Kinetic Analysis of the Interaction between Liposomes and the Complement System in Rat Serum: Re-evaluation of Size-Dependency

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The size of liposomes is considered to be an important factor in determining the liposome-complement interaction. In this study, the release of carboxyfluorescein (CF) from liposomes was measured continuously for three different diameters (800, 400 and 200 nm) by changing the liposome concentration from 1 to 1000 mmol/ml. At a low liposome concentration range (1—10 mmol/ml), small liposomes (200 nm) released CF to a similar extent (approximately 35%) as in the medium (400 nm) and large (800 nm) liposomes. The affinity ($K_m$) and capacity ($L_{max}$) of a complement system to release liposomally encapsulated CF were estimated by kinetic analysis of the liposome-complement interaction. Surprisingly, there was no remarkable size dependency in the $K_m$ and $L_{max}$ in terms of liposome number, although these parameters depended on the size of liposomes in terms of lipid concentration. These results indicated the possibility that the complement system does not discriminate according to liposome size.

Key words | complement system; liposome; kinetics; carboxyfluorescein

The interaction between liposomes and blood components is the first step after the intravenous administration of liposomes, and it is this interaction which determines the fate of liposome disposition in the body. Serum components such as antibodies, complement, or fibronectin-opsonins-are known to enhance liposome uptake by the reticuloendothelial system (RES) such as macrophages in the liver and spleen. The complement system is considered to be the first line of self-defense and differentiates between self and non-self. The complement system eliminates foreign particles by enhancing their uptake by RES as opsonins and by forming a membrane attack complex (MAC) to lyse them. The role of the complement system in the interaction between liposome and blood components has been studied extensively by many researchers, including Alving, Chonn, Devine and ourselves.

The extent of complement activation by liposomes has been shown to depend on the lipid composition, size, surface charge, lipid concentration, and species of serum. A good correlation has been shown between hepatic uptake and the extent of complement activation.

The importance of foreign particle size in the recognition by the complement system has been recognized. There have been some reports using liposomes in which hepatic uptake clearance or the disintegration of liposomes was enhanced by increasing liposome size. These results indicated that the larger the size, the higher the affinity for the complement system. However, in most studies, the effect of liposome size was examined by fixing the lipid concentration or lipid dose, which resulted in a different concentration of liposomes in terms of liposome number. Therefore, these studies could not distinguish the effect of liposome size from the effect of liposome number.

In this study, the affinity and capacity of the complement system to release encapsulated 5(6)-carboxyfluorescein (CF) was analyzed kinetically allowing a reconsideration of the effect of liposome size by changing the liposome concentration.

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MATERIALS AND METHODS

Chemicals Hydrogenated egg phosphatidylcholine (HEPC) was kindly donated by Nippon Fine Chem. Co. (Osaka, Japan). Cholesterol (CH), dicetylphosphate (DCP) and CF were purchased from Wako Pure Chem. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Kodak, Inc. (NY, U.S.A.), respectively. Other chemicals used were of analytical grade. Male Wistar rats weighing 300—400 g were purchased from Inoue Experimental Animal (Kumamoto, Japan).

Preparation of Liposomes The method of preparing liposomes was basically the same as described elsewhere. Briefly, liposomes (multilamellar vesicles) were prepared to give a lipid molar ratio of HEPC/CH/DCP=5/4/1. After hydration with CF-containing solution, liposomes were extruded through polycarbonate filters with pore sizes of 800 (large), 400 (medium) and 200 (small) nm (Nuclepore, CA, U.S.A.), eight times each. The distribution of liposome diameter was determined by a dynamic laser scattering method (NICOMP 370, Particle Sizing System, CA, U.S.A.). Free CF was removed by dialysis in phosphate buffered saline (PBS) at pH 7.4, after extrusion. The encapsulation ratios of large, medium and small liposomes were 9.4, 6.5, and 1.9%, respectively. Empty liposomes which encapsulated no CF were prepared by hydration with PBS instead of CF solution.

Preparation of Fresh Rat Serum Blood was withdrawn from rat carotid artery under light ether anesthesia and was left at room temperature for 1 h. Serum was separated by centrifugation at 3000 rpm for 10 min at 4°C. The serum obtained from each rat was pooled and stored at −80°C until the experiments.

