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Aggregation Behavior and Reactivity of Hydrophobic Vitamin B_{12} Covalently Bound to Lipid in Aqueous Media

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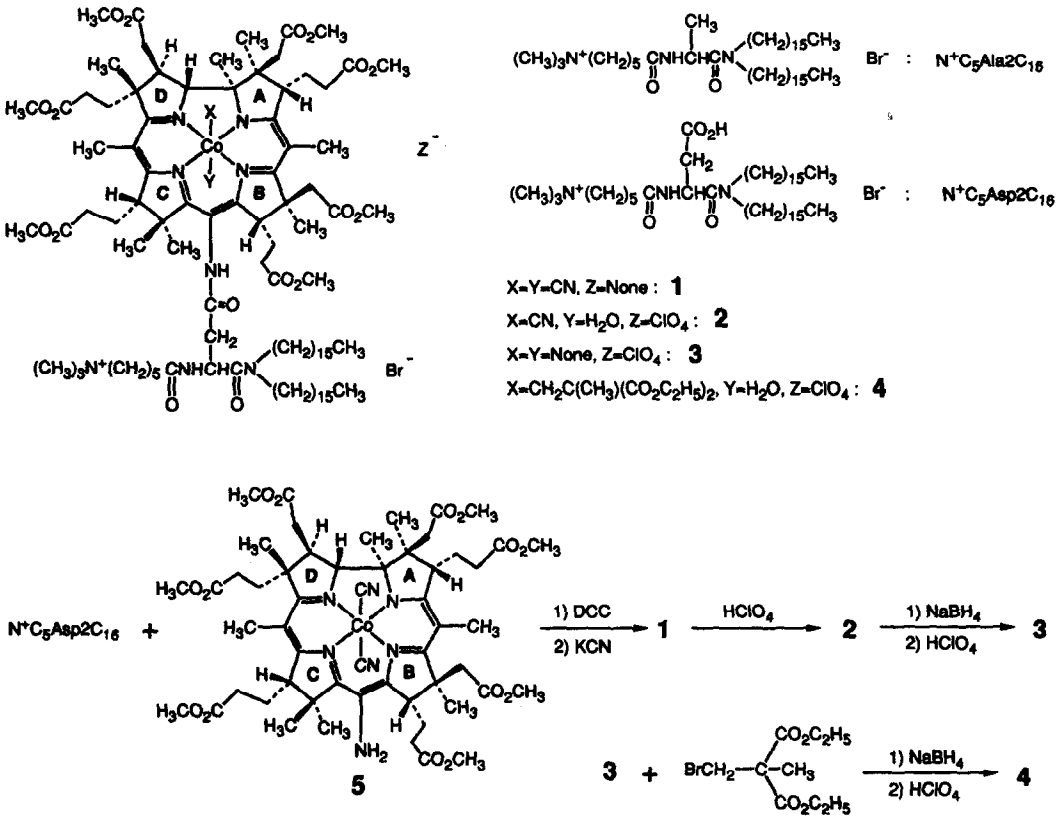
Abstract: A mixture of a hydrophobic vitamin B_{12} covalently bound to a lipid species and *N,N*-dihexadecyl-N^{α}-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide at a 1 : 50 molar ratio afforded stable single-compartment vesicles by sonication, which provided an anaerobic microenvironment in aqueous media under aerobic conditions.

Each naturally occurring holoenzyme, such as a vitamin B_{12}-dependent enzyme,[1] is composed of a specific apoprotein and a relevant cofactor. An apoprotein generally provides a binding site for specific coenzyme and substrate, which is well separated from a bulk aqueous phase. In this context, we have previously constructed an artificial vitamin B_{12} holoenzyme by combining noncovalently a synthetic bilayer membrane with a hydrophobic vitamin B_{12}.[2] In this work, we developed a novel artificial holoenzyme composed of a hydrophobic vitamin B_{12} covalently bound to a lipid species and a bilayer membrane matrix.

