] \tag{2}$$

The linear dependence of log $[T]$ on time will give the pseudo-first-order rate contant (k_{obs}) for the disintegration. The experimental conditions were set up so as to satisfy the assumptions. The turbidity changes in SDOC and SC followed pseudo-first-order kinetics, from which the values of k_{obs} were calculated.

Figure 2 shows plots of k_{obs} against the concentrations of SDOC and SC up to 25 mM. The liposomes were little damaged by the bile salts up to certain concentrations, beyond which k_{obs} increased with a tendency to reach a plateau in case of SDOC and linearly increased in the case of SC. It is clear that there was a considerable difference of the disintegration reaction between the two bile salts. The intercept on abscissa shows the minimal concentration of a bile salt required to break the bilayer structure. The bilayer to micellar transition, *i.e.* disintegration or solubilization, should occur in much lower bile salt concentrations than those used in this study since the molar ratios of the solubilization were 1:1 for SDOC and 1.8:1 for SC at equilibrium.[15] The minimal concentration should be therefore regarded as a critical concentration for bursting which was extrapolated from the rapid first-order disintegration process represented by k_{obs}. As the minimal concentration of the bile salt (MC) apparently depends on the PC concentration, it should be divided by the lipid concentration: we term the molar ratio of bile salt and PC the "critical molar ratio for disintegration" (CMRD), which would be convenient for the purpose of comparison. The CMRD values were 2.5 for SDOC and 5.8 for SC. These values indicate that to burst a small unilamellar bilayer 2.5 mol of SDOC and 5.8 mol of SC were required on a molar basis. Another useful parameter would be the slope of the dependency on bile salt concentration after the intercept, which indicates the disintegration efficiency of bile salts. The efficiency per mole of bile salt was $44\,\mathrm{mM^{-1}\,s^{-1}}$ for SDOC which was estimated from the dotted line in Fig. 2(A), and $8.6\times10^{-2}\,\mathrm{mM^{-1}}$ for SC. These values also represent the second-order rate constants. Again, it should be noted that a maximal k_{obs} was observed in SDOC.

Helenius and Simons[16] extensively reviewed the solubilization of the bilayer by surfactants, suggesting the following process. Stage I: surfactant is incorporated into the bilayer. Stage II: the bilayers are saturated with surfactant and additional surfactant induces

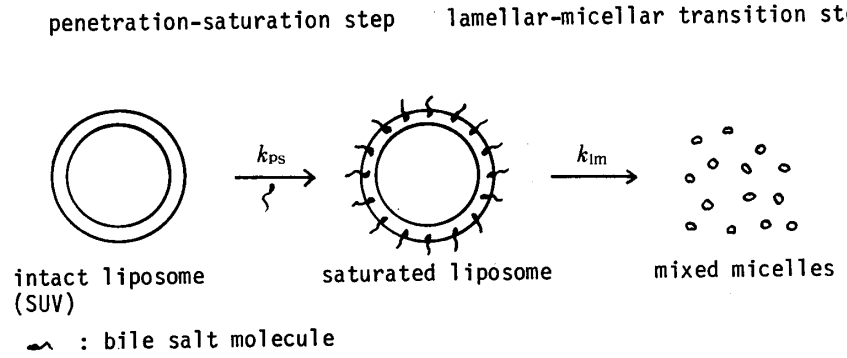

Chart 1. A Two-Step Model of the Disintegration Kinetics of Unilamellar Liposomes by Bile Salt

The physical dimensions and the number of bile salt molecules associated with a vesicle are not in conformity with the actual state.

the lamellar–micellar phase transition. Stage III: the formation of mixed micelles results in decreasing size with increasing surfactant/phospholipid ratio.