CF Release from Liposomes in Rat Serum A 1.8 ml aliquot of serum was preincubated for 10 min in a cell within a fluoro-spectrophotometer (Hitachi 650, Hitachi Co., Ltd., Tokyo, Japan) under a constant temperature at 37°C. An aliquot of liposome suspension (0.2 ml) was added to the serum and the mixture was incubated for 30 min with stirring. The fluorescence intensity (excitation=490 nm, emission=520 nm) produced by the CF released from liposomes was recorded continuously ($F_{(sample)}$). After the final record-
ing, a 0.5 ml aliquot of the incubation mixture was sampled to measure the total fluorescence intensity with 5% Triton X-100 ($F_{\text{total}}$). A control study was also done with PBS instead of serum, and the fluorescence intensity was measured with or without 5% Triton X-100. The percent release of CF from liposomes ($\% \text{Rel}(t)$), which represents the disintegration of liposomes, was calculated by the following equation:

$$\% \text{Rel}(t) = (F_{\text{sample}} - F_{\text{serum}}) / (F_{\text{total}} - F_{\text{serum}})$$  \hspace{1cm} (1)

where $F_{\text{serum}}$ represents the baseline value of serum.

**Kinetic Analysis of CF Release from Liposomes**

Description of Release Kinetics of CF from Liposomes: The time course of CF release from liposomes ($\% \text{Rel}(t)$) was described by Eq. 2 with the percent release at equilibrium ($R_{eq}$), the time to reach the half of $R_{eq}(T_{eq})$, $n$ which determines the slope, and the blank-value of fluorescence ($\alpha$).

$$\% \text{Rel}(t) = R_{eq} t^{n}(\beta t^{n} + r) + \alpha$$  \hspace{1cm} (2)

Evaluation of Affinity and Capacity of Complement System to Release Liposomally encapsulated CF: The final concentration of CF released from liposomes ($C$) at each lipid concentration ($L$) can be described as follows:

$$C = (C_{e} \times \beta L_{e}) \times L \times R_{eq}$$
$$= k \times L \times R_{eq}$$  \hspace{1cm} (3)

where $C_{e}$, $\beta$, and $L_{e}$ represent the initial concentration of CF for encapsulation, encapsulation ratio and lipid concentration when liposomes were prepared, respectively. The $k$ represents the constant ($= C_{e} \times \beta L_{e}$).

Assuming that the interaction between liposomes [L] and complement system [Pc] is a bi-molecular reaction,

$$[L]+[Pc] \rightarrow [LPc] \rightarrow \text{release of CF}$$

the relationship between the released CF concentration ($C$) and the lipid concentration ($L$) was analysed by the following equation using a non-linear least squares method.$^{14)}$

$$C = C_{\text{max}} \cdot L / (K_{n} + L)$$  \hspace{1cm} (4)

where $C_{\text{max}}$ and $K_{n}$ represent the maximum concentration of released CF per 1 ml of serum (90%) and the lipid concentration to reach the half of $C_{\text{max}}$, respectively.

The $C_{\text{max}}$ can be transformed to lipid concentration as follows:

$$L_{\text{max}} = C_{\text{max}} / ((C_{e} \times \beta L_{e}) \times \gamma) = C_{\text{max}} / (k \times \gamma)$$  \hspace{1cm} (5)

where the $L_{\text{max}}$ represents the maximum lipid concentration which can be released per 1 ml of serum (90%). The $\gamma$ represents the average maximum release percent of CF from liposome. In this analysis, the $\gamma$ was assumed to be constant (=0.35) for each size of liposome.

In this analysis, we have assumed that each liposome exists in a disintegrated or intact state at equilibrium. Therefore, the % release of CF is not only determined by $\gamma$, but also by the $K_{n}$, $C_{\text{max}}$ and liposome concentration. In this analysis, the $\gamma$ was fixed at 0.35 in this analysis based on the % release at the low liposome concentration range where there is no depletion of the complement system.

The concentration of liposomes in terms of liposome number can be calculated according to the following equaion$^{15)}$

$$N = (TLV + TVW) / (4 \pi r^{3})$$  \hspace{1cm} (6)

where the $TLV$ and $TVW$ represent the lipid and aqueous amount per 1 ml of liposome suspension, respectively. Then, the $L_{\text{max}}$ and $K_{n}$ were converted from lipid concentration to liposome number ($L_{\text{max}}(n)$, $K_{n}(n)$).

**RESULTS**

The time courses of CF release from liposomes are shown in Fig. 1. In general, the release of CF is initiated gradually, followed by a rapid increase and plateau. These curves were fitted by Eq. 2, and the parameters obtained ($R_{eq}$, $n$ and $T_{eq}$) have been summarized in Table 1. In each size of liposome, the $R_{eq}$ decreased with an increase in liposome concentration. The $n$ tended to decrease with an increase in liposome concentration, while the $T_{eq}$ increased with an increase in liposome concentration. It should be noticed that there was a similar CF release from small sized liposomes (ca. 35%) in the low concentration range (1—10 nmol/ml), and no significant difference was observed in $R_{eq}$ among the different sizes of liposomes in this concentration range. We have also confirmed the depletion of the complement system at the high liposome concentration (data not shown).

