Synthesis and Aggregation Behavior of an Amphiphilic Cobalt (III) Complex That Coordinates Higher Diacylglyceryldiamine Ligands

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ABSTRACT: An amphiphilic cobalt complex that coordinates a higher diacylglycerylethylenediamino group was prepared. They extensively aggregated in aqueous solution upon ultrasonication. Electron microscopy indicated formation of stable bilayer structures, which further formed vesicles and fibrous aggregates. We observed an intermediate state that led to a network morphology in the fusion process of small unilamellar vesicles. Differential scanning calorimetry experiments established the presence of the phase transition from gel to liquid crystal for all probes in which a C_{14} to C_{18} acyl group was bound. The phase-transition temperature was elevated with increasing carbon numbers of the acyl group.
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KEY WORDS: Amphiphilic cobalt complex, differential scanning calorimetry, fusion of liposome, higher diacylglyceryldiamine ligand, liposome, phase-transition temperature, transmission electron microscope.

Because organized molecular assemblies have great potential in various types of reactions, there has been intensive interest in many reactions catalyzed by an organized molecular aggregate. They have been extensively studied as models for an artificial enzyme catalyst (1). In particular, aggregates of metal complexes attract great interest from both fundamental and practical viewpoints. They can exhibit various functional activities, such as molecular recognition, chemical modification, and electron transfer, through the most suitable combination of metal ions and ligands. Many scientists have developed reaction system composed of metals and amphiphiles to exert useful catalytic functions (2–6).

The aggregation behavior of the metalloamphiphilic compound is an important factor in determining the extent of an aggregate-aided reaction as the result of electrostatic and hydrophobic interactions. Aggregates in an aqueous phase will provide a microenvironment that differs substantially from that of bulk water, and factors influencing these properties are of significance and are related to their potential application in membrane mimetic chemistry. The epimerization of aldoses by an amphiphilic nickel complex is an example of a reaction where micellar effects can be observed without the complicating presence of a solubilizate (7). The rate of epimerization of sugars was considerably increased with a stereospecific rearrangement by a Ni-lipophilic diamine complex, which formed a metallomicelle in an aqueous reaction system.

It is well known that these ordered arrangements of extended assembly structures, called lyotropic mesomorphs, show marked effects on the properties of the systems. The effects result in enhancement of reactivities and selectivities. It is expected that the aggregating behavior can be changed by controlling such factors as the kinds of ligands and metals, temperature, and concentration of the complexing agent. Various aggregates, such as micelles and reversed micelles, microlayer and bilayer vesicles, and lamellae, could be prepared, depending on the conditions.

To explore the structural effect on aggregative properties, we prepared the amphiphilic cobalt (III) complex (**7**) shown in Scheme 1. Because it coordinates a diamine moiety that is chemically associated with higher diacylglyceryl groups as a hydrophobic, and a tetramine group and centered cobalt (III) cation as a hydrophilic, formation of assemblies can be expected for this kind of amphiphile. The combination of pronounced hydrophobic and hydrophilic properties within one metal complex gives these molecules unique properties on dissolution in water. The information obtained on the assembly characteristics might be useful for designing functional metalloaggregates and preparing more effective reaction systems. The physicochemical properties, such as phase-transition temperatures and morphology of the assemblies in aqueous solution, were evaluated to obtain more fully refined structures at various hydrophilic and lipophilic balance conditions.

EXPERIMENTAL PROCEDURES

General measurements. Throughout this work, Merck precoated thin-layer chromatography (TLC) plates (silica gel 60F254, 0.25 mm; E. Merck, Darmstadt, Germany) were used for TLC analysis. For preparative column chromatography, Wacogel C-200 (Wako Pure Chemical Industries, Tokyo) was employed. Melting points were recorded on a melting-point

