Pergamon

Tetrahedron Vol. 51, No. 2, pp. 423-434, 1995

0040-4020/95 $9.50+0.00

0040-4020(94)00906-6

Control of Vesicle Formation by Chemical Switching

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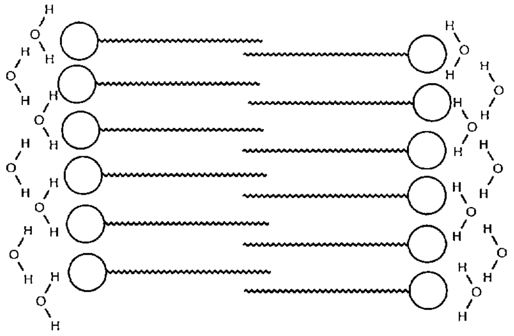
Abstract

The formation and collapse of liposomes is controlled by the existing balance between polar headgroup and hydrophobic tail. Alteration of the headgroup's charge state can be controlled, for example by redox-switching, which in turn permits regulation of aggregate formation.

1. Introduction

The bimolecular cloister commonly called cell membrane is composed of a matrix of amphiphilic molecules.[1] The membrane is formed from individual monomers that possess a polar, hydrophilic headgroup and a non-polar hydrophobic tail. In an aqueous environment, the hydrophilic headgroup orients itself to optimize interactions with bulk water. The hydrocarbon tails have little or no affinity for the polar medium and align, as best they can considering structure, with each other. When organization is favored in natural systems, an organelle or cell is formed.[2] In synthetic systems, organization of these amphiphilic monomers affords liposomes or synthetic surfactant vesicles.[3]

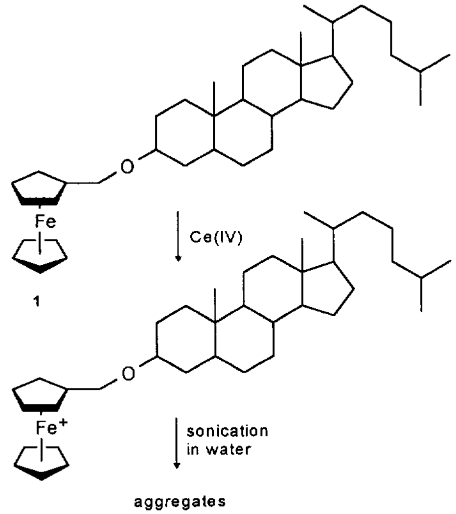
The formation of organized assemblies from a variety of synthetic monomers has been known for many years and is widely studied.[4] Many of the factors that control organization are now well understood and attempts have even been made to quantitate them.[5] It has also been recognized for some years that not only can liposomes be excellent models for biomembranes, they can also be used to envelop and deliver drugs.[6] In the latter sense, they are host molecules that deliver a biologically active guest such as a therapeutic agent. If constructed appropriately, the encapsulation of many guests is possible by a particular liposomal host. This is not molecular recognition in the sense of small molecules having complementary structures that permit selective association. The formation of the host aggregates is, however, very much a matter of molecular recognition since the head groups must be appropriate to



form an outer barrier to bulk water and the nonpolar chains must organize themselves in three dimensions into a wall of hydrocarbons that presents a barrier to the very water that attracts the polar head groups.

1.1 Switching

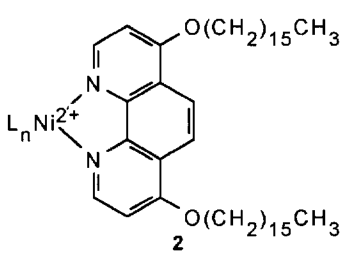
If a compound has a structure in which there is a polar head group (represented in the figure above by a circle) and a nonpolar tail (represented by a wavy line), then the compound is, by definition, amphiphilic. An amphiphile may or may not form aggregates and the formation of stable vesicles is, all things considered, an unlikely process. Such compounds as sodium dodecyl sulfate (often NaLS for sodium lauryl sulfate) dissolve in water. Aggregation of these monomers may occur when a concentration known as the critical micelle concentration (CMC) is reached. When such a concentration is attained or surpassed, globular aggregates may form which have the polar head groups turned outward toward the bulk aqueous environment and the tails turned inward to interact with and solvate each other. Different amphiphilic structures may form bilayers (a single membrane) or multiple bilayers (like an onion). The latter situation is usually referred to as "multilamellar" as opposed to "unilamellar."



