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Uptake of Transition Metal Ions Using Liposomes Containing Dicetylphosphate as a Ligand

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The uptake of Cu^{2+} was investigated using various types of liposomes composed of phosphatidylcholine (PC), cholesterol (Chol) and dicethylphosphate (DCP). DCP played a role as a ligand for Cu^{2+}. Multilamellar vesicles (MLVs) were more effective for the uptake of Cu^{2+} compared to unilamellar vesicles prepared by the extrusion technique. The uptake efficiency of MLVs for Cu^{2+} was dependent on the molar ratio of DCP in MLVs. The uptake percent of Cu^{2+} was 92% using MLVs having a PC:DCP:Chol molar ratio of 4:3:3; 95% of the total vesicle Cu^{2+} was bound to DCP of the outer membrane surface of the MLVs, and the remaining 5% of the total Cu^{2+} was distributed into the interior side of the MLVs. MLVs having a PC:DCP:Chol molar ratio of 4:3:3 were also effective as separation media for Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. The uptake efficiency of the MLVs for the transition-metal ions increased in the order $Co^{2+} < Zn^{2+} < Ni^{2+} < Mn^{2+} < Cu^{2+}$.

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Microorganisms are capable of adsorbing or accumulating heavy metal ions due to metal-attracting compositions on their cell walls or *via* metabolism-dependent intracellular metal uptake mechanisms. Therefore, the application of microbial biomass to the recovery of toxic heavy metals from an aqueous solution has been noted in recent decades. Various kinds of microorganisms were used to remove heavy metals from the environment and industrial waste water.[1,2] On the other hand, liposomes composed of natural phospholipids surfactants were used as a model of a cell, since liposomes consist of vesicular lipid bilayers that enclose a volume of aqueous solution. However, phospholipids which have markedly hydrophobic chains prevent the passage of water-soluble materials through membranes. Therefore, the transport of metal ions across the membrane of liposomes is mediated by ionophores, such as calcimycin.[3,4] On the other hand, no reports have been found on the application of liposomes to biomimetic metal-sorbing media, since liposomes are hardly permeable to metal ions.

We previously applied multilamellar vesicles (MLVs) composed of phophatidylcholine (PC), cholesterol (Chol) and dicetylphosphate (DCP) to the uptake of transition-metal ions.[5] MLVs composed of a PC:DCP:Chol molar ratio of 10:1:10 were effective for the uptake of Cu^{2+} and Zn^{2+} without ionophores. The uptake of Cu^{2+} and Zn^{2+} was probably due to complex formation between the metal ions and the phosphate of DCP. However, the MLVs were unable to adsorb Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+}. Therefore, the increase in the uptake efficiency of liposomes for those metal ions could be important in order to apply liposomes to metal-sorbing media for transition-metal ions.

In this study, the factors which affect the uptake efficiency of Cu^{2+} were investigated in terms of the pH, DCP concentration and liposome size. In addition, the Cu^{2+} content of the outer membrane surface and the interior side of the MLVs were determined. Under the optimum conditions established, MLVs were applied to the separation media of Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}.

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Experimental

Materials

Egg-yolk phosphatidylcholine (PC) and cholesterol (Chol) were obtained from Wako Pure Chemicals. Dicetylphosphate (DCP) was purchased from Sigma Chemical Co. Calcein {3,3′-bis[*N*,*N*-bis(carboxymethyl)amminomethyl]fluorescein} was bought from Dojindo Laboratories. The concentration of PC was calculated by using a molecular weight of 765.3.[6] A 1.0×10^{-2} M solution of metal ions was prepared by dissolving metal salts: $MnCl_2$, $Fe(NH_4)_2(SO_4)_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$ and $ZnCl_2$.

Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared using standard methods.[7] A mixture (16.8 μmol PC, 12.6 μmol Chol, 12.6 μmol DCP) in chloroform was added to a 100-ml round-bottom flask. Chloroform was removed by rotary evaporation at 25˚C under reduced pressure and a stream of nitrogen gas forming a lipid film on the wall of the flask. After at least 1 h *in vacuo*, a 0.9-ml portion of a 1.0×10^{-2} M 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer solution (pH 7.5) was added into the flask and whole contents were extensively mixed on a Vortex stirrer for 15 min at 25˚C to prepare MLVs.