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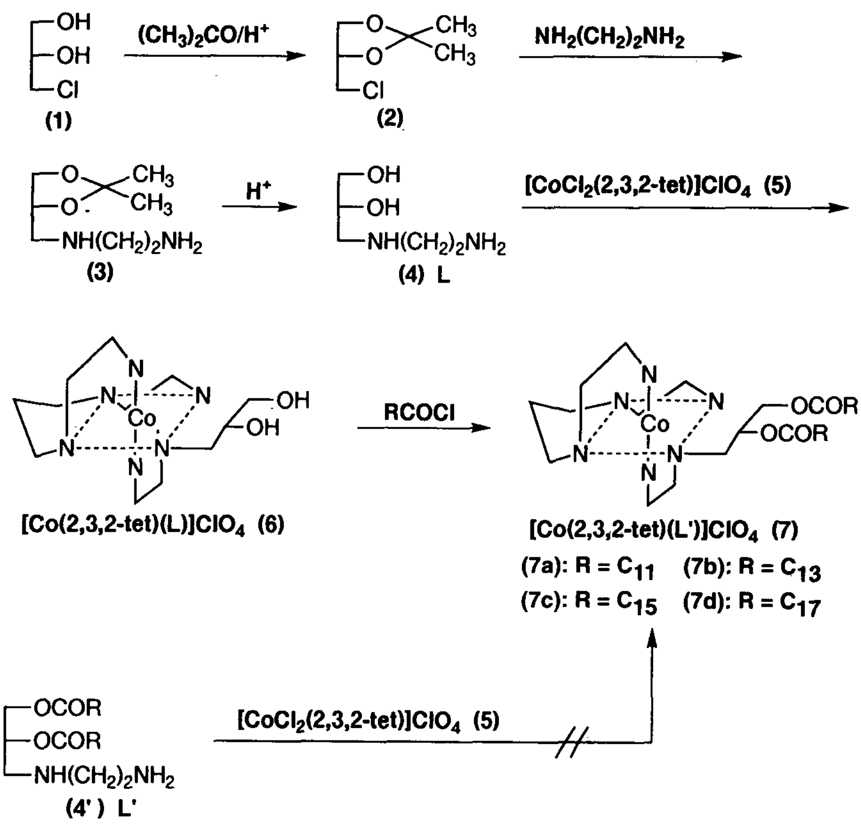

SCHEME 1

apparatus MP-J3 (Yanagikoto Co., Tokyo, Japan) and were uncorrected. Fourier-transform infrared (IR) spectra were taken with a JASCO spectrometer FT/IR-5300 (Japan Spectroscopic Co., Tokyo, Japan). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a JEOL FX 90A (JEOL, Tokyo, Japan) (90 MHz for ^{1}H NMR and 22.5 MHz for ^{13}C NMR). Chemical shifts are reported in ppm downfield (δ) from the internal standard (tetramethylsilane in $CDCl_3$; DMSO-d_6 and 2,2-dimethyl-2-silapentane-5-sulfonate in D_2O). The differential scanning calorimetry (DSC) measurements were carried out with a DSC8240 (Rigaku Co., Tokyo, Japan) at a heating rate of 2 K/min in the range of −30 to 90°C.

Raw materials. Ethylenediamine and four kinds of acid chlorides (dodecanoyl, tetradecanoyl, hexadecanoyil, and octadecanoyl chlorides) of the highest quality available were used without further purification. Cobalt chloride hexahydrate was of guaranteed grade from Wako Pure Chemical Industries, Ltd. All other reagents and solvents were of the highest commercial purity available.

Synthesis of complexes. Scheme 1 shows an outline for the preparation of the amphiphilic cobalt complex. The ligand, 1,2-dihydroxy-3-ethylenediaminopropane (**4**), and its corresponding precursor isopropylidendated derivative (**3**) were prepared in a manner similar to the synthesis of 1,2-dihydroxy-3-(N′,N′-dimethyl-ethylenediamino) propane, as previously described (8). The ligand (**4**) was a pale yellow viscous liquid. The yield of (**4**) from (**3**) was 84%. IR (neat) 3,360; 2,933; 1,468; 1,582; 1,104; 1,041 cm^{-1}; ^{1}H NMR (D_2O) δ = 2.55–2.75 (*m*, 6H, $C\mathit{H}_2NHC\mathit{H}_2C\mathit{H}_2NH_2$), 3.55 (*m*, 2H, $C\mathit{H}_2OH$), 3.8 (*m*, 1H, $C\mathit{H}OH$).

A precursor complex (**6**) was prepared from the complex *trans*-[$CoCl_2$ (2,3,2–tet)]ClO_4 (**5**) (9) and the ligand (**4**). 1.58 g (4.06 mmol) (**5**), 0.1 g activated charcoal, and 15 mL freshly distilled dimethylsulfoxide (DMSO) were placed in a 50-mL round-bottomed flask, fitted with a magnetic stirring bar and a reflux condenser. The mixture was then stirred and heated at 50°C while 15 mL DMSO solution, containing 0.67 g (5.0 mmol) diamine ligand (**4**), was added dropwise. After the mixture was stirred at 50°C for 3 h, the reaction product was filtered, and the filtrate was concentrated to dryness. The residue was dissolved in water and adsorbed on the top of an SP-Sephadex C-25 column (Na$^+$ form; Pharmacia LKB, Uppsala, Sweden). Elution with 0.5 M sodium chloride solution afforded an orange band. Concentration of the eluate from the band resulted in an orange precipitate. The crude product was dissolved in ethanol, and insoluble sodium chloride was filtered off. The corresponding perchlorate complex was obtained from the chloride of the complex by using the same SP-Sephadex column with 0.5 M sodium perchlorate as an eluent. Sodium perchlorate was filtered off in a manner similar to that above. After several cycles, an orange powder was obtained, which was recrystallized from 50% ethanol. Complex (**6**) was a reddish powder: yield 66%; IR (KBr) 3,540; 3,343; 3,297; 2,895; 1,605; 1,470; 1,142; 1,090 cm^{-1}; ^{1}H

