Liposomal Heme as Oxygen Carrier under Semi-physiological Conditions †

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A *meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinatoiron(II) complex of a 2-methylimidazole, substituted in the 1-position with a hydrophobic group, was incorporated into a lipid bilayer of phosphatidylcholine (liposomal heme). The liposomal heme reversibly bound molecular oxygen in neutral aqueous media at 37 °C, and the half-life of the oxygen adduct was half a day. The oxygen-binding affinity ($p_{\frac{1}{2}}$) for the liposomal heme was *ca.* 50 mmHg, which is similar to that of hemoglobin in blood. The incorporation and structure of the liposomal heme were confirmed by physico-chemical methods, which indicated that the hydrophobic environment of the inner region of liposome protected the oxygen adduct from its proton-driven oxidation.

The respiratory proteins, hemoglobin (hb) and myoglobin (mb), which are composed of iron(II)–porphyrin complexes and globin proteins, serve to transport and to store molecular oxygen (O_2) in a living body. If their oxygen-binding sites, the iron–porphyrin complexes, are isolated from the proteins and exposed to oxygen, the iron(II)–porphyrin complexes are immediately and irreversibly oxidized and they do not act as oxygen carriers.

Much effort has been made to mimic oxygen carriers like hb by synthesizing various modified iron–porphyrin derivatives.[1-7] These synthetic iron–porphyrin complexes have been successful in oxygen binding in aprotic solvents or in the solid state, but in aqueous media they have been irreversibly oxidized. Only hb and mb are known as oxygen carriers in aqueous media.

The reasons for the oxygen binding by hb and mb are considered to be as follows. (*i*) The iron–porphyrin complex has a five-co-ordinate structure whose sixth co-ordination site is vacant to bind molecular oxygen [equation (1)]. (*ii*) The iron–porphyrin complex is dispersed and diluted to suppress the irreversible oxidation *via* a μ-dioxo-dimer [equation (2)]. (*iii*) The iron–porphyrin complex is surrounded by the hydrophobic environment causing the proton-driven oxidation [equation (3)] to be retarded: FeP, $Fe^{II}P$, and $Fe^{III}P$ represent

Oxygenation

$$Fe^{II}PL + O_2 \rightleftharpoons LPFe{-}O_2 \quad (1)$$

Oxidation

$$LPFe{-}O_2 + Fe^{II}L \longrightarrow LPFe{-}O_2{-}FePL \longrightarrow PFe^{III}{-}O{-}Fe^{III}P \quad (2)$$

$$LPFe{-}O_2 + H^+ \xrightarrow{(H_2O)} Fe^{III}PL + HO_2\cdot \quad (3)$$

iron–, iron(II)–, and iron(III)–porphyrins respectively; L is an axial ligand such as an imidazole derivative.

Globin protein forms the five-co-ordinate iron–porphyrin complex and ' tucks ' it separately, *i.e.* globin protein protects the iron–porphyrin complex from the oxidation [equation (2)] by embedding it separately in the macromolecule, and the hydrophobic domain of the globular protein excludes water molecules and suppresses the proton-driven oxidation [equation (3)].

In order to construct a hydrophobic environment in aqueous solution, it is a possibility to use micelle or liposome instead of the globin protein. Some attempts to achieve reversible oxygen binding by solubilizing an iron–porphyrin complex in water with micelle or liposome have been reported,[8,9] but they resulted in a questionable oxygenation or irreversible oxidation.

Recently we reported that the *meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinatoiron(II) (heme) complex of 1-lauryl-2-methylimidazole (lmi) (lauryl = dodecyl) incorporated into liposome of phosphatidylcholine [abbreviated as ' liposomal heme ', Figure 1 ($R = C_{12}H_{25}$)] bound molecular oxygen reversibly under semi-physiological conditions (at pH 7.0 in aqueous media at 37 °C).[10] The present paper describes oxygen-binding ability and features of incorporation of the liposomal heme under semi-physiological conditions.

† *Non-S.I. unit employed:* 1 mmHg = (101 325/760) N m^{-2}.

