Liposomal Membranes. V. Interaction of Zinc(II) Ion with Egg Phosphatidylcholine Liposomes

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The efficiency of encapsulation of several divalent metal ions, mainly Zn(II) ion, into egg lecithin liposomes has been investigated with particular attention to the stability of liposomal membranes against the transition metal(II) ions. The stronger interaction of metal ions to lecithins led to the less formation of liposomes. The addition of a suitable amount of nonionic detergent, 2-[ω-hydroxypentadeca(oxyethylene)-α-yl]-1,3-bis(dodecyloxy)propane ($2C_{12}15G$, see text), enhanced the encapsulation of Zn(II) and Cu(II) ions in liposomes. The stoichiometric measurement for the binding of Zn(II) ion to single compartment liposomes of egg lecithin suggested that a Zn(II) ion is interacting with three or four lecithin molecules on the average in both halves of liposomal bilayers. Even under the circumstances, the bilayer structure of liposomes was certainly kept stable, which was electron micrographically visualized.

Since Bangham and his coworkers reported on the bilayer structure of liposome and its resemblance to biological membranes, liposomes have attracted much attention as a suitable model of biomembranes.[1] The interaction of various metal ions with liposomal membranes has been also extensively investigated from this standpoint. The spontaneous permeability and the transmembrane movement[2-4] of metal ions, such as K^+, Na^+,[5,6] Ca^{2+}, or Mg^{2+} ion,[7] as affected by the hydrogen ion concentration,[8] the ionic strength,[9] several antibiotics providing ionophore activity,[8,10,11] and detergents[12] have been studied. Transition metals, Cr, Mn, Fe, Co, Cu, Zn, and Mo, are biologically called the "essential trace metals" since they are essentially required for enzymes or coenzymes to hold the activity.[13] The lack of these metal ions, in fact, induces specific diseases, and that the administration of the metal ions can therapeut these diseases is well known.[14,15] More recently, Gregoriadis and his coworkers have proposed that liposomes are able to be utilized as a drug carrier for therapeutic purposes.[16] Since the liposomes can encapsulate drugs,[17-19] proteins,[20] or enzymes,[21] it can be administered intravenously or orally to patients of various kinds of disease.[22] The most expedient point of liposomes as a drug-carrier is based on the following two unique features: (1) Since liposomes are constructed of amphiphilic lecithins, liposomes can also encapsulate both hydrophilic and lipophilic drugs.[19] Encapsulating water soluble drugs into the interior core of vesicles eases the permeability of these drugs across the hydrophobic cell membranes, while encapsulation of lipophilic drugs in the bulk liposomal bilayers makes the drugs easy to move in aqueous blood stream.[22] (2) If liposomes were constructed of natural phospholipids, they are biodegradable and easily metabolized. And this will promise that toxicological and immunological effects of liposomes are minimized *in vivo*.

From these aspects, we have investigated on the interaction with and the encapsulation in liposomes for several divalent metal ions which are, in a sense, thought to be a kind of hydrophilic and therapeutic drugs. This work will afford a basic knowledge on the stability of biomembranes against several divalent metal ions in one hand and on the efficiency of encapsulation of the metal ions into liposomes in other hand.

Experimental

Egg phosphatidylcholine (egg PC) was isolated and purified from fresh egg yolk according to the method in literature[23] with minor modification. Divalent metal nitrates were commercially available as analytical grade. Water used in this work was very carefully purified by deionization and redistillation using glass apparatus.

Formation of Liposomes Encapsulating Divalent Metal Ions. Liposomes were usually prepared from a mixture of egg PC and 40 mol % of chloesterol. Thirty mg of egg PC and 10 mg of cholesterol were dissolved in 1—2 ml of dry chloroform and placed in a 50 ml vol round bottom flask. Chloroform was removed off using a rotary vacuum evaporator under nitrogen atmosphere to form a thin film. The film was disperesd with 4 ml of aqueous 20 mM acetate buffer solution (pH 5.1) containing 0.1 M NaCl and 250 mM of aqueous metal ion solution by shaking on a Vortex mixer at 20 °C for 15 min. The resulting milky suspension is mostly consisted of multicompartment liposomes,[24] which was submitted to a Sephadex G-50 column (1.8×30 cm) to remove off free metal ions[10,25] and decomposed lecithins. To obtain single compartment liposomes the above milky suspension was sonified under stream of nitrogen at 2—5 °C for 15 min at 30 s intervals using a Tomy UR-200P probe-type sonifier. The sonified liposome solution (about 1.0—2.0 ml) was passed through a Sepharose 4B column (1.8×30 cm) to remove both free metal ions[10,25] and multicompartment liposomes. The amount of metal ions entrapped in liposomes was determined by atomic absorption analysis, fluorometric analysis,[26] or other spectrophotometric techniques.[27] Determination of lipid concentration was accomplished by the modified Allen's method.[28] Reproducibility of liposome formation which was ascertained by lecithin assay was rather good within about ±6% in error.