NMR ($\mathrm{D_2O}$) δ = 2.10 (2H, *m*, $\mathrm{CH_2}\mathit{CH}_2\mathrm{CH_2}$), 2.35–3.30 (18H, *m*, $\mathit{CH}_2\mathrm{NH_2}$, $\mathit{CH}_2\mathrm{NH}$), 3.50–3.70 (2H, *m*, $\mathit{CH}_2\mathrm{OH}$), 4.15 (1H, *m*, C*H*OH).

Synthesis of complex (**7**) was made by direct acylation of complex (**6**). Preparation of the hexadecanoyl homologue (**7c**) is shown here as an example. After the finely ground complex (**6**) (1.00 g, 1.54 mmol) was dissolved in 15 mL dimethylacetamide (DMAc), 1.85 g (18.3 mmol) of triethylamine was added. Then, a mixture of hexadecanoyl chloride (1.02 g, 3.70 mmol) and DMAc (5 mL) was added dropwise to the solution with stirring. Stirring was continued overnight at room temperature. The solvent, DMAc, and excess triethylamine were removed by evaporation *in vacuo*. Acetone (150 mL) was added to the residue. A powdery orange precipitate was collected by filtration with suction, washed with acetone, and air-dried. The filtrate was concentrated to dryness. The residue was dispersed in ether and centrifuged. After several cycles, an orange precipitate was collected and combined with the preceding acetone-insoluble material. The crude combined product was dissolved in a minimum amount of methanol and then adsorbed on the top of an SP-Sephadex C-25 column. The column was eluted with a mixed solvent of ethanol and chloroform (3:2, vol/vol). The colored fractions were collected. The concentration of collected effluent on a rotary evaporator resulted in an orange precipitate, which was purified by recrystallization from a mixed solvent of ethanol and chloroform in a 50:50 volume ratio. The yield of (**7c**) from (**6**) was 21%. IR (KBr) 3,073, 2,920, 2,851, 1,742, 1,093 $\mathrm{cm^{-1}}$; ^{1}H NMR (DMSO–$\mathrm{d_6}$ + $\mathrm{D_2O}$) δ = 0.85 (6H, *t*, *J* = 7.5 Hz, $\mathit{CH}_3 \times 2$), 1.24 (48 H, *br s*, $\mathrm{CH_3}\,(\mathit{CH}_2)_{12} \times 2$), 1.49 (4H, *m*, $\mathrm{COCH_2}\mathit{CH}_2 \times 2$), 1.75 (2H, *m*, $\mathrm{NCH_2}\mathit{CH}_2\mathrm{CH_2N}$), 2.00–3.15 (22H, *m*, $\mathit{CH}_2\mathrm{NH_2}$, $\mathit{CH}_2\mathrm{NH}$, $\mathrm{CO}\mathit{CH}_2\mathrm{CH_2} \times 2$), 3.85–4.25 (2H, *m*, $\mathit{CH}_2\mathrm{O}$), 4.80 (1H, *m*, C*H*OH); found: C, 49.69%; H, 8.82%; N, 8.10%. Calcd. for $\mathrm{C_{44}H_{94}Cl_3CoN_6O_{12}}$; C, 49.65%; H, 8.90%; N, 7.89%. The elemental analyses of other complex homologues were also in fair agreement with the theoretical value.

Preparation of vesicles for transmission electron microscopy (TEM). Small unilamellar vesicles (SUV) were prepared by a standard technique, as follows (8,10,11). The dried amphiphilic complex (**7**) (10–20 mg) was dissolved in 2 mL water. The mixture was shaken by vortexing for 30 s at room temperature and then allowed to equilibrate for 2 min. This cycle of vortexing–equilibrating repeated three times. The resulting 0.5–1% aqueous solution was ultrasonicated for 10 min in a Bransonic 52 bath-type sonicator (Branson Ultrasonic Corp., Danbury, CT). With this method, sonication resulted in perfectly clear solutions. Temperature was maintained 10°C above the corresponding phase-transition temperature (Tc). The water used for the preparation of vesicles was carefully prepared to remove all colloidal impurities. Deionized water was further distilled in a quartz distilling apparatus. The conductivity and surface tension of the water used were less than 1 μS/cm and more than 71 dyne/cm, respectively.