Experimental

Materials.—(*a*) *Synthesis of metalloporphyrins. meso*-5,10,15,20-Tetra(*o*-pivalamidophenyl)porphyrinatoiron(III) bromide was prepared as described in refs. 11 and 12. *meso*-5,10,15,20-Tetra(*o*-pivalamidophenyl)porphyrinatozinc(II) chloride, used as a fluorescence probe, was prepared by the reaction of *meso*-5,10,15,20-tetra-(*o*-pivalamidophenyl)porphyrin with zinc chloride in *NN*-dimethylformamide (dmf) at 80 °C for 1 h and purified by column chromatography [Wakogel C-200, diethyl ether–light petroleum (4 : 1)]: t.l.c., $R_f = 0.3$; $\lambda_{max.}$ (benzene) = 423, 515, 553, and 592 nm.

(*b*) *Synthesis of 1-substituted-2-methylimidazoles.* 1-Lauryl-2-methylimidazole was prepared by the reaction of lauryl bromide with 2-methylimidazole at 200 °C: b.p. 150 °C (1.5 mmHg) (Found: C, 76.1; H, 12.9; N, 11.0%; C : N, 6.91 : 1. Calc. for $C_{16}H_{30}N_2$: C, 76.8; H, 12.0; N, 11.2%; C : N, 6.86 : 1); M^+/e 250; δ_H (100 MHz, in $CDCl_3$, standard $SiMe_4$) 0.95 (3 H, s, lauryl CH_3), 1.30—1.85 [20 H, lauryl $-(CH_2)_{10}-$], 2.30 (3 H, s, imidazole 2-CH_3), 3.90 (2 H, t, imidazole-CH_2), 6.90 (1 H, d, imidazole 4- or 5-CH), 6.95 (1 H, d, imidazole 4- or 5-CH). 1-Stearyl- and 1-trityl-2-methylimidazole were prepared in a similar manner as above. 1-Stearyl-2-methylimidazole (stearyl = octadecyl): m.p. 44—45 °C (Found: C, 79.0; H, 12.7, N, 8.30. Calc. for $C_{22}H_{42}N_2$: C, 79.0; H, 12.6; N, 8.4%); δ_H (100 MHz, in $CDCl_3$, standard $SiMe_4$) 0.88 (3 H, s, stearyl CH_3), 1.26 [30 H, s, stearyl $-(CH_2)_{15}-$], 1.70 (2 H, t, imidazole-CCH_2), 2.37 (3 H, s, imidazole 2-CH_3), 3.80 (2 H, t, imidazole-CH_2), 6.80 (1 H, d, imidazole 4- or 5-CH), 6.89 (1 H, d, imidazole 4- or 5-CH). 1-Trityl-2-methylimidazole: m.p. 217.0—219.8 °C (Found: C, 85.25; H, 6.15; N, 8.45. Calc. for $C_{23}H_{20}N_2$: C, 85.15; H, 6.20; N, 8.65%); M^+/e 324; δ_H (100 MHz, in $CDCl_3$, standard $SiMe_4$) 1.67 (3 H, s, imidazole 2-CH_3), 6.76 [1 H, d, imidazole 4- or 5-H], 6.86 [1 H, d, imidazole 4- or 5-CH], 7.00—7.40 (15 H, m, Ph_3). 1,2-Dimethylimidazole