Thus-prepared MLVs suspensions were extruded through a polycarbonate filter to prepare unilamellar vesicles. Polycarbonate filters with pore sizes of 100, 400 and 1000 nm were obtained from Avestin Inc. The filters were mounted in a LiposoFast™-Basic (Avestin Inc.) fitted with two 0.5 ml Hamilton syringes. We subjected samples to 20 passes through a single filter. A numerical subscript indicated the pore size of the polycarbonate filter employed. Thus, a VET_{1000} indicated that liposomes were extruded through a polycarbonate filter

with a pore size of 1000 nm. The size distributions of the MLVs and VETs were estimated by a laser scattering particle size distribution analyzer (LA-910, Horiba, Japan).

Uptake of metal ions using liposomes

The uptake procedure of metal ions with liposomes consisted of pipetting 0.9 ml of liposome suspensions and a 0.1 ml of a 1.0×10^{-2} M metal ion solution into a glass cuvette. The mixture was shaken for 30 min by a water bath-incubator at 25˚C. Next, the separation of free metal ions and liposomes containing metal ions was performed on a Sephadex G-50 column (column size, 10 × 300 mm). The column was equilibrated with 10 mM Mops buffer (pH 7.5); the flow rate was 18 ml/h. A 275-µl portion of the mixture was applied to the column. The amounts of liposomes and metal ions eluted from the column were determined by measuring the phosphorus and metal ion in each fraction tube by an inductively coupled plasma atomic emission spectrometer (ICP-AES) with an ultrasonic neblizer (ICPS-1000IV, Shimadzu, Japan).[8] The uptake percent is referred to as the mole ratio of the metal ions detected in the fraction of liposomes to the total moles of the metal ions in a liposome suspensions applied to the column.

Measurement of the trapping efficiency of calcein in MLVs

Calcein-trapped MLVs were prepared according to the procedure, except that a 1.0-ml portion of a 5.0×10^{-8} M calcein solution was added into the flask in place of the buffer solution in the swelling of the lipid film.[7] A 50-µl portion of MLVs suspensions was diluted with a 2.0-ml portion of the buffer solution in a 1-cm quartz cell. The fluorescence intensity (F_1) was measured at exitation and emission wavelengths of 490 and 513 nm, respectively. Subsequently, a 5-µl portion of a 1.0×10^{-2} M of Co^{2+} solution was injected into the cell. Vigorous agitation by a magnetic stirrer was continued during the fluoresence measurement. The fluorescence intensity after the addition of a Co^{2+} solution was defined as F_2. Next, a 20-µl portion of 10% Triton X-100 was added into the cell, thus destroying those MLVs containing calcein. The thus-obtained fluorescence intensity (F_3) represents the equilibrium concentration of free calcein. The trapping efficiency of calceine in MLVs was calculated from:

$$\text{trapping efficiency \%} = [F_2 - (F_3 \cdot r)]/[F_1 - (F_3 \cdot r)] \times 100$$

where r is the dilution factor due to the Triton X-100 solution, 1.01 in the present case.

Results and Discussion

Uptake of Cu^{2+} using MLVs and the recovery of MLVs containing Cu^{2+}

We carried out the uptake of Cu^{2+} with MLVs composed of a PC:DCP:Chol molar ratio of 4:3:3. The mixture of free Cu^{2+} and MLVs containing Cu^{2+} was applied to a Sephadex G-50 column. Typical elution profiles of phosphorus and Cu^{2+} are shown in Fig.1. One peak was observed in the elution profiles of phosphorus and Cu^{2+}. When MLVs containing Cu^{2+} were eluted from the column, the elution volume of Cu^{2+} coincided with that of phosphorus. Therefore, the peak observed in the elution profile is ascribed to the elution of MLVs containing Cu^{2+}. Free Cu^{2+} could be eluted from the column in the range from 20 ml to 30 ml.[5] However, no peak based on the elution of free Cu^{2+} was observed in the elution profile. This is because free Cu^{2+} could be adsorbed on the column.