TEM observation. TEM observation was carried out on an HU12AS electron microscope (Hitachi, Ltd., Tokyo) at an operating voltage of 75 k V. Negatively stained samples were prepared by a standard technique (12,13). A copper grid covered with formvar was further coated with a thin carbon film (thickness: 50~80 Å) by vapor deposition to enhance the affinity for the amphiphilic cobalt complex. Staining of the complex vesicles was done with 0.5% uranyl acetate solution. A drop of the complex vesicle solution was applied on the copper grid, and the excess solution was blotted off, followed by the addition of a drop of uranyl acetate solution. The grid was left in the air for a few minutes to dry after blotting off the excess staining solution. Representative photos are shown in Figures 1–3.

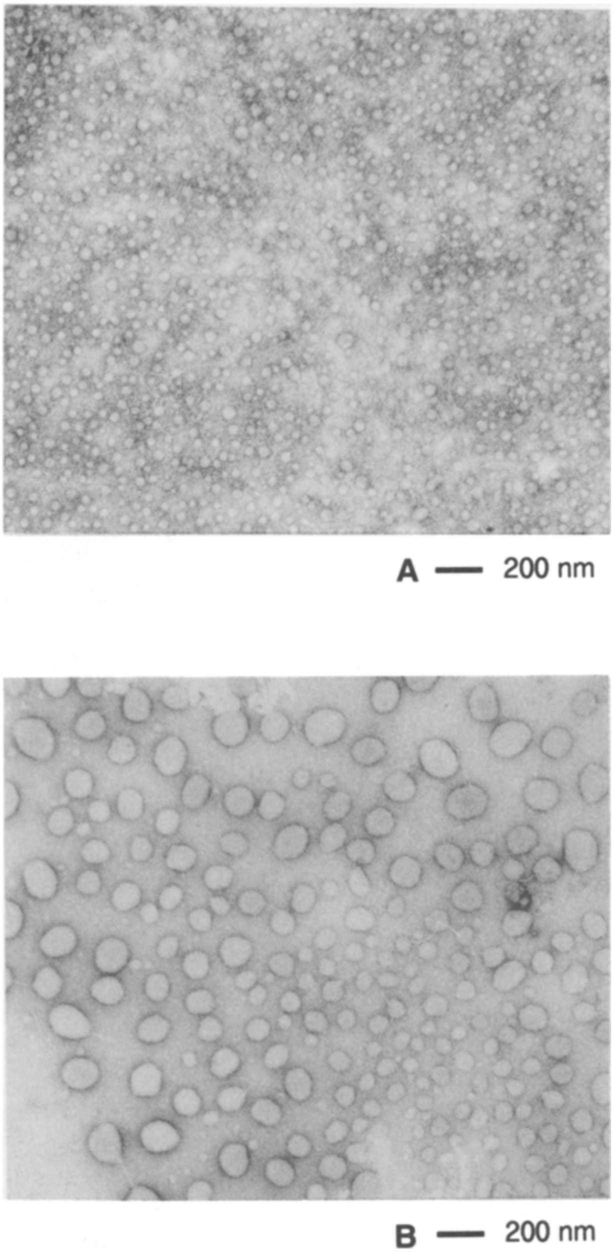

FIG. 1. Transmission electron microscopic views of the cobalt complex vesicles stained with uranyl acetate after using the drop method: A), ditetradecanoyl homologue; B), dioctadecanoyl homologue.

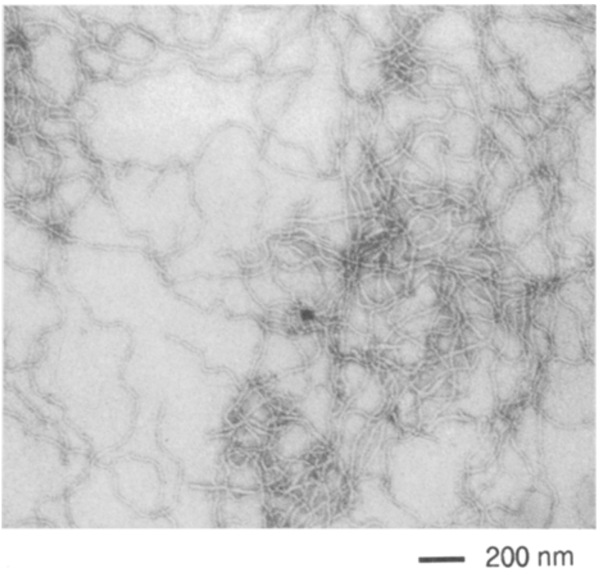

FIG. 2. Transmission electron microscopic views of the cobalt complex aggregates stained with uranyl acetate after using the drop method. Fibrous structure from ditetradecanoyl homologue.