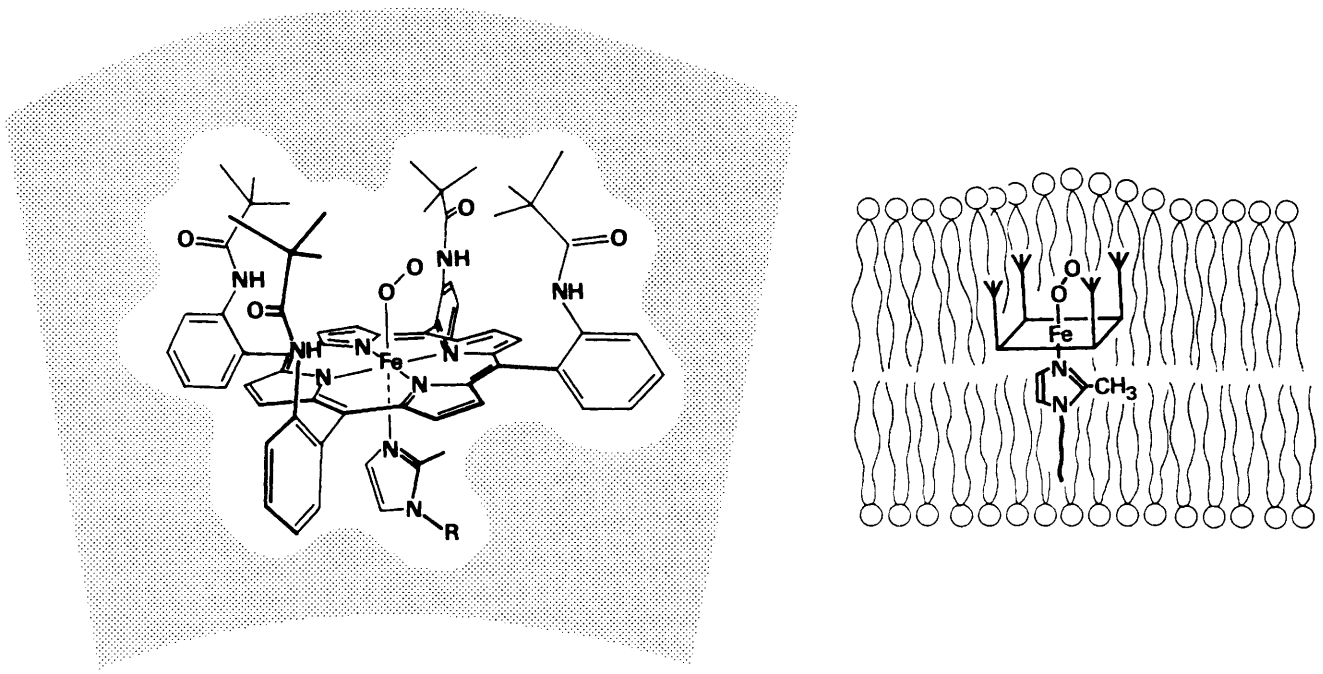

Figure 1. Liposomal heme

(commercial grade) was purified by recrystallization from benzene–light petroleum.

(*c*) *Other materials.* Dimyristoylphosphatidylcholine (dmpc), dipalmitoylphosphatidylcholine (dppc), egg yolk lecithin (eyl), lyso egg yolk lecithin (lyso-eyl), and cholesterol were purchased from Sigma (special grade). Emulgen PP 250 (block copolymer of ethylene oxide and propylene oxide, average molecular weight 30 000), Triton-X 100 [*p*-$C_9H_{19}C_6H_4O(CH_2CH_2O)_nH$], and cetyltrimethylammonium bromide (ctab) were used without further purification. The europium salt, $Eu(NO_3)_6{\cdot}6D_2O$, and sodium phosphotungstate, $2Na_2O{\cdot}P_2O_5{\cdot}12WO_3{\cdot}18H_2O$, were purchased from Sigma (special grade).

Preparation of Liposomal Heme.—The liposome-embedded iron(II) porphyrin (liposomal deoxy-heme) was prepared by modifying the normal method of liposome preparation.[13] The iron(III)–porphyrin complex (1 μmol) was reduced in the presence of 1-lauryl-2-methylimidazole (50 μmol) under nitrogen by mixing the toluene solution with aqueous sodium dithionite, and then carbon monoxide gas was bubbled through the mixture. The toluene phase was collected, dried with molecular sieves (4A), and added to the toluene solution of phospholipid (200 μmol) saturated with carbon monoxide. By evaporating the solvent under reduced pressure, thin films were prepared on the glass wall of a large round flask. This was dried *in vacuo* for *ca.* 1 h at 90 °C to remove carbon monoxide, giving the iron(II)–porphyrin complex. Oxygen-free phosphate buffer (pH 7.0, 20 cm^3) was added, and the mixture was then shaken by a Vortex mixer. It was ultrasonicated and homogenized in an ice–water bath under nitrogen. Thus prepared liposomal deoxyheme solution was incubated at room temperature for 2 h. The red transparent solution showed a u.v.–visible absorption spectrum with maxima at 438, 535, and 562 nm which agreed with the five-co-ordinate deoxyheme of the corresponding complex in toluene.[11]