There are obviously several natural tendencies that may be exhibited by amphiphiles. Rather than adhering to existing structures and proclivities, however, it has been our interest to control the aggregation of various monomers by chemically altering the amphiphilic balance between headgroup and tail. It should be noted that both Grätzel *et al.*[7] and Saji *et al.*[8] have altered molecular and aggregation properties by oxidation and reduction. We have reported three structurally distinct examples of this phenomenon. In the first of these,[9] cholestanyl ferrocenylmethyl ether, **1**, was oxidized to the corresponding ferrocenium salt. Sonication in water afforded vesicles that were found to be multilamellar. Reduction of these aggregates returned ferrocene to its normal, relatively nonpolar, state and the aggregates collapsed.

1.2 Alternate Switching

Another system which exhibits redox-switchable properties is based upon 1,10-phenanthroline.[10] In this case, the switching mechanism is somewhat different. In particular, 4,7-dihydroxy-1,10-phenanthroline was alkylated with hexadecyl bromide to give the bis(ether). Both the silver and nickel complexes were formed (**2**). Sonication afforded aggregates which were characterized primarily by laser light scattering. Collapse of the vesicles was accomplished by treatment with a dilute solution of the reducing agent sodium dithionite ($Na_2S_2O_4$). In this case, the metalloamphiphilic monomer exhibits a tendency to form aggregates and alteration of its charged headgroup so diminishes the amphiphilicity that aggregation cannot be sustained.



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The third, structurally distinct, system that exhibits switchability is the bis(hexadecylamine) salt of silver acetate (**3**). We demonstrated that these monomers could aggregate into vesicles of approximately 1200Å in size and be collapsed by contact with a dilute aqueous formaldehyde solution. The deaggregation involves the classical Tollens reaction in which formaldehyde is oxidized to formate as Ag^+ is reduced to Ag^o.

$AcO^-Ag^+[H_2N(CH_2)_{15}CH_3]_2$

3

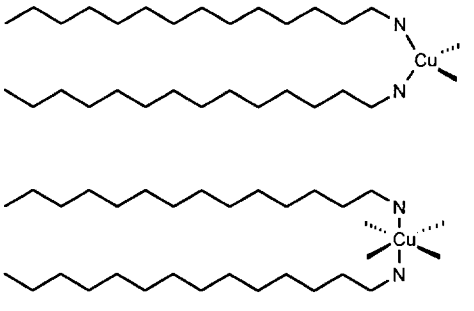
1.3 Reversibility of aggregate formation

An important goal of this work is to demonstrate a "cycle" of vesicle formation. The overall sequence of events involves formation of vesicles from monomers. The type of switching used is determined by whether the monomer is itself an amphiphile or if it must be converted into one by a chemical step. The ferrocenylsteroid, **1**, is non-amphiphilic but can be oxidized to form active monomers. Metalloamphiphiles **2** and **3** form aggregates when sonicated. Collapse of the metalloamphisomes formed from $\mathbf{1}^+$ can be induced by reduction (*i.e.* $\mathbf{1}^+ + e \rightarrow \mathbf{1}$). In the case of **2** and **3**, the amphiphiles must be converted into a non-amphiphilic form before aggregate collapse can occur. Reformation by chemical steps of $\mathbf{1}^+$, **2**, or **3** will lead to active amphiphiles but the monomers do not spontaneously form aggregates without sonication or other application of energy. A cycle, then, is the sequence monomer → amphiphile → aggregate → monomer in the case of $\mathbf{1}^+$ and amphiphile → aggregate → monomer in the case of **2** or **3**. In the latter cases, if the monomer or "pro-amphiphile" is isolated and converted into the corresponding amphiphile, aggregates can be formed as before.