The relationship between liposome concentration ($L$) and final CF concentration ($C$) is shown in Fig. 2. The $C$ increased against $L$ in a saturable manner in general. The $C_{\text{max}}$

![Fig. 1. Time Courses of CF Release from Liposomes](image)

The release of CF from liposomes was measured continuously in rat serum. The final concentration of liposomes were as follows: $\bigcirc$, 1; $\bigtriangleup$, 10; $\Delta$, 100; $\bigtriangledown$, 1000 nmol/ml. The solid lines represent the fitted curves based on Eq. 2. The diameters of liposomes were (A): 800 nm, (B): 400 nm and (C): 200 nm.
and $K_m$ were obtained using the non-linear least squares method.\textsuperscript{16} Next, the $C_{\text{max}}$ was transformed into liposome concentration ($I_{\text{max}}$) according to Eq. 5 and is shown in Table 2. These parameters were described in terms of lipid concentration. The numbers of liposomes per 1 ml of liposome suspension were calculated according to Eq. 6 described by Pigion and Hunt\textsuperscript{35} (Table 3). The $C_{\text{max}}$ and $K_m$ were converted in terms of liposome number based on Table 3. As summarized in Table 2, there was no remarkable effect of liposome size on either $I_{\text{max}}(n)$ or $K_m(n)$, which suggests that the complement system recognizes various sizes of liposomes with a similar affinity and that each size of liposome consumed the complement to a similar extent.

**DISCUSSION**

A kinetic model has been postulated for quantitative analysis of the complement system.\textsuperscript{16} The model assumes that the one hit theory of immune hemolysis is applicable and that the rate of lysis is directly proportional both to the concentration of the complement and to the fraction of cells lysed throughout the course of the reaction.\textsuperscript{16} The parameters derived from the model can be quantitatively related to the complement concentration and can be used as the basis for a quantitative assay of complement activity. More sophisticated models for both the classical\textsuperscript{17} and alternative\textsuperscript{18} pathways have also been developed based on the kinetic behavior of individual components of the system. In this study, a sigmoidal curve was applied to describe the release kinetics of CF from liposomes for simplicity, and the reader should note that the parameters used in this study have no direct relevance to the molecular reactions in the complement activation, since complement system-composed of twenty plasma proteins and regulated by inhibitors and activators-is too complicated to describe the cascade reaction with a few kinetic parameters.

The $R_{eq}$, which is usually used to represent the extent of complement activation, depended on liposome concentration for each size of liposome. In this study, the interaction between liposomes and complement system was examined in a wide range of liposome concentrations, and it was shown that even small sizes of liposomes (200 nm in diameter) can release CF to a similar extent (ca. 35%) as those from medium (400 nm) and large (800 nm) sized liposomes in the low concentration range. This result is inconsistent with previous results in which the activation of the complement system is dependent on the size of the liposomes and showed no activation by small (200 nm) liposomes.\textsuperscript{7,19,20} This discrepancy re-

### Table 1. Effect of Liposome Size and Lipid Concentration on Each Parameter of Sigmoidal Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Size (nm)</th>
<th>Concentration of liposomes (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>$R_{eq}$</td>
<td>800</td>
<td>34.9</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>30.6</td>
</tr>
<tr>
<td>$T_{50}$</td>
<td>200</td>
<td>34.7</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>2.5</td>
</tr>
<tr>
<td>$n$</td>
<td>200</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} n.d. represents a release of CF that was not detected. The $R_{eq}$ and $T_{50}$ were fitted based on Eq. 2 for each liposome. $R_{eq}$ and $T_{50}$ represent the extent of complement activation at each lipid concentration and the time required to reach 50% of equilibrated value, respectively. The $n$ represents the sigmodial coefficient.

### Table 2. Kinetic Parameters for CF Release from Liposomes in Rat Serum

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Size (nm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>200</td>
</tr>
</tbody>
</table>

1) In terms of lipid concentration (nmol/ml)

$T_{\text{max}} = 380 \pm 9.3$  
$K_m = 340 \pm 22$

2) In terms of liposome number (number/ml)

$I_{\text{max}}(n) = 4.1 \pm 0.10E+9$  
$K_m(n) = 4.6 \pm 0.29E+9$

\textsuperscript{a} $I_{\text{max}}$ and $K_m$ in terms of lipid concentration were fitted based on Eq. 4 and 5 for each size of liposomes. The $I_{\text{max}}$ and $K_m$ in terms of liposome number were converted based on the relation shown in Table 2.