Measurements.—(*a*) *Spectroscopic.* Electronic absorption spectra were measured with Shimazu UV-240 and Hitachi UV-320 spectrometers. Oxygen-binding affinity was determined by spectroscopic measurement of the oxygen-binding equilibrium curve as in ref. 14. For reference, the oxygen-binding equilibrium curve of the red cell suspension [14] was measured with the above mentioned apparatus; it was consistent with the curves reported in refs. 14 and 15 which supports validity of the measurement in the present experiment.

The structure of the liposomal heme was estimated in D_2O by ^1H and ^{31}P n.m.r. spectroscopy (JEOL JNM FX-100). The e.s.r. spectrum was measured at −196 °C, using a JEOL FE-3X spectrophotometer, on the spin-labelled heme prepared by bubbling nitrogen oxide gas through the liposomal heme solution. The fluorescence spectra of the liposomal heme were measured using excitation at 413 nm with a fluorescence spectrophotometer (Japan Spectroscopic JASCO FP-550); *meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinato-(1-lauryl-2-methylimidazole)zinc(II) (50 μmol) was used as fluorescence probe.

(*b*) *Other measurements.* The gel permeation chromatography (g.p.c.) elution curve was measured with a Sepharose 4B column (Pharmacia Fine Chemical, 2.2 × 70 cm). The liposomal heme was also separated from the aqueous media by ultracentrifugation (45 000 r.p.m., 90 min at 40 °C, ultracentrifuge Hitachi 65p-7). Transmission electron microscopy (t.e.m.) (Hitachi H-500) of the liposomal heme was carried out by the negative staining method using sodium phosphotungstate. The particle size of the liposomal heme was also measured by a nano-sizer (Coulter Electronics). Gel and liquid-crystal phase transitions of the liposomal heme were measured by a differential scanning calorimeter (Seiko SSC-560U; 5 °C min^{-1}).

Results and Discussion

The visible absorption spectrum of the liposomal heme is shown in Figure 2(*a*). The spectrum of the five-co-ordinate deoxy-heme complex ($\lambda_{max.}$ = 535 and 562 nm) changed to that assigned to the oxygen adduct [11] ($\lambda_{max.}$ = 546 nm) on

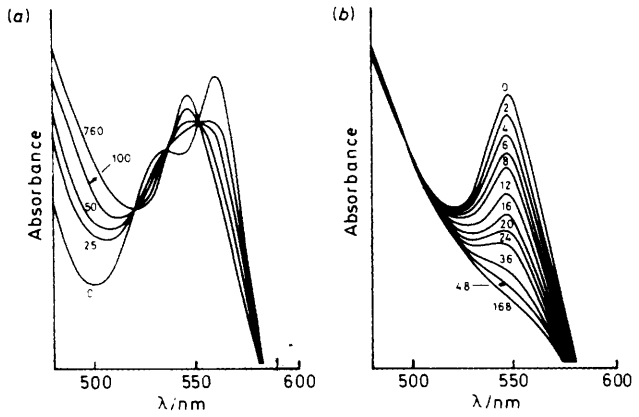

Figure 2. Visible absorption spectra ([Fe] = 0.05 mmol dm^{-3}, [lmi] = 2.5 mmol dm^{-3}; at pH 7.0 in aqueous media at 37 °C) of (*a*) the liposomal deoxy-heme and its oxygen adduct at $p(O_2)$ = 0, 25, 50, 100, and 760 mmHg; (*b*) the degradation of its oxygen adduct after 0, 2, 4, 6, 8, 12, 16, 20, 24, 36, 48, and 168 h