The vesicle systems studied are designed to be deaggregated by altering a specific chemical property of the monomer. For example, ferrocenylsteroid **1** is oxidized to form a positively-charged metalloamphiphile. Collapse of the vesicles formed from $\mathbf{1}^+$ could be accomplished by a variety of vigorous means, but were effected by reduction ($\mathbf{1}^+ \rightarrow \mathbf{1}$). The reducing agents used for this reaction were $Na_2S_2O_4$ and $NaBH_4$. The collapse of vesicles in the presence of salts may be attributed to salt-induced lysis rather than to a specific chemical switch. We therefore demonstrate, using a novel metalloamphiphile system, that switching can be accomplished with a purely organic trigger by a clear, chemical mechanism.

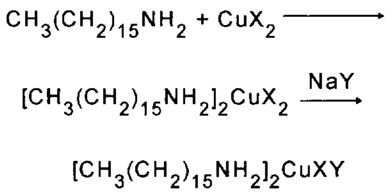
2. Results and Discussion

2.1 Formation of a novel copper amphiphile



The salt diamminecopper(II) acetate, $(H_3N)_2Cu^{2+}(CH_3COO^-)_2$, is known.[11] Conceptually, extension to a metalloamphiphilic monomer is straightforward: the ammonia ligands are replaced by two molecules of hexadecylamine. The structure of the resulting complex is not necessarily obvious, however, since $(H_3N)_2Cu^{2+}Cl_2$ for example exists in structurally and magnetically non-equivalent forms designated as α and β.[12] Many copper compounds exist in distorted polyhedral arrangements[13] ranging from square planar to tetrahedral to octahedral. For our purposes, the potential amphiphile $[CH_3(CH_2)_{15}NH_2]_2CuX_2$ will have chains extending in the same direction and proximate to each other irrespective of the copper geometry. The figure above shows schematically what is apparent from an examination of CPK molecular models, namely that the two chain positions differ little from tetrahedral to *trans*-octahedral. This might contradict one's intuition that the only acceptable geometries are those in which copper is tetrahedral, *cis*-square planar, or *cis*-octahedral. An examination of models also makes clear that if the two polymethylene chains align, the identities of the charged groups attached to copper are sterically relatively unimportant so long as they are reasonably small. Thus chloride, bromide, or acetate anions are expected to have a similar effect on the compound's overall amphiphilicity.

In principle, reaction of hexadecylamine with either $CuCl_2$ or $Cu(OAc)_2$ could give the complex $(C_{16}H_{33}NH_2)_2Cu^{2+}X_2$ in which X is acetate or chloride or one of each. When hexadecylamine is treated with $CuCl_2$ and NaOAc, any of three copper



$$CH_3(CH_2)_{15}NH_2 + CuX_2 \longrightarrow [CH_3(CH_2)_{15}NH_2]_2CuX_2 \xrightarrow{NaY} [CH_3(CH_2)_{15}NH_2]_2CuXY$$

amphiphiles may result. We have isolated two (**4** X_2 = Cl,OAc and **5** X_2 = Cl_2) of the three possible compounds by the following approach. A methanol solution of $CuCl_2 \bullet 2H_2O$ was added to a methanol solution of sodium acetate. To the combined solution was added a methanol solution of hexadecylamine. Metathesis was accomplished using sodium acetate in methanol. Precipitation of a blue-green mixture of compounds occurs from which can be obtained by careful crystallization $(C_{16}H_{33}NH_2)_2CuXY$ where X = Y = Cl and X = Cl, Y = OAc (see experimental section). Three crystallizations from methanol gave the chloride acetate complex, **4**, as a light blue solid which softened at 85 °C and then melted at 99-101 °C, in 40% yield. Bis(hexadecylamine)copper(II) dichloride can be prepared by treating copper(II) chloride dihydrate with two equivalents of hexadecylamine in methanol. Both compounds were used in the present studies.