Electron Micrography of Liposomal Membranes. Liposome solution for electron micrographic measurement were negatively stained with 2% (by wt.) aqueous sodium tungstophosphate solution. A drop of the stained liposome solution was carefully put on a copper sheet-mesh using 1 μl-microsyringe, and excess sample solution was taken off with a scrap of filter paper. The sheet-mesh bearing samples was kept overnight in a vacuum desiccator and run on a JEOL JEM 100 V electron microscope.

Assay of Divalent Metal Ions; Zn(II), Cu(II), Mn(II), and Ca(II) Ions. To 0.5—1 ml of sample solution in a 3 ml-vol. Kjeldahl flask containing 0.01—250 mM metal(II) ions

was added 1 ml of 60% (v/v) perchloric acid, and heated to dryness, which was repeated thrice. After final evaporation of perchloric acid, it was diluted with 10 mM perchloric acid up to the concentration corresponding to about 10 μM of the metal(II) ions and measured on a Simadzu AA-620 atomic absorption flame spectrophotometer by regular procedures using the specific standard solution for respective metal ions.

Results and Discussion

Interaction of Liposomal Membranes with Divalent Metal Ions. The interaction of divalent metal ions and SeO_3^{2-} ion[29] with liposomal membranes of egg PC containing cholesterol was investigated focusing on the two points; (1) the amount of metal ions interacting with lecithin molecules in liposomes, $(M)_{lip}/(lecithin)_{lip}$, and (2) the % yield of lecithin molecules as recovered as liposomes after sonication and gel-filtration in the presence of metal ions. Table 1 shows the encapsulation efficiency of metal ions into liposomes. The efficiency was scarcely altered by the metal(II) ions except SeO_3^{2-} (Table 1). The stronger interaction of metal ions to liposomal membranes leads to the more destruction of liposomes, that is, the less recovery of lecithins as liposomes. Similarly, harder ultrasonication in the presence of metal ions increases the chance for metal(II) ions to interact with lecithins, but resulting in the lower lecithin recovery. The positively charged surface of egg PC liposomal membranes drastically depresses the $(Zn^{2+})_{lip}/(lecithin)_{lip}$-value comparing with the neutral surface. Contrary to what would be expected, on the other hand, the negatively charged surface hardly affect the interaction of the metal ion with membranes. The anionic phosphate moiety of phosphatidylcholines itself may present an effective binding site for metal ions. Table 1 also reveals the effect of chelating agents, EDTA and perhydrodibenzo-18-crown-6, on the encapsulation of zinc(II) ion into liposomes. EDTA was added as an aqueous solution along with zinc(II) ions during the dispersion of lipid, while dicyclohexyl-18-crown-6 was employed as a chloroform solution together with lipids during the thin lipid-film formation. The result shows that the adoption of EDTA or perhydrodibenzo-18-crown-6 rather decreases the encapsulation of zinc(II) ions. In the presence of EDTA or the crown compound, however, the lecithin recovery increased comparing with that in the absence of these chelating agents. Clearly from the fact that most of these agents adopted first are recovered during the gel-filtration, both chelating agents seem to be hardly encapsulated in liposomes. Therefore, metal ions bind predominantly to these agents in bulk aqueous solutions rather than to lecithins, which leads to saving liposomes from the destruction.

Table 1. Encapsulation of different divalent metal ions into multicompartment liposomes of egg lecithin at 25.0 °C[a]

Metal(II) ion	$\frac{(M)_{lip}}{(Lecithin)_{lip}}$[b]	Encapsulation[c] %	Lecithin[d] recovery %
None	—	—	85
Cu(II)	0.57	0.59	56
Mn(II)	0.23	0.23	81
Ca(II)	0.52	0.65	77
SeO_3^{2-}	0.03	0.03	78
Zn(II)	0.38	0.63	73
Zn(II)[e]	0.32	0.48	83
Zn(II)[f]	0.15	0.15	59
Zn(II)[g]	0.48	0.99	56
Zn(II)[g,h]	0.32	1.02	59
Zn(II)[g,i]	0.38	1.22	62
Zn(II)[g,j]	0.35	1.33	72