Table 1. Lifetimes (half-life, τ) of the oxygen adduct of the liposomal hemes and the heme complex solubilized by surfactants at pH 7 in aqueous media at 37 °C [a]

Substituent of 2-methylimidazole	Phospholipid or surfactant [b]	τ [c]/h
1-CH_3	dmpc (1.3)	0
1-CPh_3	dmpc (1.3)	10
1-$(CH_2)_{17}CH_3$	dmpc (1.3)	12
1-$(CH_2)_{11}CH_3$	dmpc (1.3)	12
1-$(CH_2)_{11}CH_3$	dppc (1.3)	13
1-$(CH_2)_{11}CH_3$	eyl (1.3)	12
1-$(CH_2)_{11}CH_3$	eyl + lyso-eyl (1 : 1)	1
1-$(CH_2)_{11}CH_3$	eyl + cholesterol (1 : 0.1)	12
1-$(CH_2)_{11}CH_3$	ctab[1] (4)	0.1
1-$(CH_2)_{11}CH_3$	Triton-X 100 (4)	0.5
1-$(CH_2)_{11}CH_3$	Emulgen PP 250 (5)	0

[a] [Fe] = 0.05 mmol dm^{-3}, [imidazole] = 2.5 mmol dm^{-3}. [b] Concentrations (percentage by weight) of phospholipid or surfactant are given in parentheses. [c] For the oxygen adduct with visible absorption maxima at 422 and 546 nm.

exposure to oxygen, through isosbestic points at 520, 536, 552, and 589 nm. The spectrum of the oxygen adduct changed to that of the CO adduct (540 nm) on bubbling through carbon monoxide and returned to that of deoxy-heme on bubbling through nitrogen. The oxy-deoxy cycle could be repeated dozens of times.

The oxygen adduct was slowly degraded to the iron(III)-porphyrin complex [Figure 2(*b*)] and this degradation obeyed first-order kinetics. The lifetime (half-life, τ) of the oxygen adduct of the liposomal heme was half a day at pH 7.0 in aqueous media at 37 °C.

The lifetimes of the oxygen adduct measured for the liposomal hemes composed of various imidazole ligands and phospholipids are summarized in Table 1. Only the liposomal hemes complexed with a hydrophobic group-substituted imidazole, *e.g.* 1-lauryl-, 1-stearyl-, and 1-trityl-2-methylimidazole, give a stable oxygen adduct. Table 1 also shows that a stable oxygen adduct is observed for the heme complex solubilized with the liposome of phosphatidylcholine or its mixture with cholesterol. The only unstable oxygen adducts were observed for the heme complex solubilized with micelles of synthetic surfactants such as Triton-X 100 and ctab; the heme complex solubilized with non-micelle forming surfactant such as Emulgen PP 250 resulted in rapid irreversible oxidation of heme on exposure to oxygen. The heme complex solubilized with the mixture of eyl and lyso egg yolk lecithin, which could not form liposome, did not give the stable oxygen adduct. The use of a phospholipid and a hydrophobic imidazole was necessary to prepare the liposomal heme with oxygen-binding ability.

Table 2. Oxygen-binding affinity of the liposomal heme at 37 °C

Heme		$p_{\frac{1}{2}}$/mmHg
Liposomal heme	dmpc	49
	eyl	51
Hemoglobin (blood)		27 [a]
Myoglobin		0.9 [b]

[a] Ref. 17. [b] Ref. 18.