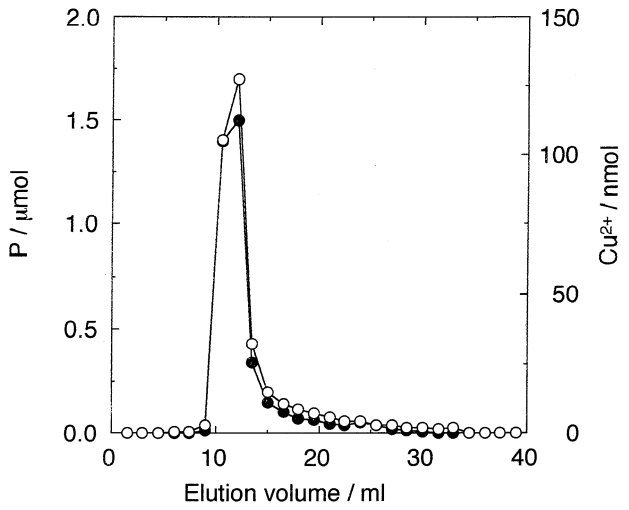

Fig. 1 Elution profiles of MLVs containing Cu^{2+} obtained by gel-filtration. Conditions: column, Sephadex G-50 (10 × 300 mm); mobile phase, 10 mM Mops buffer (pH 7.5); MLVs suspensions, 275 µl; flow rate, 0.3 ml/min; fractionation, 5 min/tube. (○), phosphorus; (●), copper.

Next, MLVs containing Cu^{2+} were mixed after collecting the fraction eluted from 10.5 to 15 ml. We then determined the amount of Cu^{2+} incorporated into the MLVs. The uptake percent of Cu^{2+} was 92% when MLVs composed of a PC:DCP:Chol molar ratio of 4:4:3 were used. The amount of phosphorus was also determined by ICP-AES. The amount of phosphorus in MLVs containing Cu^{2+} was almost equal to that in MLVs suspensions applied to the column. Therefore, MLVs containing Cu^{2+} were essentially excluded from the column.

Optimum conditions for the uptake of Cu^{2+}

The effects of the mixing time, pH and liposome size on the uptake percent of Cu^{2+} were investigated using MLVs composed of a PC:DCP:Chol molar ratio of 4:4:3. The effect of the mixing time on the uptake percent was examined over the range of 5 – 60 min. The uptake percent was almost constant after 10 min. Thus, the optimum mixing time was chosen to be 30 min. We then examined the dependence of the pH on the uptake percent of Cu^{2+} in the pH range from 7.0 to 8.0. The uptake percent of Cu^{2+} was constant in the pH range examined. The optimum pH was thus chosen to be 7.5. Next, the effect of the liposome size on the uptake percent of Cu^{2+} was investigated using VET_{100}, VET_{400}, VET_{1000} and MLVs. As shown in Fig. 2, the MLVs were more effective for the uptake of Cu^{2+} compared to various sizes of the VETs. The mean diameters of VET_{100}, VET_{400}, VET_{1000} and the MLVs were 155, 272, 440 and 12000 nm, respectively. This result indicates that the surface area of MLVs was remarkably greater than those of the VETs. Therefore, the increase in the surface area of the liposomes could accelerate the interaction between DCP and Cu^{2+}, thus resulting in an increase in the uptake percent of Cu^{2+}. However, the reason for the differences in the uptake percent between VETs is still not clear. Thus, MLVs were chosen to be used as a separation media.

The uptake percent of Cu^{2+} was 18% using MLVs composed of a PC:DCP:Chol molar ratio of 10:1:10.[5] The result suggested that the uptake percent of Cu^{2+} could be affected by the composition of DCP in MLVs suspensions. We then examined the effect of the molar ratio of DCP in MLVs on the uptake percent of Cu^{2+}. The total mol of PC, DCP and Chol dissolved

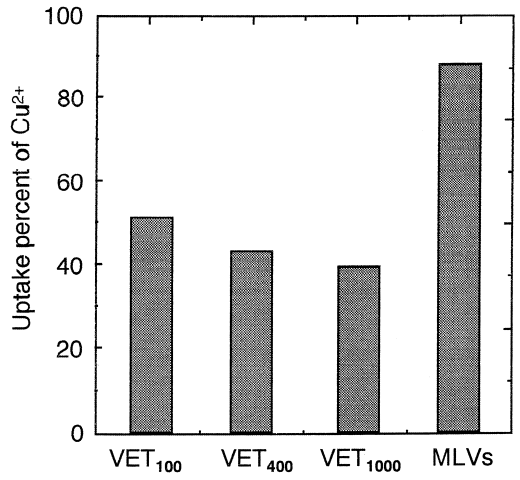

Fig. 2 Effect of the liposome size on the uptake percent of Cu^{2+}. Conditions: liposome suspensions, 0.9 ml; solution of Cu^{2+}, 0.1 ml; mixing time, 30 min.