2.2 Aqueous self-assembly

Upon mild ultrasonic dispersion in water (*see* Experimental), either copper(II)hexadecylamine complex yields a sky-blue suspension of metalloamphisomes (apparent hydrodynamic diameters shown in Table 1). For **4**, in 0.0100 M tris(hydroxymethylamino)methane hydrochloride ["Tris" or "tromethane" hydrochloride $(HOCH_2)_3CNH_2$], at pH 8.0, dynamic laser light scattering analysis using a single exponential term yielded the unimodal diameter at 344±100 nm (3440±1000 Å). The multiexponential superposition gave an apparent hydrodynamic size of 402±150 nm (4020±1500 Å) by intensity and 440±170 nm (4400±1700 Å) by weight, respectively. The intensity distribution suggests that the suspension of metalloamphisomes is radially monodisperse. Because of a concern that the primary amino group of tromethane might directly interact with Cu^{2+}, the above experiment was reproduced using $(HOCH_2)_3CNH(CH_2)_3SO_3H$ (TAPS, buffer pH = 8.55) as buffer. Dynamic laser light scattering as above yielded a unimodal diameter of 241±68 nm (2410±680 Å). The cumulant method gave an apparent size of 275±76 nm (2750±760 Å) and by weight: 278±74 nm (2780±740 Å).

Table 1 also lists the apparent hydrodynamic diameter of **4** in tris(hydroxymethylamino)methane buffer at pH 10.0. Analysis of the autocorrelation function according to a single exponential term yielded the unimodal diameter at 232±74 nm (2320±740Å). The cumulant method gave the apparent size as 243±110 nm (2430±1100 Å) by intensity, and 180±120 nm (1800±1200 Å) by weight, respectively. Again, the radial distribution appears to be monodisperse.

In 10 mM tris(hydroxymethylamino)methane at pH 10.0, the acetate counterions may be displaced from the endo- and exovesicular surfaces by hydroxide ions.

Coordination of electrostatically dense hydroxide ions to the hydrophilic headgroups screens intermonomeric cation-cation electrostatic repulsions. The area occupied by the surfactant headgroups is expected to decrease. If all other factors are held constant, a smaller headgroup area should decrease the colloidal diameter compared to that of vesicles suspended in nearly neutral pH.

Table 1. Effect of pH on the hydrodynamic diameter distribution of bis(hexadecylamine)copper(II) metalloamphisomes[a]

		Diameter, in Å		
			Cumulant Distribution	
Compound and Buffer System Employed	pH	Unimodal Diameter	By Intensity	By Weight
4, 0.0100 M Tris•HCl	8.0	3440±100	4020±1500	4400±1700
4, 0.0100 M Tris	10.0	2320±740	2430±1100	1800±1200
4, 0.0100 M TAPS	8.55	2770[b]	2970±570	2980±560
5, 0.0100 M TAPS	8.55	2410±680	2750±760	2780±740

a. The protocol to prepare vesicles is described in the experimental section. b. The instrument reported "narrow" variability limits.

2.3 Redox disassembly protocol

Copper ions normally exist either in the +1 or +2 oxidation state. The cuprous state is accessible by reaction of cupric ions with reducing agents such as sodium dithionite ($Na_2S_2O_4$). In the case of cupric metallovesicles (**4**$_n$), it is useful to measure and compare the radial distribution in the absence and in the presence of the reducing agent. For example, the radial distribution of 1×10^{-5} M bis(hexadecylamine)copper(II) chloride,acetate metallovesicles in 0.0100 M tris(hydroxymethylamino)methane hydrochloride at pH 8.0, is as follows: 169 nm (2%), 300 nm (47%), 533 nm (49%), and 949 nm (2%) respectively. Thus, the average hydrodynamic diameter is about 420±130 nm (4200±1300Å). Furthermore, in the presence of 2×10^{-4} M external Na_2SO_4, which is not a reducing agent, the size distribution remained the same, within experimental error, as follows: 169 nm (14%), 300 nm (30%), 533 nm (44%) and 949 nm (12%), respectively. Thus the average hydrodynamic diameter of about 460±180 nm (4600±1800Å) is the same as that measured in the absence of Na_2SO_4. Addition of Na_2SO_4 does not cause osmotic rupture, or electrolyte-induced flocculation of the vesicles.

