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# Double Fluorescent Labeling Method Used for a Study on Liposomes (II) — On the particle size of Liposomes Containing Carboxyfluorescein or Calcein —

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By using the double fluorescent labeling method of the previous report,[1] the size of liposome entrapping carboxyfluorescein (CF), which was prepared by the sonification method, was compared with that of calcein (CAL). As a result, it was revealed that the size of CF–liposomes was always smaller than the size of CAL–liposomes in every preparations and the entrapment efficiencies of CF–liposomes were always lower than those of CAL–liposomes. The results obtained in this study suggest that the diameter and the entrapment effciency of lipospmes would be influenced by the molecular size and structure of drugs entrapped in liposomes. Therefore, it will be necessary to consider the above–mentioned evidence when liposomes are prepared and used for a drug delivery system(DDS).

**Keywords** : liposomes ; double fluorescent labeling method ; carboxyfluorescein ; calcein ; particle size

The method in which it is able to determine concurrently the transition of two kinds of liposomes in the blood circulation has been devised and reported as a double fluorescent labeling method[1]. On the previous study[1], many experiments for the preparations of liposomes containing carboxyfluorescein (CF) and liposomes containing calcein (CAL) were carried out, and it was found that the entrapment efficiencies of the CAL–liposomes were always higher than those of CF–liposomes. As it was considered that the entrapment efficiencies of liposomes might be influenced by the drug entrapped, the following study was carried out to elucidate the cause of the phenomenon.

## Materials and Methods

**Reagents** CF was purchased from Eastman Kodak, Inc., and purified by the method of Ralston et *al.*[2] CAL was purchased from Wako Pure Chemical Industries, Ltd., and used untreated. Phosphatidylcholine (PC) was extracted from the yolk and purified by the modification of the methods of Rhodes et *al.*[3] and Bangham et *al.*[4] Cholesterol (Chol) was purchased from Wako Pure Chemical Industries, Ltd., and used unereated. Sepharose 2B and DEAE Sephadex A–25 were purchased from Pharmacia LKB Biotechnology and all other reagents used were of the highest grade commercially available.

**Preparation of CF–Liposomes and CAL–Liposomes** CF–liposomes and CAL–

liposomes were prepared by the previous method.[1] That is, 30, 18 or 6 μmoles of Chol were added to 30 μmole of PC respectively and three kinds of thin lipid films were prepared in a pear type flask with 50 ml as volume. 2 ml of 0.1 M CF solution (pH7.4) or 0.086 M CAL solution (pH 7.4) were added to the thin lipid film and vortexed with a mixer to prepare multilamellar vesicles (MLV). The MLV was sonificated and free CF or CAL was removed by using a DEAE Sephadex A-25 column. 3.2 ml of the liposome suspension were obtained. All six kinds of liposomes were prepared by the same method.

**Measuring the Fluorescent Self-Quenching and Entrapment Efficiencies of CF-Liposomes and CAL-Liposomes** 0.1 ml of the above CF-liposomes suspension was diluted by 5151 times with 0.1 M Tris-HCl buffer (pH8.5) containing 0.15 M sucrose and 0.1 mM ethylenediaminetetraacetic acid (EDTA). Both the fluorescent intensities (FI) of CF in the diluted solution (A) and the modified solution (B) which relased CF from liposomes by adding a tenth volume of 10% Triton X100 to the solution (A) were measured by the previous method.[1] The percent values of fluprescent self-quenching (FSQ) were calculated from (FI of B / 0.9 - FI of A) ÷ FI of B / 0.9 × 100. The entrapment efficiencies (EE) of CF-liposomes were calculated as the ratio of CF entrapped into liposomes to total CF used for the liposome preparation. FSQ and EE of CAL-liposomes were determined by the same procedure to CF-liposomes.

**Comparison of the Liposome Size by Using the Gel Filtration Mehod** In order to compare to the sizes of both CF and CAL liposomes, the liposomes mixture with the same fluorescent intensity on CF and CAL was prepared by the use of liposomes composed of 30 μmol / 30 μmol to PC / Chol. Actually, 1.77ml of the CF-liposome suspension and 2.90 ml of the CAL-liposome suspension were mixed and concentrated up to 2.05 ml by using centriflo ultrafiltration membrane corn (CF25 ; Amicon Div., W. R. Grace & Co.). 0.75ml of the solution concentrated was set on a Sepharose 2B column (1.4 × 30cm), saturated with phosphate buffered saline (PBS ; 137 mM NaCl, 2.6 mM KCl, 6.4 mM $KH_2PO_4$, 1.4 mM $Na_2HPO_4$) and eluted by PBS with flow rate of 5 ml / hr. All procedures were run at 4 ℃ . Twenty-six fractions of 2 ml were harvested. The fluorescent intensities of CF and CAL contained in the each fraction were measured by pervious method.[1]

## Results and Discussion

Egg PC and Chol as lipids of liposomes were used and three kinds of liposomes composed of 30 μmol / 30 μmol, 30 μmol / 18 μmol, and 30 μmol / 6 μmol to the ratio of PC / Chol were used. Vales of the EE and the FSQ of three kinds of CF-liposomes and three kinds of CAL-liposomes are summarized in Table I. It shows that the EE of CAL-liposomes are always higher than those of CF-liposomes, and their individual differences in EE became larger with the higher contents of the Chol. These data in Table I suggest that the mean diameter of

Table 1 Entrapment Efficiency and Fluorescence Self-Quenching of Liposomes Containing CF or CAL

| PC/Chol (μmoles) | Entrapped Material | EE(%) | FSQ(%) |
|---|---|---|---|
| 30 / 30 | CF | 1.08 ± 0.15 | 96.0 ± 0.8 |
| | CAL | 1.40 ± 0.09 | 94.0 ± 0.8 |
| 30 / 18 | CF | 0.73 ± 0.03 | 94.2 ± 1.7 |
| | CAL | 0.85 ± 0.08 | 91.1 ± 2.7 |
| 30 / 6 | CF | 0.55 ± 0.02 | 95.0 ± 2.4 |
| | CAL | 0.63 ± 0.06 | 91.4 ± 3.7 |

a) Values represent mean ± s. d. in 2-11 experiments.