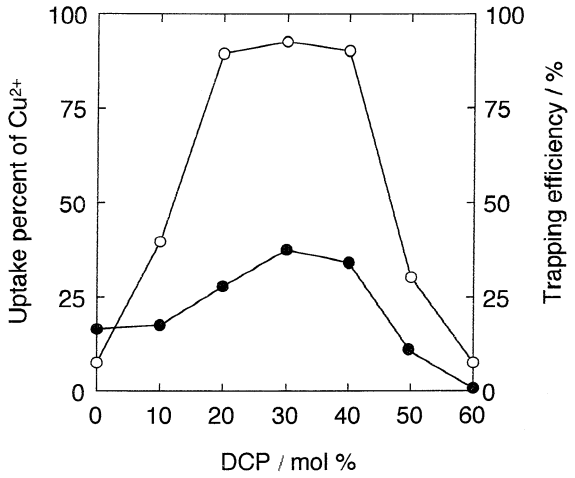

Fig. 3 Effect of the molar composition of DCP in MLVs on the uptake percent of Cu^{2+} and the trapping efficiency of calcein. Conditions: total mol of PC, Chol and DCP was kept constant at 42 μmol and the mol% of Chol was kept constant at 30 mol%. (○) uptake of Cu^{2+}; (●) trapping efficiency of calcein.

in chloroform was kept constant at 42 μmol, and a molar ratio of Chol was kept constant at 30%. On the other hand, DCP was dissolved in chloroform in the range of 0 – 60% at a molar ratio. The optimization curve for the molar ratio of DCP in MLVs is shown in Fig. 3. The uptake percent of Cu^{2+} exhibited a broad maximum at 30 mol% of DCP. On the other hand, the uptake percent decreased above 40 mol% of DCP. We then measured the trapping efficiency of calcein in MLVs according to the procedure in order to confirm the formation of liposomes in the range of 0 – 60% at a molar ratio of DCP. The trapping efficiency exhibited a broad maximum at 30 mol% of DCP and decreased remarkably above 40 mol% of DCP. Therefore, the decrease in the uptake percent above 40 mol% of DCP can probably be attributed to the decrease in the yield of the MLVs. The optimum mol% of DCP dissolved in chloroform was chosen to be 30 mol%.

Distribution of Cu^{2+} into MLVs

The uptake of Cu^{2+} was due to complex formation between

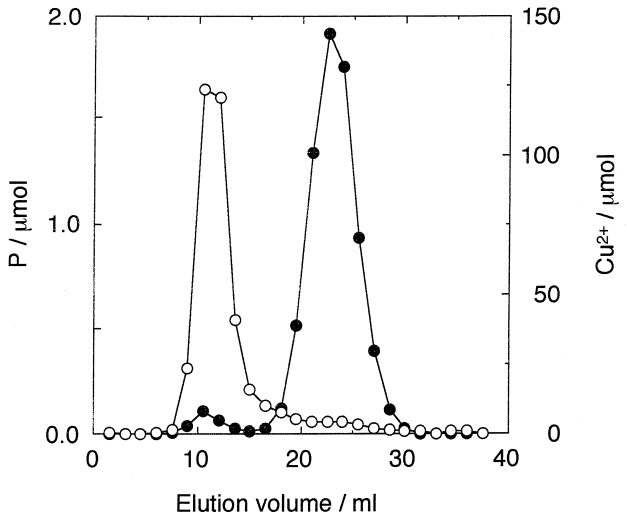

Fig. 4 Elution profiles of the Cu^{2+}-EDTA complex and MLVs containing Cu^{2+} by gel-filtration. Conditions: column, Sephadex G-50 (10 × 300 mm); mobile phase, 10 mM Mops buffer (pH 7.5); MLVs suspensions, 275 μl; flow rate, 0.3 ml/min; fractionation, 5 min/tube. (○), phosphorus; (●), copper.