a) 40 μmol of egg lecithin with 26 μmol of cholesterol was dispersed in 4.0 ml of 20 mM acetate buffer containing 0.1 M NaCl and 250 mM of metal(II) nitrates at 25.0 °C. After sonication (25 W for 15 min) and gel-filtration (Sephadex G-50), concentrations of metal(II) ions and lecithin were independently determined as described under Experimental. All the values are average of duplicated or triplicated runs. b) Error which stems from assay of metal ions was as follows: ± 6.0% for Zn(II), ±0.5% for Mn(II), ±2.5% for Cu(II), and ±9.0% for Ca(II). c) Encapsulation $\% = 100 \times (M)_{lip}/(M)_{total}$. d) Lecithin recovery $\% = 100 \times (lecithin)_{lip}/(lecithin)_t$. Experimental error was within ±6.0%. e) To ensure the anionic surface charge on the liposomal membranes, 5.6 μmol of dihexadecylhydrogenphosphate was added during the lipid thin film formation. f) To ensure the cationic surface charge on the membranes, 5.6 μmol of hexadecylamine was employed during the course of thin-film formation. g) Sonicated at 60 W for 15 min. h) 10 μmol of EDTA was added. i) 25 μmol of EDTA was added. j) 10 μmol of crown ether was added.

Table 2. Effect of nonionic detergent, $2C_{12}15G$, on the encapsulation of Zn(II) ions in egg lecithin liposomes[a]

Liposomes Lecithin μmol	$2C_{12}15G$ μmol	$\frac{(Zn(II))_{lip}}{(Lecithin)_{lip}}$	Encapsulation %
40	0	0.38	0.63[b]
38	2	0.37	0.93
36	4	0.39	1.26
32	8	0.35	0.72
28	12	0.27	0.83
20	20	0.03	0.13

a) The nonionic detergent was mixed with egg lecithin in the absence of cholesterol during the course of thin film formation. Liposome solutions were sonicated at 25 W for 15 min. b) The same data as that listed in Table 1.

The effect of nonionic detergent, $2C_{12}15G$,[30] on the encapsulation of Zn(II) ion is listed in Table 2. The detergent

$$CH_3\{CH_2\}_{11}OCH_2 \diagdown$$
$$\qquad\qquad CH\{OCH_2CH_2\}_{15}OH$$
$$CH_3\{CH_2\}_{11}OCH_2 \diagup$$

2-[ω-hydroxypentadeca(oxyethylene)-α-yl]-1,3-bis(dodecyloxy)propane ($2C_{12}15G$)

is bearing long ethylene oxide moiety as the hydrophilic part, which is expected to behave just like as an acyclic

antibiotic ionophore.[11] Intercalating up to about 10 mol% of $2C_{12}15G$ in liposomes without cholesterol enhanced the encapsulation of the metal(II) ion different from cases of the former two chelating agents. Leakage of the nonionic detergent from the liposomal bilayers to the exterior aqueous phase can be neglected under the present conditions. The totally similar effect was observed also for the encapsulation of Cu(II) ion (not shown). Addition of more detergent, however, rather decreased the efficiency of encapsulation of metal ions. Electron micrographic studies indicated that adopting the detergent up to 10 mol% against lecithins still holds reliable bilayer structure of multicompartment liposomes, but the presence of more detergent than 20 mol% gradually causes the physico-chemical damage on the bilayer structure (Fig. 1). This is very comparable to the efficiency on the metal ion encapsulation. Interestingly, in addition, as increasing the content of the detergent, the bilayer thickness determined on the electron micrograph pictures also increased: 30 Å for 100% lecithin liposomes, 34 Å for liposomes containing 10 mol% of $2C_{12}15G$, 37 Å for 20 mol%, 39 Å for 30 mol%, and 45 Å for more than 50 mol%.

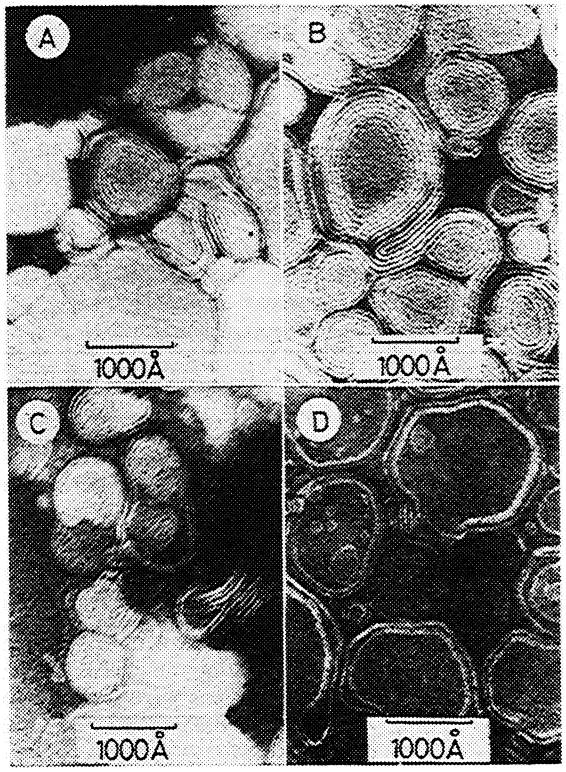

Fig. 1. Electron micrographs of egg lecithin multicompartment liposomes containing nonionic detergent, $2C_{12}15G$, at different concentrations (A, 0 mol%; B, 10 mol%; C, 30 mol%; and D, 50 mol%) in the presence of Zn (II) ion.