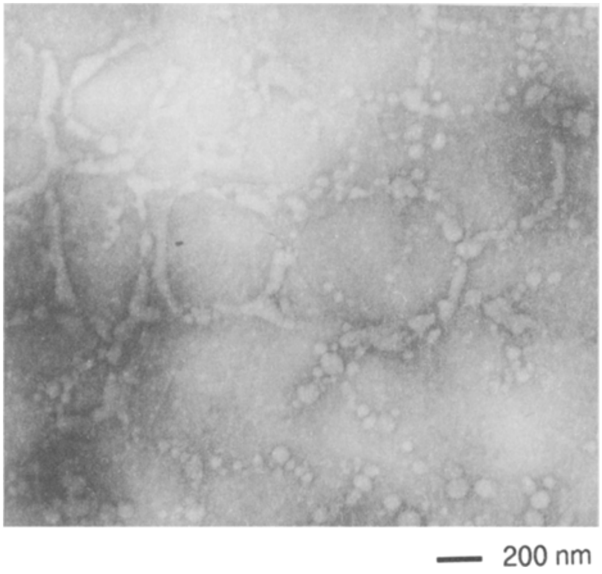

FIG. 3. Transmission electron microscopic views of the cobalt complex aggregates stained with uranyl acetate after using the drop method. Fusion of small sphere vesicles into network structure.

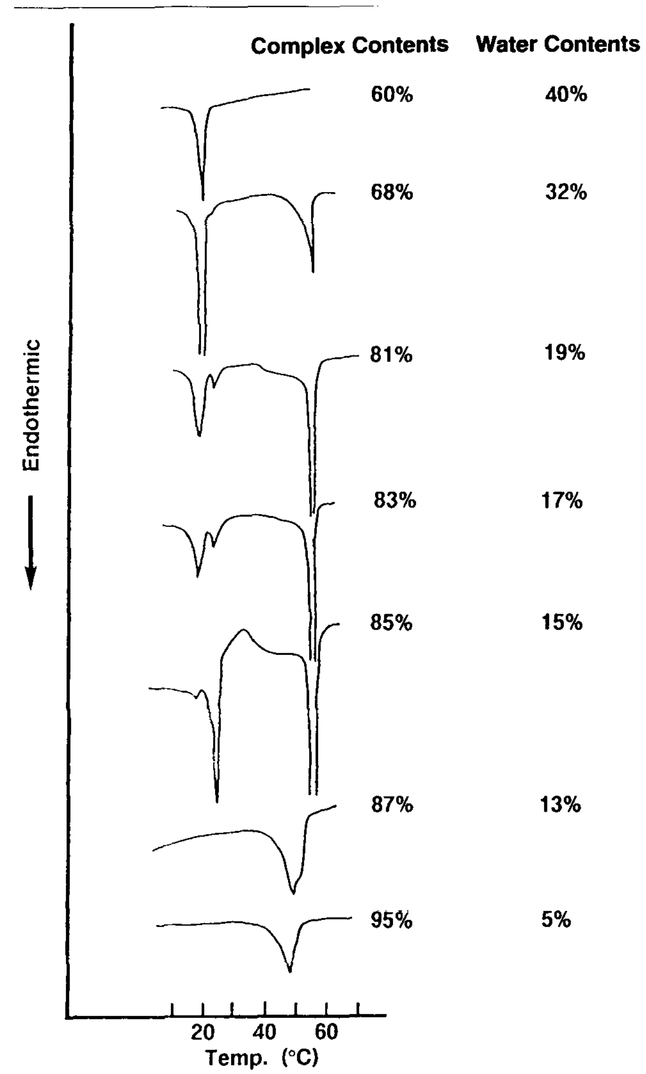

FIG. 4. Differential scanning calorimetry curves of cobalt complex (C_{14} homologue) at various water contents.

Thermal analysis. The amphiphilic cobalt (III) complex/water system was prepared over the concentration range $0.95 > c > 0.05$. Before thermal analysis, heating and cooling of the aluminum pan containing the sample were repeated three or four times a cross the presumed Tc to homogenize the mixing state of the greasy sample. Tc and the enthalpy difference (ΔH) in the phase transition between gel and liquid crystal were studied. Tc was defined as the temperature at which the slope of the forearm of the main peak intersected the baseline in the DSC curve (Fig. 4). Results are summarized in Table 1.