In marked contrast to the above, addition of 2×10^{-4} M sodium dithionite under anaerobic conditions causes the metalloamphisomes to collapse into an amorphous, heterogeneous suspension of ill-defined composition. Note that the use of a strong electrolyte as the reducing agent is limited to conditions of high dilution such as those described in Table 2. At higher concentration, osmotic lysis or electrolyte induced flocculation may take place. Clearly, the use of a neutral, polar organic molecule circumvents this difficulty and simplifies the experimental protocol.

Table 2. Salt effect on the hydrodynamic diameter distribution of bis(hexadecylamine)copper(II) metalloamphisomes[a]

		External salt (2×10^{-4} M)[b]	
		Na_2SO_4	$Na_2S_2O_4$
Diameter, nm (Å)[c]	Frequency, %[d]	Frequency, %	Frequency, %
169 (1690)	2	14	0
300 (3000)	47	30	0
533 (5330)	49	44	0
949 (9490)	2	12	0

a. The analyte was prepared (at 25 °C) by dissolving 100 μL of stock vesicle suspension (7.32×10^{-4} M surfactant) in deoxygenated Tris•HCl buffer (3.00 mL, 0.0100 M, pH 8). b. A 6 μL aliquot of 0.100 M Na_2SO_4 or deoxygenated $Na_2S_2O_4$ was added to the vesicle suspension and incubated for 0.5 h. c. Light scattering determined as described in Experimental. d. According to a multiexponential superposition by weight.

Hydroxylamine and hydrazine (see Experimental) are two neutral molecules that may behave as reducing agents. Oxidation of hydroxylamine may be described as follows:

$$NH_2OH \rightarrow NOH + 2H^+ + 2e$$

$$2\ NOH \rightarrow HON{=}NOH \rightarrow N_2O\uparrow + H_2O$$

Oxidation of hydroxylamine affords two NOH radicals that combine to yield hyponitrous acid. This, in turn, decomposes instantly into nitrous oxide and water (see equations). Excess sodium acetate, present in the methanol solution, increases the polarity of the medium which minimizes dioxygen solubility in it. When approximately two equivalents of hydroxylamine hydrochloride were added to **4** under anaerobic conditions, the broad absorption band which characterizes the cupric complex was rapidly (<5 sec) bleached. Exposure of the system to atmospheric oxygen (open cuvette, stirring for 0.5 h) regenerated the blue cupric state. The oxidation and reduction of the metal cation is thus shown to be reversible. Formation of $(C_{16}H_{33}NH_2)_2Cu^{2+}$ from the regenerated metal salt completes a cycle of switched vesicle formation and collapse.

From the above information, the mechanism of anaerobic lysis of **4**-metallovesicles involves electron transfer to reduce cupric ion in the headgroup to the cuprous state. This decreases both headgroup charge density and hydrophilicity. As a result, the vesicular surfaces probably become dehydrated and the polymetallic assemblies are degraded by coalescence into a heterogeneous suspension of amorphous aggregates. This deaggregation phenomenon is reversible to the extent that subsequent aeration regenerates the initial metalloamphiphile.

3. Conclusion

This report concerned the reversible disassembly of electroactive bis-(hexadecylamine)copper(II) metalloamphisomes in alkaline media, in the presence of an external reductant such as dithionite, hydroxylamine or hydrazine. The latter are polar, neutral organic molecules which reduce the electrophilic vesicles smoothly and preclude the possibility of electrolyte-induced flocculation which may take place at high concentrations of intervesicular sodium dithionite. As anticipated, oxidation of hydroxylamine occurs with the simultaneous evolution of a brown gas presumed to be nitrous oxide. In the case of hydrazine, the colorless gas evolved is probably dinitrogen. Deaggregation involves the reduction $Cu^{2+} \rightarrow Cu^{+}$. Subsequent aeration regenerates the initial Cu^{2+} oxidation state from which metalloamphiphile **4** may reform. Salt-induced or other stringent environmental effects do not operate in this system.