### Table 3. Number of Liposomes with Different Diameters

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Encapsulation ratio (%)</th>
<th>Number\textsuperscript{a} (number/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>9.4</td>
<td>4.4E+11</td>
</tr>
<tr>
<td>400</td>
<td>6.5</td>
<td>2.6E+12</td>
</tr>
<tr>
<td>200</td>
<td>1.9</td>
<td>1.0E+13</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Number of liposomes was calculated according to the Eq. 6 for liposome suspension at 40 µmol lipid/ml.

![Fig. 2](image)

**Fig. 2.** The Relationship between the Concentration of Liposomes and Released CF from Liposomes

The final concentration of released CF (C) was analyzed by Eq. 4. The solid lines represent the fitted curves based on the parameters in Table 2. The diameters of liposomes; (A): 800 nm, (B): 400 nm and (C): 200 nm.
sulted from the difference of liposome concentration between this study and previous studies where the concentration of liposomes was usually fixed at 2000 nmol/mL. In the high lipid concentration, there should be complete depletion of the complement system in the case of small liposomes due to their excess numbers.

There seems to exist an upper limit in the $R_{eq}$ for each size of liposome, since there was no increase in the $R_{eq}$ in the lower liposome concentrations, even for large liposomes. The mechanism of this plateau of marker release has been the subject of considerable speculation. One major hypothesis is that only the outer few lamellae of the liposomes are attacked by complement. The strongest evidence in support of this hypothesis is that small unilamellar liposomes release essentially 100% of their trapped marker. The other hypothesis involves the transient formation of a MAC, which will be closed or lost before all CF is released. In this study, multilamellar vesicles (MLV) were used. Therefore, the % release of CF should be higher in the smaller liposomes, if CF releases from the most outer phase of MLV. However, there was no size-dependency in the CF release from liposomes in our experiments (Fig. 3). These results suggested that the size-independent release of CF might result from the size-dependent disintegration of the liposomal membrane in depth, i.e. the more lipid bilayers were attacked by the complement system in the larger liposomes.

There is a tendency for parameter $T_{50}$ to increase with an increase in liposome concentration. The $T_{50}$ is considered to reflect the density of MACs formed on liposome membranes, because this density does not determine the extent of CF release, but the rate of CF release from liposomes. The parameter $n$ is also considered to be related to the rate of CF release, which is principally determined by the density of MAC formed on the liposomes. The decrease in both $R_{eq}$ and $n$ with the increase of liposome concentration suggests that some components of the complement system are depleted, and the density of MAC per liposome is decreased with the increase of liposome concentration.

Figure 3 clearly shows that there was no size effect in the CF release from liposomes if we plot the percent release against liposome concentration in terms of liposome number. This result strongly indicates that the complement system is not recognizing the size of liposomes. This result seems to be inconsistent with previous reports which have shown that larger liposomes were preferentially attacked by the complement system in vitro, in situ, and in vivo. Previous studies have shown that blood circulation time can be prolonged by decreasing the size of liposomes and that the hepatic uptake clearance of liposomes decreases with a decrease in liposome size. This tendency was consistent with the results in the interaction between liposomes and serum. The discrepancy might result from the differing experimental conditions in which the dose or concentration of liposomes was fixed in terms of liposomal lipid in previous studies, while dosage differed in terms of liposome number in this analysis. There can be 20-fold difference in terms of liposome number between small and large sized liposomes at the same lipid concentration.

Large sized liposomes can bind more complements than smaller ones, if we assume that the same surface bound complements per unit surface area of liposomes. This predicts that the large liposomes can deplete the complement system at a lower liposome number. However, there was no difference in the capacity of liposomes to release CF by the complement system (Table 2). The binding of the third component of complement system (C3), which is the key component in the complement activation, is size-dependent. However, the complement-dependent release of CF is size-independent in this study. This discrepancy can be explained by assuming that the size-dependency in CF release disappears after the binding of C3 on the liposome surface, possibly due to the formation of MAC. The precise molecular mechanism involved in the size-independence in the CF release from liposomes by complement system requires further investigation. The assay system for the ninth components of complement system on the liposome surface has been under investigation in our laboratory.

Finally, it was shown that the activation of the complement system is not dependent on the size of liposomes. The size-dependency in the interaction between liposomes and the complement system should be re-examined in terms of liposome number.

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