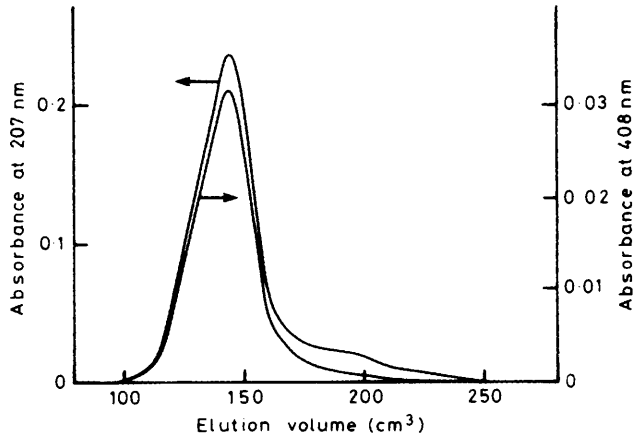

Figure 3. Elution curves of the liposomal heme solution by g.p.c. detected at 207 nm based on dmpc and at 408 nm based on the heme

The oxygen-binding affinity ($p_{\frac{1}{2}}$: oxygen pressure at half oxygen-binding for the heme) of the liposomal heme was determined by the oxygen-binding equilibrium curve measurement and values are listed in Table 2. For dmpc- and eyl-liposomal heme, $p_{\frac{1}{2}}$ is 49 and 51 mmHg at 37 °C, respectively, which agree with the $p_{\frac{1}{2}}$ value (38 mmHg at 25 °C) for the corresponding heme complex in toluene.[16] The $p_{\frac{1}{2}}$ values of the liposomal hemes are close to that of hb in blood,[17] but considerably different to that of mb.[18] This suggests that the liposomal heme has the potential to act as the oxygen carrier under physiological conditions which transports oxygen from lungs [$p(O_2)$ = *ca.* 110 mmHg] to mb in muscle tissue as does hb. The oxygen binding was also measured in a mixture of the serum of rat blood and saline (1 : 1 v/v) at 37 °C. The liposomal heme showed the same reversible oxygen-binding ability with a life-time for the oxygen adduct of 9 h and $p_{\frac{1}{2}}$ = 46 mmHg. This means that proteins and other components in serum do not affect the oxygen-binding ability of the liposomal heme.

Incorporation of the heme complex in the lipid bilayer of liposome was studied for the liposomal heme composed of lmi and dmpc as follows.

The incorporation was first confirmed by g.p.c. (Sepharose column) monitored by the absorptions at 207 and 408 nm based on the phospholipid and the heme, respectively. The curves coincide with each other, which means that the heme complex is included in the liposome. It is known that a multilamellar and large liposome is eluted faster with a sharp

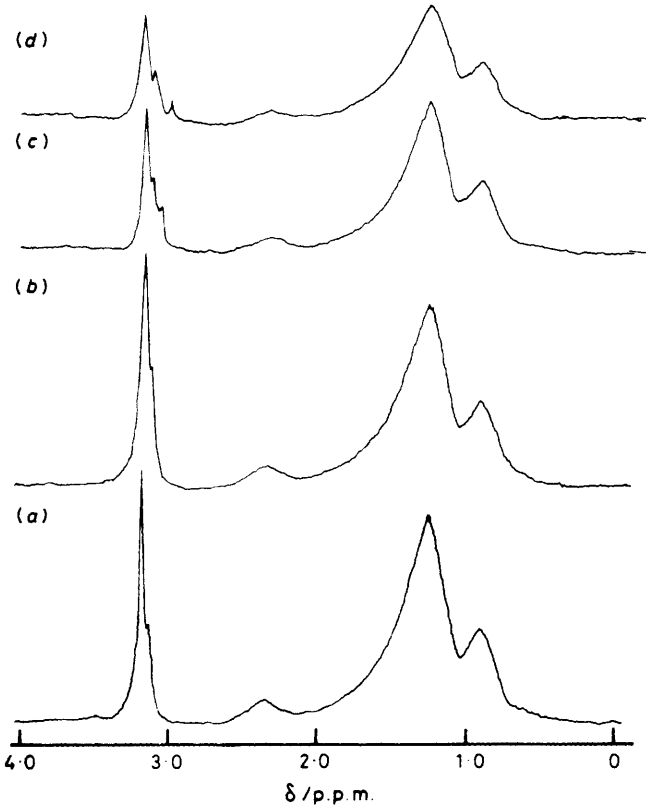

Figure 5. Hydrogen-1 n.m.r. spectra of the dmpc liposomal heme with added Eu^{3+}: $[Eu^{3+}]$ = (*a*) 0, (*b*) 2.5, (*c*) 6.1, (*d*) 8.7 mmol dm^{-3}; [Fe] = 0.22, [Imi] = 11, [dmpc] = 44 mmol dm^{-3}