An important goal in our development of redox-switched vesicle formation is that, in principle at least, polymetallic vesicles (hosts) may be utilized to discharge occluded solutes (guests) selectively at target sites; for example, under physiological conditions. We have shown here that mild and selective manipulation of a simple monomer may dramatically alter its aggregation state. This in turn may be a trigger for guest release in this macroscopic host-guest complex.

4. Experimental Section

Reagents and Chemicals. ^{1}H-NMR were recorded on a Gemini 300 or Varian VXR-400 NMR spectrometer in $CDCl_3$ solvents and are reported in ppm (δ) downfield from internal $(CH_3)_4Si$. ^{13}C-NMR were recorded on a JEOL FX90Q or Varian VXR-400 NMR Spectrometer or as noted above. Infrared spectra were recorded on a Perkin-

Elmer 1310 Infrared Spectrophotometer and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas Hoover apparatus in open capillaries and are uncorrected. (Thin layer chromatographic TLC) analyses were performed on aluminum oxide 60 F-254 neutral (Type E) with a 0.2 mm layer thickness or on silica gel 60 F-254 with a 0.2 mm layer thickness. Preparative chromatography columns were packed with activated aluminum oxide (MCB 80-325 mesh, chromatographic grade, AX 611) or with Kieselgel 60 (70-230 mesh).

All reagents were the best grade commercially available and were distilled, recrystallized, or used without further purification, as appropriate. Molecular distillation temperatures refer to the oven temperature of a Kugelrohr apparatus. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percents.

The dynamic light scattering measurements were carried out by using a Coulter Model N4MD spectrophotometer equipped with a 4 mW helium-neon laser source, operated at 632.8 nm. The detection angle was fixed at 90°.

Preparation of bis(hexadecylamine)copper(II) chloride acetate, 4. A 50 mL Erlenmeyer flask was charged with $CuCl_2 \bullet 2H_2O$ (0.50 g, 2.93 mmoles) and methanol (10 mL). A bright green solution resulted. Sodium acetate (1.0 g, 12.2 mmoles) was dissolved in methanol (10 mL), the solutions were mixed, and a light blue color developed. A solution of hexadecylamine (1.44 g, 6.00 mmoles) in methanol (20 mL) was then added dropwise with stirring. A sky-blue, dense precipitate formed which was collected by vacuum filtration. The crude mixture of bis(hexadecylamine)copper(II) $(X^-)_2$ complexes was recrystallized 3× from hot methanol (50 mL) which contained about 1.0 g of sodium acetate. Filtration and overnight drying *in vacuo* (0.001 mm Hg; 0.80 g) gave **4** as a sky-blue solid (0.82 g, 1.20 mmoles, 40%, mp soften at 85 °C, then melt 99-101 °C). A ^1H-NMR spectrum obtained in $CDCl_3$ showed a triplet at δ0.90 and a large and broad singlet centered at δ1.28. The integral ratio of these two peaks was expected to be approximately 10:1 as observed for a sample of starting hexadecylamine but instead was found to be approximately 6:1. This anomalous integral ratio was observed for two different samples of this compound prepared with hexadecylamine from different commercial sources. IR (KBr): 3263, 2922 (s), 2849, 1569 (s), 1468, 1407, 1169, 1055, 1017, 720, 627 cm^{-1}. *Anal.* calcd for $C_{34}H_{73}N_2O_2CuCl$: C, 63.71%; H, 11.48%; N, 4.22%. Found: C, 63.67%; H, 11.43%; N, 4.34%.

Preparation of bis(hexadecylamine)copper(II) dichloride, 5. To a solution of $CuCl_2 \bullet 2H_2O$ (0.50 g, 2.9 mmol) in methanol (20 mL) was added dropwise with stirring (ambient temperature) a solution of hexadecylamine (1.44 g, 6.0 mmol) in methanol (20 mL). A color change from green to blue/green was apparent as soon as the first drops of amine solution were added. The solution was clouded by blue solid. The solution became more blue as soon as the final drops of amine solution were added.