Novel hydrophobic vitamin B_{12} derivatives were prepared by following reaction steps shown in Scheme 1. All the products were characterized and identified by ^{1}H-NMR, IR, and electronic spectroscopy as well as by elemental analyses. Complex **1** was prepared by condensation of *N,N*-dihexadecyl-N^{α}-[6-(trimethylammonio)hexanoyl]-L-aspartamide ($N^+C_5Asp2C_{16}$), which was synthesized by a method similar to that adopted for the preparation of *N,N*-dihexadecyl-N^{α}-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide ($N^+C_5Ala2C_{16}$),[3] with a hydrophobic vitamin B_{12} (**5**), which was prepared after a method used for preparation of an analogous compound,[4] as described below.

A dry dichloromethane solution (5 mL) of $N^+C_5Asp2C_{16}$ (498 mg, 0.61 mmol) and *N,N'*-dicyclohexylcarbodiimide (DCC; 126 mg, 0.61 mmol) was stirred for 0.5 h at 0 °C, and **5** (673 mg, 0.61 mmol) was added to the solution which was subsequently stirred for 6 h at 0 °C and then overnight at room temperature. Precipitates (*N,N'*-dicyclohexylurea; DCurea) were removed by filtration, the filtrate was evaporated to dryness in vacuo, and ethyl acetate (80 mL) was added to the residue. After the solution was allowed to stand in a refrigerator overnight, the resulting precipitates (DCurea) were removed by filtration. The filtrate was evaporated to dryness to afford a purple solid. The crude product was purified by TLC on silica gel (Kiesel gel 60) with chloroform–methanol–water (16 : 6 : 1 by volume). The second purple fraction was evaporated to dryness, and the residue was dissolved in dichloromethane. The solution was treated with aqueous potassium cyanide and evaporated to dryness to afford a purple solid. All the counterions of the product were converted to the bromide ion by ion-exchange chromatography on a column of Amberlite IRA-401. The product was reprecipitated from benzene upon addition of hexane to afford a purple powder: yield 647 mg (54%); λ_{max}(CH_3OH) 284, 308, 318, 370, 422, 514, 552, and 592 nm. Found: C, 61.31; H, 8.51; N, 7.11%. Calcd for $C_{99}H_{162}BrCoN_{10}O_{17}\cdot 2H_2O$: C, 61.32; H, 8.42; N, 7.22%.

Complex **1** was converted to the corresponding divalent cobalt complex (**3**) via formation of complex **2** in the same manner as reported previously.[5] An alkylated complex (**4**) was prepared as follows. An aqueous phosphate buffer [15 mL, pH 7, 0.01 mol dm^{-3}, μ 0.10 (KCl)] was added to a methanol solution (30 mL) of **3**



Scheme 1.

(70 mg, 3.6 x 10^{-5} mol), and the solution was deoxygenated by bubbling nitrogen gas through it for 30 min. The following operations were carried out in the dark. Sodium tetrahydroborate (70 mg, 1.85 x 10^{-3} mol) was added to the deoxygenated solution with vigorous stirring under nitrogen atmosphere. When the solution turned dark green, 2,2-bis(ethoxycarbonyl)-1-bromopropane[6] (103 mg, 3.74 x 10^{-4} mol) was added to it. The resulting solution was stirred for 8 min at room temperature, and 60% (w/w) aqueous perchloric acid (3 mL) was carefully added to it in order to decompose an excess amount of sodium tetrahydroborate. The resulting mixture was extracted with dichloromethane, and the extract was washed with distilled water. After being dried over sodium sulfate, the extract was evaporated to dryness in vacuo at room temperature. The residue was reprecipitated from benzene upon addition of hexane to afford a brown powder: yield 69 mg (89%); $\lambda_{max}(CH_2Cl_2)$ 266, 300, 418, and 464 nm. Found: C, 58.66; H, 8.29; N, 5.24%. Calcd for $C_{106}H_{179}BrClCoN_8O_{26}\cdot H_2O$: C, 58.57; H, 8.39; N, 5.15%.