CAL–liposomes would be larger than of CF–liposomes, and the following experiments to confirm the fact were carried out.

First, the diameters of CF–liposome and CAL–liposome, which were composed with 30 $\mu$mol / 30 $\mu$mol of PC / Chol, were compared by using a molecular sieve chromatography of a Sepharose 2B column.

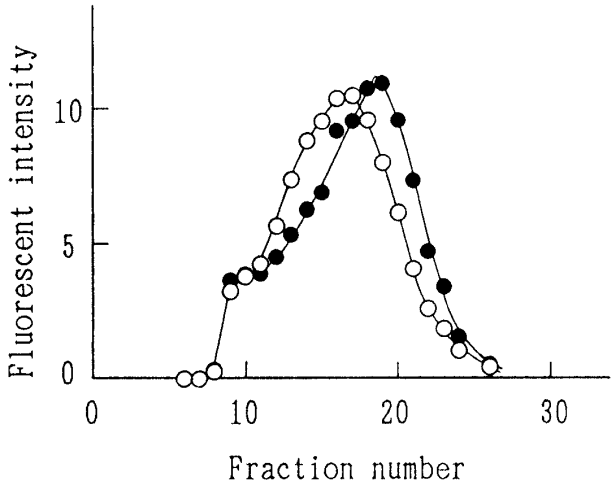


Figure 1 Elution Profiles of Liposomes Containing CF (●) or CAL (○) on Sepharose 2B Coiumn

The CAL–liposome was eluted earlier than that of CF–liposome as shown in Fig. 1. Therefore, it was proved that the diameter of CAL–liposome was larger than that of CF–liposome. Similar elution profiles of CF–liposomes and CAL–liposomes were also observed in other compositions of PC / Chol, and the smaller contents of Chol tended to make smaller the intervals in the eluting order between CF–liposomes and CAL–liposomes. The detailed reason on the difference of liposome sizes which rises from entrapping CF or CAL is not clear. However, it may be possible to consider as follows. The molecular weight of CAL is 622.54 and that of CF is 376.32. Therefore, CAL has 1.65 folds molecular weight to CF. CAL has two dicarboxymethyl aminomethyl groups as side chains and a more complex molecular shape than CF, as shown in Fig. 2.

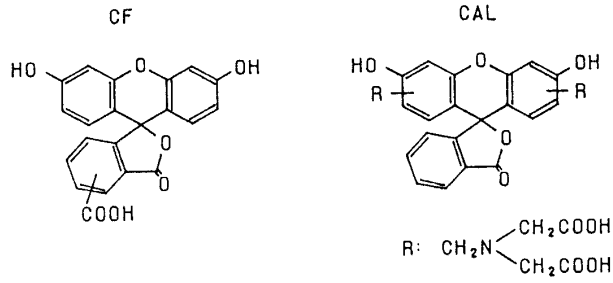


Figure 2 Chemical Structures of Caroxyfluorescein (CF) and Calcein(CAL)

Also it is well known that Chol reduces the fluidity of a bilayer membrane of liposomes.[5] The hydrated thin lipid film on liposomes preparation was strongly vortexed in the entrapping drug solution, and then was sonificated. A part of CF or CAL might be mingled into a lipid bilayer membrane under these procedures. Therefore, it is thought that some CAL molecules are able to remain in the bilayer membrane, more CAL than CF. It supports the above postulate that the self–quenching vaiue of free CAL solution is slightly larger than that of free CF solution (not shown), and the self–quenching values of CAL–liposomes are nevertheless smaller than those of CF–liposomes (Table I). That is to say, it is considered that the self–quenching values were suppresed by the fluorescence illuminating from the fluorescent substance in or on the bilayer lipid membrane of the liposomes' surface. Moreover the fact that CAL molecular is intercalated in the bilayer membrane on the surface of liposomes can be presumed from the following experimental results. When the DEAE Sephadex A–25 chromatography to remove the free CF or CAL on liposomes purification was used, CF–liposomes were freely passed through the column, but CAL–liposomes were passed with tailing in the column. This phenomenon indicates that the surface of CAL–liposomes has been charged weakly negative and also

supports the fact that the CAL molecular remained in or on the bilayer membrane. All of the facts obtained in this study suggest that CAL may make the liposomes' size larger than CF, and further cause the entrapment efficiencies of the liposomes to be larger. 0.1M CF solution and 0.086M CAL solution were used in this study because these solution are isotonic to the blood and available to the future studies in vivo.

Recently, Shinoda et al. reported the changing of the liposomes' size by adding a hydrophobic material, cepharanthine, as a membrane stabilizing agent.[6] They proved that the size of liposomes containing cepharanthine, which were prepared by sonification method or removal detergent of sodium cholate method, had significantly larger diameters than liposomes not containing cepharanthine. The results of both Shinoda's and our studies have proved that the liposome size will be influenced by the nature of drugs entrapped. It is well known that the liposomes' size affects the distribution of liposomes as well as a drug delivery system (DDS) in vivo.[7-13] Therefore, in the case of liposomes preparation for DDS, it must be kept in mind that the entrapped drugs influence the liposomes' size. Double fluorescent labeling method[1] is useful for such a study of liposomes.

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