Stirring was continued for five minutes at room temperature and the solution was then filtered. The product (1.1 g, 60%) was collected as a sky-blue solid that had mp 105-122 °C. Washing the crude product with several small portions of $CHCl_3$ inmproved the mp to 118-123 °C. The NMR spectrum obtained in CD_3OD showed a methyl group triplet at δ0.74, a large, broad singlet centered at δ1.12, and a broad peak presumably due to the amine hydrogens at δ2.6. The only other peaks apparent in the spectrum were singlets at δ3.1 and δ4.7 presumably due to protio material in the deuteriomethanol. We note that the integral ratio for the methyl and methylene groups, which should have been 10:1 was 6:1 as above.

An identical reaction was conducted at reflux overnight to be certain that the product was the thermodynamically stable isomer if more than one could form. The material obtained from the overnight reaction was purified by repeated washing with chloroform. The melting point after this treatment was 121-124 °C. IR (KBr): 3269, 3241, 3222, 2957, 2918 (s), 2849, 2371, 1595, 1471 (s), 1171, 719, 627 cm^{-1}. Anal. calcd. for $C_{32}H_{70}N_2CuCl_2$: C, 62.26; H, 11.43; N, 4.54%. Found: C, 62.47; H, 11.21; and N, 4.38%.

Vesicle preparations from 4 and 5. Vesicles were prepared from **4** at pH 8.0 as follows.[14] A 25 mL test tube was charged with **4** (5 mg, 7.32 µmoles) and tris(hydroxymethyl)aminomethane hydrochloride buffer solution (10.0 mL, 0.0100 M). The resulting mixture was dispersed (sonicator bath) at 43±2 °C for 30 min to yield a sky-blue metalloamphisome suspension at 7.32×10^{-4} M surfactant concentration.

Vesicles were prepared similarly at pH 10.0 except that the buffer contained 0.0100 M tris(hydroxymethyl)aminomethane and 0.00500 M NaCl. The latter electrolyte was added to maintain the same approximate ionic strength as in the preceding suspension.

An additional preparation was undertaken as above with **5** at pH 8.55 using tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS)[15]/NaOH buffer. Results were similar to those described above and observed values are recorded in Table 1.

Hydroxylamine reduction. A 200 µL aliquot of the stock vesicle suspension (146.4 nmoles of surfactant) was dissolved in 3.00 mL deoxygenated 0.0100 M tris(hydroxymethyl)aminomethane at pH 10.0, which contained 0.00500 M NaCl. A stock solution of hydroxylamine was prepared in methanol, as follows: 5.00 mL of 1.00 M KOH in methanol was mixed with 5.00 mL 1.00 M hydroxylamine hydrochloride in methanol and the resulting KCl precipitate removed by gravity filtration. A 20 µL aliquot of the stock 0.500 M solution of hydroxylamine (10 µmoles) was added to the vesicle suspension. After mixing, the analyte was allowed to incubate for 30 minutes at ambient temperature. A heterogeneous suspension of aggregates was obtained which could not be characterized by dynamic light scattering. In addition, gas bubbles (presumably N_2O) were observed to grow on the surface of the cuvette. When hydrazine, instead of hydroxylamine, was used as the reducing agent, the gas that evolved was presumably N_2.

Monomer reduction in methanol. The electronic absorption spectrum of deoxygenated 4.0×10^{-3} M bis(hexadecylamine)copper(II) acetate in methanol, in the presence of 0.200 M sodium acetate, was recorded using a Hewlett Packard, Diode Array spectrophotometer. To a 3.00 mL aliquot of the stock surfactant solution was added 25 μL of 1.0 M hydroxylamine hydrochloride solution in methanol (about two equivalents) under an inert atmosphere. The sky-blue color discharged almost immediately. Subsequent aeration (open cuvette) using atmospheric oxygen regenerated the cupric chromophore during 30 min of moderate bubbling. The UV-visible spectrum, which showed a broad peak at *ca.* 680 nm, was lost during reduction and fully restored upon oxidation.

Acknowledgments We thank the NIH for a grant (GM-36262) that supported this work.

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(*Received* 2 *April* 1994)