The aggregation behavior of **1** alone and a mixture of **1** and $N^+C_5Ala2C_{16}$ was examined by means of differential scanning calorimetry (DSC; MicroCal MC-2) and electron microscopy (JEOL JEM-200B). An aqueous solution of compound **1** (2 x 10^{-4} mol dm^{-3}) did not show any clear DSC peak, indicating that compound **1** alone does not form stable bilayer vesicles. Because of the fact that the solution demonstrated a surfactant property by generating foams after shaking, **1** alone seems to form micelle-type aggregates in an aqueous medium. On the other hand, a mixture of **1** (4.0 x 10^{-6} mol dm^{-3}) and $N^+C_5Ala2C_{16}$ (2.0 x 10^{-4} mol dm^{-3}) at a 1 : 50 molar ratio in the dispersion state showed a clear DSC peak caused by a phase transition from

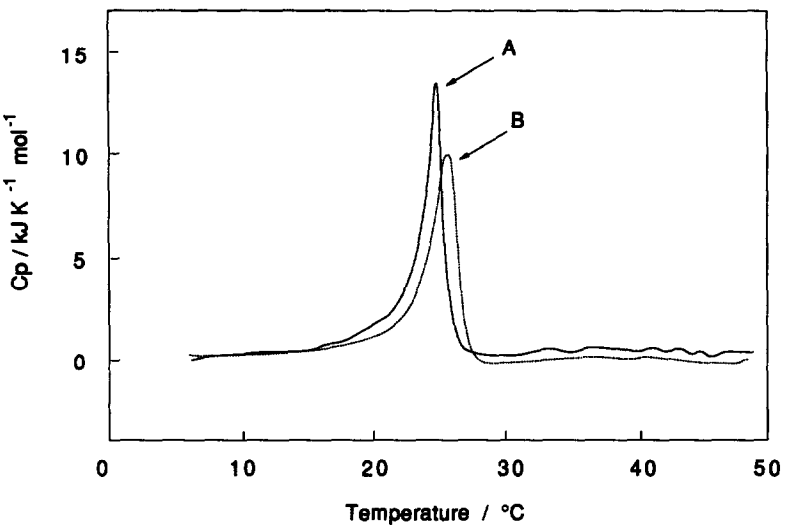
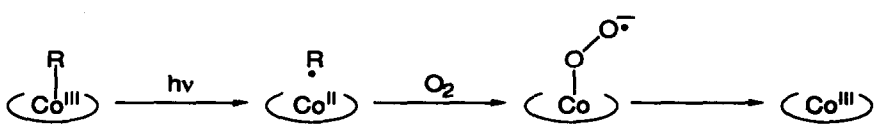


Fig. 1. DSC thermograms for dispersion samples in aqueous phosphate buffer (pH 7, μ=0.10 with KCl): A, $N^+C_5Ala2C_{16}$ (2.0×10^{-4} mol dm^{-3}); B, **1** (4.0×10^{-6} mol dm^{-3}) and $N^+C_5Ala2C_{16}$ (2.0×10^{-4} mol dm^{-3}).

the gel to the liquid-crystalline state as shown in Fig. 1. The phase transition parameters evaluated by DSC are as follows: temperature at a peak maximum (T_m), 25.7 °C; enthalpy change (ΔH), 29.6 kJ mol^{-1}. As for the $N^+C_5Ala2C_{16}$ vesicle alone (2.0×10^{-4} mol dm^{-3}), the following parameters were obtained: T_m = 24.9 °C, ΔH = 32.0 kJ mol^{-1}. The DSC thermograms and parameters indicate that the mixture of **1** and $N^+C_5Ala2C_{16}$ at a 1 : 50 molar ratio forms stable bilayer membranes, even though the molecular packing mode for formation of the aggregates is somewhat loose as compared to that for the $N^+C_5Ala2C_{16}$ vesicle alone. Transmission electron microscopy was applied on samples negatively stained with uranyl acetate. An aqueous dispersion composed of **1** (1.0×10^{-4} mol dm^{-3}) and $N^+C_5Ala2C_{16}$ (4.9×10^{-3} mol dm^{-3}) involved lamella-type aggregates.[3] After the dispersion sample was sonicated with a probe-type sonicator at 30 W for 3 min, single-compartment vesicles ranging from 300 through 700 Å in diameter were observed.