RESULTS AND DISCUSSION

Synthesis of the complex. As shown in Scheme 1, our essential synthetic approach was based on the direct acylation of the two hydroxyl groups of complex (**6**) in coexistence with plural amino groups. Various kinds of solvents were examined in seeking appropriate conditions to acylate only the hydroxyl groups in the complex. Generally, some aprotic polar solvents, such as DMSO, dimethylformamide (DMF), DMAc, and acetonitrile (AN), were more suitable for this reaction than were hydrocarbon and ether solvents, such as hexane, chloroform, ether, dioxane, and tetrahydrofuran. Particularly, DMAc was the most satisfactory solvent among the tested solvents with respect to reactivity and solubility of the substrate and other reactants. To elevate the yield of complex (**7**), solubility of reactant complex (**6**) in a solvent was improved. Modification of the counter anion of (**6**) from chloride to perchlorate ion raised the solubility of the complex in DMAc. The actual increase in yield was accomplished by a solubility rise, and the result was that the solubility increment showed a much greater effect than those resulting from other conditions. For example, elongating reaction time and increasing reaction temperature merely led to an increase in unfavorable products, which could not be identified.

It was confirmed that the amino groups of the ligand that were coordinated to the center metal, cobalt, were well protected against acylation. A typical IR spectrum of product (**7**) did not show an amide group, but it did show an ester functional group at 1,742 cm^{-1}. Despite this impressive endeavor, the direct coordination of a higher-acylated bulky ligand, such as N-(2,3-diacyloxypropyl)-ethylenediamine (**4′**, **L′**), into complex (**5**) was unsuccessful. Because both reactants, diamine (**4′**) and complex (**5**), could be easily prepared in advance, if the above reaction was possible, a new preparation method would be developed.

The hydroxyl groups were successfully acylated without acylation of the coexisting amino groups, even though the yield was unsatisfactory. A convergent route for the synthesis of the double-chained amphiphilic complex, containing two higher acyl groups, has been developed. It could be used for the synthesis of analogues that coordinated an optically active ligand with the same structure as (**4**) by a similar method starting with the relevant optically active isopropylidenechloride (**2**) (8).

Aggregate morphology. To confirm the formation of aggregates, the vortexed and subsequently ultrasonicated aqueous solutions that contained the amphiphilic cobalt complexes were observed by TEM because their structure could be elucidated best directly by microscopic observation. The cobalt complexes aggregated in different forms depending on their acyl chainlength. As some photos in Figures 1–3 show, spherical and ellipsoidal particles of various magnitudes, fibrous images, and rodlike deformed vesicles were observed.

The influence of the hydrophilic and hydrophobic balance in an amphiphile on the aggregation state of the cobalt complex was studied. Didodecanoyl homologues showed no sharp aggregates because their hydrophobicity may be too poor to form aggregates. On the other hand, the homologues higher than ditetradecanoyl steadily produced numerous types of aggregates, such as spherical and oval particles, with various diameters ranging from 20 to 200 nm, and elongated fibrous aggregates.

Figures 1A and 1B show that spherical unilamellar vesicles with different diameters were obtained. The micrographs of the specimen contained a high population of spherical particles ranging from 20–200 nm in diameter. They were indistinguishable from dimyristoyl lecithin vesicles as reported, for example by Sundler *et al.* (10), Menger *et al.* (11), and Schullery *et al.* (12), in terms of their magnitudes and shapes. The C_{14} homologue formed smaller structures, with diameters ranging from 20–50 nm and appeared to be relatively monodisperse. C_{18} formed a second set of characteristic sizes with the most probable diameter showing a greater range of variation. According to Figure 1A, the diameters of the vesicles were widely distributed in this system. Under conditions where an ultrasonicating bath was used for preparing vesicles, even if the sonic radiating time was the same, the reproducibility of the vesicle size was poor. Among the three different chainlengths, however, the most probable diameter showed an increasing trend with a lengthening acyl chain (13). The fundamental unit of these aggregates seemed to have a bilayer structure, similar to that of the biomembrane that mainly consists of lecithins, as could be seen from the fact that the layer thickness was 4–5 nm. These images were similar to those found for vesicles prepared from various other kinds of artificial amphiphilic compounds that contained plural hydrophobic groups (14–16). Unequivocally, unilamellar vesicles were observed, and neither oligonor multilamellar structures were ever seen in these systems.