In the disintegration kinetics followed by turbidity changes, the sequence of the surfactant action may be simplified to two stages, *i.e.* the surfactant penetration step, which is dependent on the surfactant concentration (P-S step), and the lamellar–micellar transition, which is independent of the surfactant concentration (L-M step).[17] The disintegration rate is governed by either the P-S step or L-M step or may be a function of both. We assumed that the k_{obs} dependence on the SDOC concentration is a typical case in which the monotonously increasing k_{obs} reflects both steps and tends to level off corresponding to the L-M step.

We shall next extend the disintegration kinetics expressed by Eq. 2 to a more detailed model containing the step mechanism mentioned above. The disintegration process of unilamellar liposomes is schematically depicted as follows (Chart 1), where k_{ps} is the pseudo-first-order rate constant for the P-S step and k_{lm} is the first-order rate constant for the L-M step. $[T]_i$ and $[T]_{sat}$ are the turbidities of the intact liposomes and the saturated liposomes, respectively. The total turbidity (T_{tot}) is expressed by

$$[T]_{tot}=[T]_i+[T]_{sat} \tag{3}$$

Differentiating Eq. 3 with time gives

$$\frac{d[T]_{tot}}{dt}=\frac{d[T]_i}{dt}+\frac{d[T]_{sat}}{dt} \tag{4}$$

where

$$-\frac{d[T]_i}{dt}=k_{ps}[T]_i=k_{b,ps}[BS][T]_i \tag{5}$$

and

$$\frac{d[T]_{sat}}{dt}=k_{ps}[T]_i-k_{lm}[T]_{sat} \tag{6}$$

Accordingly, we have

$$-\frac{d[T]_{tot}}{dt}=k_{lm}[T]_{sat} \tag{7}$$

Because turbidity changes of $[T]_i$ and $[T]_{sat}$ could not be separately determined, we assumed quasi-state conditions for $[T]_{sat}$. We have the following equation from Eq. 6.

$$[T]_i=\frac{k_{lm}[T]_{sat}}{k_{ps}} \tag{8}$$

Combination of Eqs. 3, 7 and 8 and rearrangement give

$$-\frac{d[T]_{tot}}{dt}=\frac{k_{ps}k_{lm}}{k_{ps}+k_{lm}}[T]_{tot} \tag{9}$$

therefore

$$k_{obs}=\frac{k_{ps}k_{lm}}{k_{ps}+k_{lm}} \tag{10}$$

The principles represented by Eq. 10 are demonstrated in Fig. 2(A), in which the disintegration rate is influenced by both the P-S and L-M steps. The plateau represents the region where the L-M transition process is rate-determining and is independent of the surfactant concentration. Equation 10 was therefore rearranged in a manner analogous to the

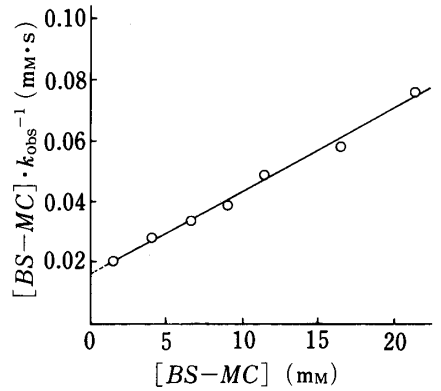

Fig. 3. Plot According to Eq. 11 for the Dependency of the Pseudo-First-Order Disintegration Rate Constant (k_{obs}) on the Concentration of SDOC

TABLE I. The Values of the Second-Order Rate Constant ($k_{b,ps}$) for the Penetration– Saturation Steps and the First-Order Rate Constant (k_{lm}) for the Lamellar–Micellar Transition Step

Bile salt	$k_{b,ps}$ ($mM^{-1}s^{-1}$)	k_{lm} (s^{-1})
SDOC	4.4×10	2.8×10^{2}
	$(5.9\times10)^{a)}$	$(3.6\times10^{2})^{a)}$
SC	8.6×10^{-2}	—$^{b)}$

a) Values estimated from Eq. 11. *b*) Not determined.