Complex **4** was prepared for clarification of the reactivity of an alkylated complex having a cobalt–carbon bond in the vesicle. Photolysis of complex **4** was carried out in both dichloromethane and the vesicle under aerobic conditions. The aerobic photolysis reaction is ordinarily expected to proceed as shown in eq. 1. In dichloromethane, the electronic spectrum changed as shown in Fig. 2A by the photolysis; absorption maxima at 350 and 500 nm are characteristic of the Co(III) state.[7] This result indicates that the Co(II) species is autoxidized to the Co(III) species in dichloromethane after homolytic cleavage of the cobalt–carbon bond under irradiation with visible light. On the other hand, the electronic spectrum underwent a change as shown in Fig. 2B in the vesicle; absorption maxima at 316 and 470 nm are characteristic of the Co(II) state.[7] The latter spectral change apparently indicates that the Co(II) species formed by aerobic photolysis is protected from



(1)

autoxidation that affords the Co(III) species in the vesicle. Therefore, we conclude that the present bialyer vesicle provides an efficient anaerobic microenvironment in aqueous media even though the reaction is carried out under aerobic conditions.

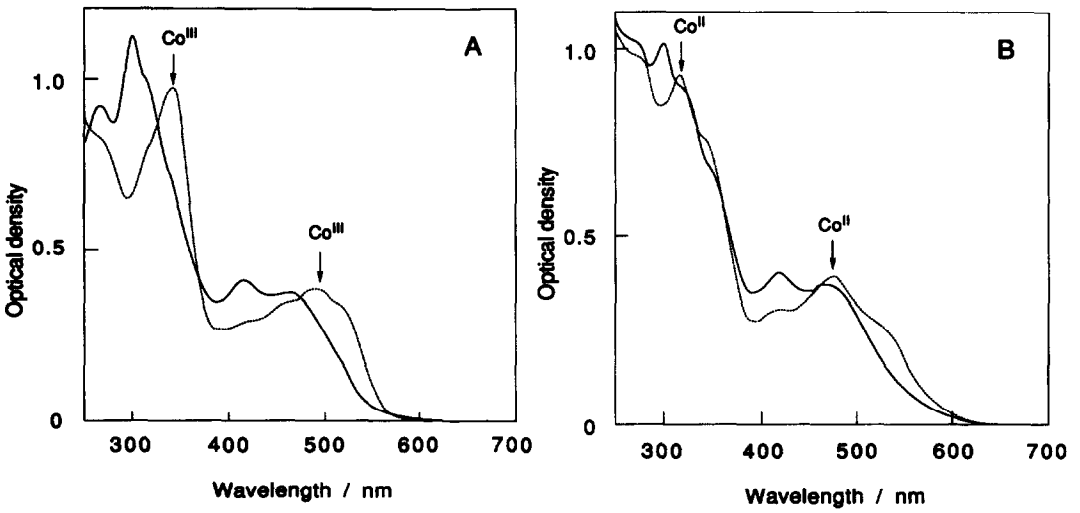


Fig. 2. Electronic spectra for the aerobic photolysis of an alkylated hydrophobic vitamin B_{12} (4) before photolysis (solid lines) and after irradiation with a 500-W tungsten lamp at a distance of 40 cm for 5 min (broken lines): A, 4 (5.0×10^{-5} mol dm^{-3}) in dichloromethane; B, $N^+C_5Ala2C_{16}$ (2.5×10^{-3} mol dm^{-3}) and 4 (5.0×10^{-5} mol dm^{-3}) in aqueous phosphate buffer (pH 7).

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