Cu^{2+} and phosphate of DCP. Therefore, most of the Cu^{2+} could be localized on the surface of the MLVs, though Cu^{2+} was partly permeated into the interior side of the MLVs.[5] We then determined the amount of Cu^{2+} on the outer membrane surface of the MLVs and on their interior side.

First, the total amount of Cu^{2+} distributed into the MLVs was determined after collecting MLVs containing Cu^{2+} by a Sephadex column according to the procedure. Next, a 0.1 ml portion of a 2.0×10^{-3} M EDTA solution was added into the solution of the MLVs containing Cu^{2+}, and the mixture was incubated for 30 min at 25°C. EDTA was allowed to react with Cu^{2+} bounded to the phosphate ion of DCP on the outer membrane surface of the MLVs, since EDTA exists as anionic species under the experimental conditions, and liposomes are hardly permeable to ionic species. The thus-formed Cu^{2+}-EDTA complex and MLVs containing Cu^{2+} on the interior side were separated by the Sephadex column. Figure 4 shows typical elution profiles of phosphorus and Cu^{2+}. Two peaks appeared in the elution profile of Cu^{2+}. When MLVs containing Cu^{2+} were eluted from the column, the elution volume of Cu^{2+} could coincide with that of phosphorus. Therefore, the first and second peaks were ascribed to the elution of MLVs containing Cu^{2+} and the Cu^{2+}-EDTA complex, respectively. MLVs containing Cu^{2+} was mixing by collecting the fraction eluted from 9.5 ml to 15 ml. We then determined the amount of Cu^{2+} incorporated into the interior side of the MLVs. Consequently, it was found that the 95% of the total vesicle Cu^{2+} was bound to DCP of the outer membrane surface, and that the remaining 5% of the total Cu^{2+} was distributed into the interior side of the MLVs. The effect of the mixing time on the formation of the Cu^{2+}-EDTA complex was examined over the range of 10 – 60 min. The amount of Cu^{2+} bound to DCP of the outer membrane surface was independent of the mixing time examined. The distribution of Cu^{2+} into the interior side of the MLVs corresponded to a result previously obtained in which the permeation of Cu^{2+} into the inner phase of MLVs was confirmed by the fluorescence intensity of the calcein-time profile.[5]

Uptake of transition-metal ions using MLVs

We previously investigated the uptake of Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} using MLVs having a PC:DCP:Chol molar ratio of 10:1:10.[5] The uptake percent of Cu^{2+} and Zn^{2+} was 18 and 6%, respectively. However, the uptake was only little observed in the case of Mn^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+}. Therefore, MLVs composed of a PC:DCP:Chol molar ratio of 10:1:10 were unsuitable for the separation media of Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+}. On the other hand, the uptake efficiency of Cu^{2+} was remarkably influenced by the concentration of DCP. We then examined the uptake of Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} using MLVs composed of a PC:DCP:Chol molar ratio of 4:4:3.

The uptake percents of Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} were 56, 24, 47 and 33%, respectively. The uptake efficiency of Zn^{2+} increased remarkably compared to that using MLVs composed of a PC:DCP:Chol molar ratio of 10:1:10. In addition, MLVs composed of a PC:DCP:Chol molar ratio of 4:4:3 were used as the separation media for Mn^{2+}, Co^{2+} and Ni^{2+}. However, in the case of Fe^{2+}, MLVs were condensed by mixing the Fe^{2+} solution. The difference in the uptake efficiency between metal ions could be ascribed to the difference in the ability for complex formation between the metal ions and the phosphate of DCP.

In conclusion, MLVs containing DCP were effective for the uptake of Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. The uptake efficiency for metal ions was dependent on the molar ratio of DCP in MLVs, since DCP played a role as a ligand for metal ions. A large portion of Cu^{2+} reacted with DCP on the surface of MLVs. Therefore, these results suggested that MLVs containing DCP could be applied to biomimetic metal-sorbing media.

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