Langmuir-type equation to give Eq. 11. Since k_{ps} is the pseudo-first-order rate constant and the minimal surfactant concentration required to disintegrate the bilayer is given by MC, the bile salt concentration is replaced $[BS-MC]$. stated in one form it is:

$$\frac{[BS-MC]}{k_{obs}}=\frac{[BS-MC]}{k_{lm}}+\frac{1}{k_{b,ps}} \tag{11}$$

where $k_{b,ps}$ is the second-order rate constant in the P-S step. A plot of $[BS-MC]/k_{obs}$ against $[BS-MC]$ enables us to calculate the values of k_{lm} and $k_{b,ps}$. This is useful to estimate these parameters separately.

Figure 3 shows a plot according to Eq. 11 illustrating the dependency of k_{obs} on the concentration of SDOC. The estimated values are listed in Table I. The estimated value of k_{lm} for SDOC agrees with the experimental value in order of magnitude. Because of reciprocal treatment of the data, the plot might result in some overestimation caused by slight scattering of the original values. However, it should be noted here that SDOC was characterized by a considerably faster reaction rate than that of SC, and Eq. 11 based on the model may be applicable to such a case.

As can be seen in Fig. 2(B), the dependence of k_{obs} on the SC concentration appeared to be linear within the range of the bile salt concentration examined, for which Eq. 11 is no longer available and rather $k_{obs}=k_{ps}$ holds because $k_{lm}\gg k_{ps}$ may be assumed in Eq. 10. The value of $k_{b,ps}$ was therefore calculated from the slope, giving 8.6×10^{-2} mM s^{-1}.

There was a dramatic difference of the disintegrating action between SDOC (dihydroxy bile salt) and SC (trihydroxy bile salt). When bile salts penetrate into the lipid bilayer, hydrophobic interaction would be a predominant force. The results therefore indicate that the dihydroxy bile salt molecules penetrate into the bilayer structure about 5×10^{2} faster than the trihydroxy bile salt; this is probably determined by the difference of the molecular surface available for hydrophobic interaction. This is consistent with the fact that the critical micelle concentration of SDOC is 4—6 mM in H_2O, which is lower than that of SC (13—15 mM in H_2O),[18] indicating that hydrophobic character of SDOC is greater than that of SC. In addition, it is likely that the position and the number of the OH groups of the bile salts contribute to the considerable difference of the reaction rate through steric hindrance to penetration.

Bile contains a variety of bile salts and the total concentration of bile salts in gall bladder bile is relatively high, ranging from about 200 mM in humans, and 360 mM in rabbits to 25 mM

in guinea pigs.[19,20] When bile is released into the intestinal tract, the bile salts may be diluted to some extent, but nevertheless, orally administered liposomes pass into bile salt-rich fluids. Thus, the present results suggest that drug-loaded liposomes are transformed almost instantly into mixed micelles, which contribute to the solubilization of drugs, especially lipophilic and water-insoluble drugs such as vitamin K_1.[15]

We previously reported that blood coagulation recovery was enhanced by liposomally-associated vitamin K_1 administered orally to warfarin-induced hypoprothrombinemic rabbits.[21] The vitamin-loaded liposomes used were multilamellar, but it is very unlikely that the enhanced recovery was exclusively due to the absorption of an intact liposomal form of the vitamin.[22,23] Multilamellar liposomes are disintegrated from the outermost bilayer successively in the same manner as unilamellar liposomes and therefore the apparent half-life of the disintegration-mixed micelle formation process will be longer.[7,14] Yet, as shown in the present results, it is very probable that the disintegrating action of bile salts toward even multilamellar liposomes would be complete within a few seconds. The resulting mixed micelles with much reduced sizes would have easier access to the absorptive epithelium.[24] The disintegration rate of liposomes is therefore essentially a negligible factor in the whole absorption process, in agreement with the observation of enhanced recovery of blood coagulation on oral administration of liposomal vitamin K_1.

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