As shown in Figure 2, at the same time, elongated tubular aggregates emerged as clear figures. According to electron microscopy, the external and intermal radii of the tube were homogeneous and were 25–40 and 20–30 nm, respectively; the thickness of the wall was approximately 4–5 nm, compatible with the vesicle's wall size (Fig. 1A), introduced above, and in naturally-occurring lecithins. If the radius of the rodlike micelle corresponds approximately to the extended length of the hydrocarbon chain of the acyl group in an amphiphile, the diameters of the fibrous assemblies obtained in this work are about two or three times thicker than that of the rodlike micelle, where hydrophilic groups constitute the surfaces of the cylinders and the hydrophobic groups comprise their interior's. The diameters of the fibrous assemblies prepared in this study have suggested that the aggregates were not rodlike micelles (17,18) but were tubelike bilayer aggregates that surrounded a definite internal volume.

Figure 3 shows the intermediate stage that produces network structures composed of cross-linked aggregates. This sample was prepared from C_{16} homologues and was incubated at room temperature for 15 h after the sample solution was sonicated. The newly prepared network structures seemed to be a result of fusion processes of the corresponding isolated vesicles. Each unilamellar vesicle contacts another at the junction, and the resulting dimer vesicles develop into rod-shaped vesicles. These elongated vesicles further aggregated with each other, fused again, and then constructed networks. The diameters of the fibrous aggregates in a network structure and the original vesicles were almost same.

The development of vesicles into the formation of a huge fibrous-shaped vesicle could be compatible with an alteration in the fusion of lecithin bilayer membranes (19,20). The present picture shows the fusion of a totally manmade artificial bilayer vesicle. It will be used for preparing molecular organizations that possess various functions.

Phase-transition behavior. Phase transition of the cobalt complexes was observed by DSC. As an example, the endothermal heat vs. temperature diagram of the cobalt complex (ditetradecanoylated homologue) is illustrated in Figure

4. Enthalpy of the phase transition was calculated from the peak area of the diagram. In the range of about 15 to 35% water content, plural peaks appeared and disappeared. Their shapes and magnitudes changed in accordance with their water contents. This shows that various morphologies were equally probable at different water contents (21). This thermal behavior is summarized in Figure 5. At this stage, we could not identify the presence of the pre- and sub-phase transition in addition to the main-phase transition as determined for phosphatidyl choline (22).

When a water content greater than 35% was available, the sample showed one endothermic peak at relatively constant temperature regardless of its water content. The temperature appearing in this high-water region is defined as Tc between gel and liquid. It decreased slowly and slightly from 17 to 12°C as the concentration of water increased. Tc values were somewhat lower than that of lecithin, which possesses the same acyl groups.

Tc values of the complex are summarized in Table 1. The thermal-phase transition was somewhat similar to that of dipalmitoyl lecithin, although the temperature was quite different. As Table 1 shows, Tc of each amphiphilic Co (III) complex gradually increased with increasing hydrocarbon chainlength. The hydrophobic interaction between long hydrocarbon chain moieties in the amphiphiles is essential for aggregation in an aqueous solution. At the same time, the interaction between head groups is another significant factor, which determines the packing state of the aggregate. The Co (III) complex has a strong positive charge, as the structure itself shows. These differences in Tc between the amphiphilic metal complex and the representative double-chain vital lipid, lecithin, are due to the strong electrostatic repulsion around the central metal ion.

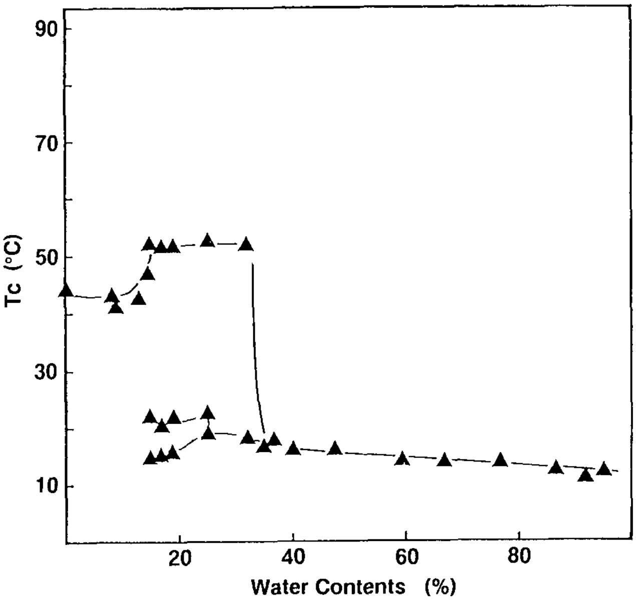

FIG. 5. Phase diagram of cobalt complex (diC_{14} homologue)/water system.

TABLE 1
Temperatures and Enthalpy Differences During the Phase Transition Between Gel and Liquid Crystal of the Amphiphilic Co Complexes

Acyl groups	Tc[a] (°C)	ΔH (kcal/mol)
C_{12}	—	—
C_{14}	12.0	4.81
C_{16}	36.6	6.75
C_{18}	51.5	8.66

[a] Phase-transition temperature.