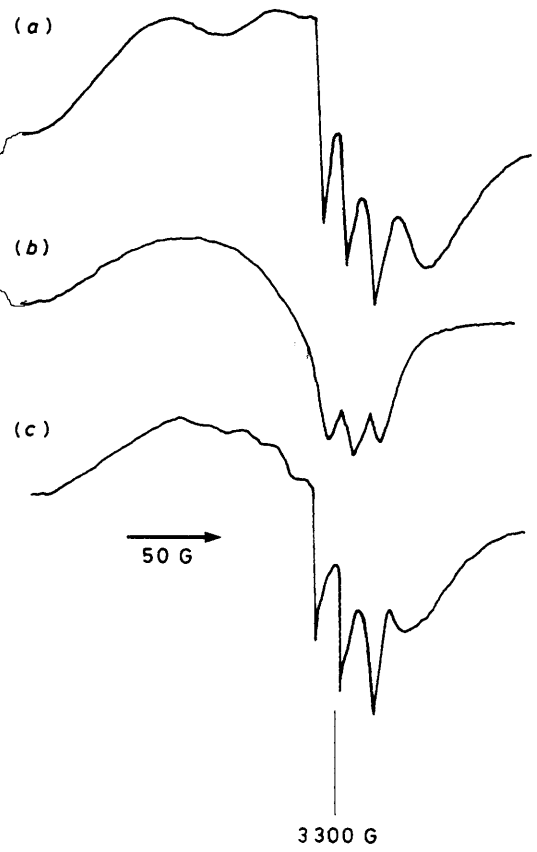

Figure 6. E.s.r. spectra of the nitrogen oxide-labelled heme complex at −196 °C: (*a*) NO adduct of the heme–Imi complex in dmf; (*b*) NO adduct of the heme–Imi complex dispersed in aqueous media; (*c*) dmpc liposomal heme in aqueous media

pattern than a single-walled and small liposome (a broadened one).[19] The elution pattern in Figure 3 suggests that the heme is included in a multilamellar liposome.

The liposome solution was also checked by ultracentrifugation; the supernatant did not contain both the phospholipid and the heme. This indicated also that the heme complex is included in a relatively large and multilamellar liposome.

The electron microscopic photograph (t.e.m.) is shown in Figure 4. The liposomal heme looks like a multilamellar liposome and the diameter is *ca.* 800 Å. The average particle size of the liposomal heme was also measured by the nano-sizer; the diameter was determined to be *ca.* 760 Å. We note here that the liposomal heme can be prepared as fine particles (diameter < 0.1 μm) by controlling sonication conditions and that the liposomal heme is expected to pass through small capillaries of the human body.

The formation of liposome was confirmed by n.m.r. measurements. When europium ion (Eu^{3+}) is added to a liposome solution, Eu^{3+} interacts with the choline groups of the outward facing phospholipid and shifts the n.m.r. signal of the choline methyl groups upfield.[20,21] The same shift was observed for the liposomal heme with the addition of Eu^{3+} [Figure 5(*a*)—(*d*)]. This result implies liposome formation for the liposomal heme solution. The liposome formation was also supported by the sharp resonance in the ^{31}P n.m.r. spectrum [0.8 p.p.m.], which is caused by the spherical geometry of phosphatidyl group.[22]

The Fourier-transform ^1H n.m.r. spectrum of the liposomal heme at 37 °C showed an absorption signal at −0.1 p.p.m. assigned to the pivaloyl group of the heme as well as the signals of dmpc. However, at 20 °C only the absorption of the choline group of the phospholipid was observed. This suggests that above the gel–liquid-crystal phase transition temperature (T_c) the heme complex is molecularly dispersed in the bilayer of the dmpc liposome.