Adsorption of Zinc(II) Ion on the Liposomal Bilayers. Electrophoretic mobility and ^{31}P-NMR measurements were combined by McLaughlin and his coworkers[31] to test whether the combination of Henry, Boltzmann, and Grahame equations is capable of describing the adsorption of the divalent metal ions to phosphatidylcholine membrane, where they assumed a 1:1 stoichiometry for the binding of the divalent ions to phosphatidylcholine. In this work, amounts of each Zn(II) ion being in the interior of vesicles and adsorbing onto the outer surface of vesicles were independently determined by following procedures. After the gel-filtration of the single compartment liposome solutions containing Zn(II) ions, which were prepared according to the regular technique as described under Experimental, the total amount of metal ions interacting with lecithins ($(Zn(II))_{lip}$) was determined: it was 6.27 μmol. Then, the liposomes were washed by adding about 0.02 M EDTA aqueous solution[10] (about 100 times excess to total metal ions entrapped in liposomes) and contributed again to the gel-filtration in order to remove off Zn(II) ions adsorbed only on the outer surface of membranes.[10] At this stage both amounts of metal ions adsorbing on the outer surface ($(Zn(II))_{lip\cdot out}$) and locating in the interior core of vesicles ($(Zn(II))_{lip\cdot in}$) could be determined: they were 3.78 and 1.79 μmol, respectively. Hence, the yield of Zn(II) ions recovered during the above procedures was about 90%. Same experiment was repeated twice. The result also indicates that about 70% of Zn(II) ions in liposomes are binding onto the outer half of liposomal bilayers. To ascertain the result, on the other hand, the blank single compartment liposomes containing no metal ions were independently prepared. And Zn(II) ion was then added from the exterior of liposomes. After the gel-filtration to remove free metal ions off, the amount of Zn(II) ions, which corresponds to those bound only to the outer surface of liposomes ($(Zn(II))_{lip\cdot out}$), was determined: it was 4.13 μmol. This value is in good agreement with the preceding one (3.78 μmol) within experimental error (±10%). Finally, the resulting liposome solutions were washed by 0.02 M EDTA and gel-filtered again. Hence, the amount of Zn(II) ions remained in liposomes was determined: it was 0.36 μmol. This may mean that only 8.7% of Zn(II) ions permeates into the liposomes as a complex with EDTA and/or lecithins during the course of washing the outer surface of liposomes by EDTA.

TABLE 3. NUMBER OF Zn(II) ION BINDING ONTO RESPECTIVE HALVES OF BILAYERS OF EGG LECITHIN SINGLE COMPARTMENT LIPOSOMES

$(Zn(II))_{lip.out}$ μmol	$\frac{(Zn(II))_{lip.out}}{(lecithin)_{lip.out}}$	$(Zn(II))_{lip.in}$ μmol	$\frac{(Zn(II))_{lip.in}}{(lecithin)_{lip.in}}$
4.13(3.78)[a]	0.30—0.34	1.79	0.30—0.41

a) The parenthesized value was obtained from the codispersion experiment.

The area ratio of the outer surface to the inner surface of the single compartment liposomes is in proportion to the ratio of the number of lecithin molecules involving in respective two halves of bilayers. Assuming that the average size of single compartment liposomes is 250—300 Å[22,24] in diameter, the thickness of bilayers is 40—50 Å, and a vesicle is consisted of 3000—4000 molecules of phosphatidylcholines,[22] thus, one can estimate the

number of Zn(II) ions interacting with lecithins of both halves of bilayers (Table 3). The estimation suggests 1 to 3—4 stoichiometry for the zinc(II) ion interacting with phosphatidylcholine in both halves of liposomal bilayers. Alternatively, if assumed that all the Zn(II) ions locating in the interior water pool of liposomes are free, the estimation suggests that the initial aqueous solutions of metal ion added when forming liposomes is diluted about two-fold. But, this seems unlikely. Surprisingly, even under the circumstances, the bilayer structure of vesicles was certainly kept stable as visualized by electron micrography.

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