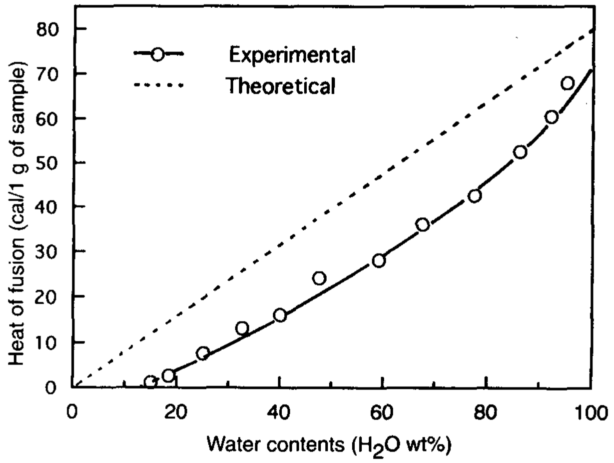

FIG. 6. Variation in heat absorbed at melting point of ice vs. water content for Co complex (diC_{14} homologue)/water system.

Despite the existence of a prescribed amount of water in the amphiphile/water system, the peak due to the fusion of ice at around 0°C was not observed when the water content was below 15%. Variation in the heat absorbed at the fusing point of water is illustrated graphically in Figure 6. The results of the DSC curve demonstrated that the added water existed not as free water but as bound water. The ratio of amphiphile to bound water was about 1:5.5 by weight; that is, 1 amphiphilic molecule was associated with 10 water molecules, which did not form ice on cooling even to −30°C.

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REFERENCES

1. Fendler, L.H., *Membrane Mimetic Chemistry*, John Wiley & Sons, New York, 1982.
2. Zimmerman, C.S., and B.F. Duerr, *J. Org. Chem. 57*:2215 (1992).
3. Daeid, N.N., and K.B. Nolan, *Inorg. Chem. Acta 211*:55–60 (1993).
4. Takagi, K., N. Miyake, E. Nakamura, H. Usami, and Y. Sawaki, *J. Chem. Soc., Faraday Trans. I 84*:3475 (1988).
5. Sutton, P.A., and D.A. Buckingham, *Acc. Chem. Res. 20*:357 (1987).

6. Sagawa, T., H. Ishida, K. Urabe, K. Yoshinaga, and K. Ohkubo, *J. Chem. Soc., Perkin Trans. 2*:1 (1993).
7. Osanai, S., R. Yanagihara, K. Uematsu, A. Okumura, and S. Yoshikawa, *J. Chem. Soc., Perkin Trans 2*:1937 (1993).
8. Osanai, S., T. Okada, T. Yamamura, and S. Yoshikawa, *J. Jpn. Oil Chem. Soc. 41*:293 (1992).
9. Brubaker, G.R., and D.S. Schaefer, *Inorg. Chem. 10*:811 (1971).
10. Sundler, R., N. Duezguenes, and D. Papahadjopoulos, *Biochim. Biophys. Acta 649*:751 (1981).
11. Menger, F.M., J.-J. Lee, P. Aikens, and S. Davis, *J. Colloid Inter. Sci. 129*:185 (1989).
12. Schullery, S.E., C.F. Schmidt, P. Felgner, T.W. Tillack, and T.E. Thompson, *Biochem. 19*:3919 (1980).
13. T.J. Racey, M.A. Singer, L. Finegold and P. Rochon, *Chem. Physics Lipids 49*:271 (1989).
14. Lukac, S., and A. Perovic, *J. Colloid Interface Sci. 103*:586 (1985).
15. Murakami, Y., A. Nakano, and H. Ikeda, *J. Org. Chem. 47*:2137 (1982).
16. Kunitake, T., and Y. Okahata, *J. Am. Chem. Soc. 99*:3860 (1977).
17. Attwood, D., and A.T. Florence, *Surfactant Systems,* Chapman and Hall, New York, 1983.
18. Rosen, M.J., *Surfactants and Interfacial Phenomena*, John Wiley & Sons, New York, 1978.
19. Vail, W.J., and J.G. Stollery, *Biochim. Biophys. Acta 551*:74 (1979).
20. Lucy, J.A., *Nature 227*:814 (1970).
21. Kodama, M., M. Kuwabara, and S. Seki, *Biochim. Biophys. Acta 684*:59 (1982).
22. Chen, S.C., and J.M. Sturtevant, *Proc. Natl. Acad. Sci. USA 77*:5060 (1980).

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