The e.s.r. spectra of the heme complex labelled with nitrogen oxide are shown in Figure 6. The spin-labelled heme complex in homogeneous dmf solution gave a triplet signal in the *z* direction and weak signals in the *x* and *y* directions [Figure 6(*a*)], as reported previously.[23,24] On the other hand, the spin-labelled heme complex solubilized in water showed only the signal in the *z* direction [Figure 6(*b*)], which was often observed in the solid state. The spectrum of the spin-labelled heme complex solubilized in water by the liposome [Figure 6(*c*)] is similar to that in the homogeneous solution. This result also suggests that the heme complex is molecularly dispersed and well solubilized in the bilayer of the liposome.

A d.s.c. thermogram of the liposomal heme was measured to estimate the phase transition of the liposomal heme. The dmpc-liposome showed the endothermic peak at 23.7 °C, which corresponds to the gel–liquid-crystal phase transition temperature (T_c) of the liposome and agreed with that reported previously.[25] For the liposomal heme, the peak was broadened and shifted to the lower temperature of 21.5 °C. This suggests that the incorporation of the heme complex into the bilayer of the liposome causes partial disorder in the alkyl chain orientation of the phospholipid molecule.

The Zn–porphyrin complex was used as fluorescent probe and incorporated in the liposome. The fluorescence spectrum, with maxima at 614 and 664 nm, agrees with those in aprotic,

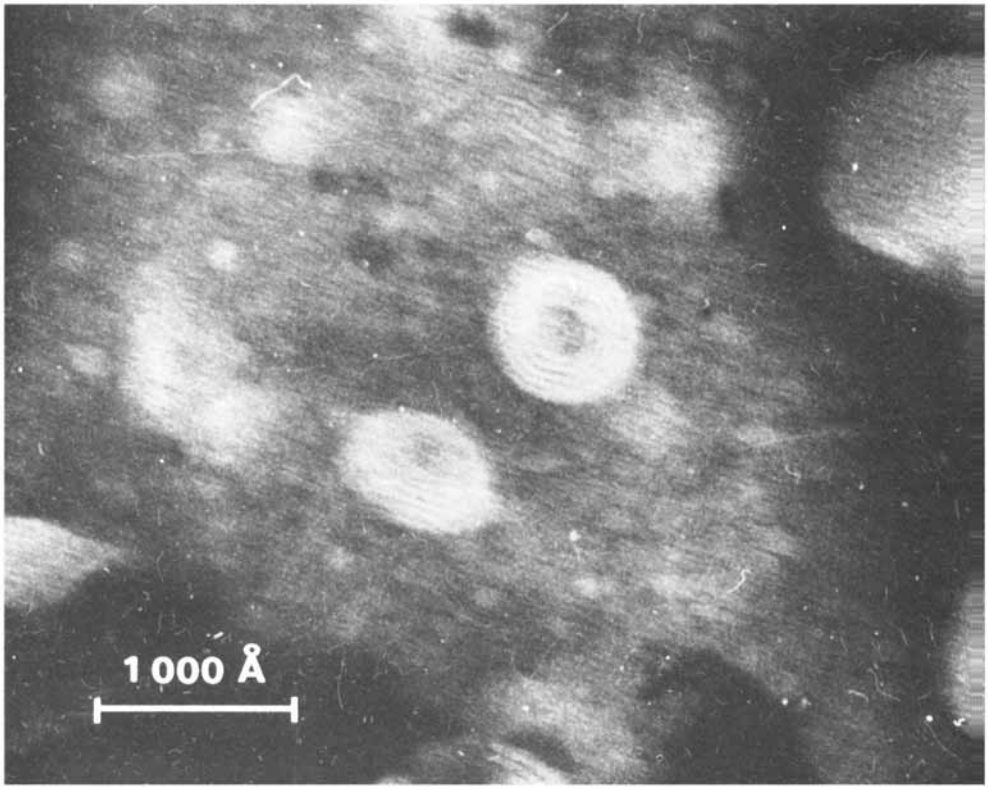

Figure 4. T.e.m. photograph for the dmpc liposomal hen

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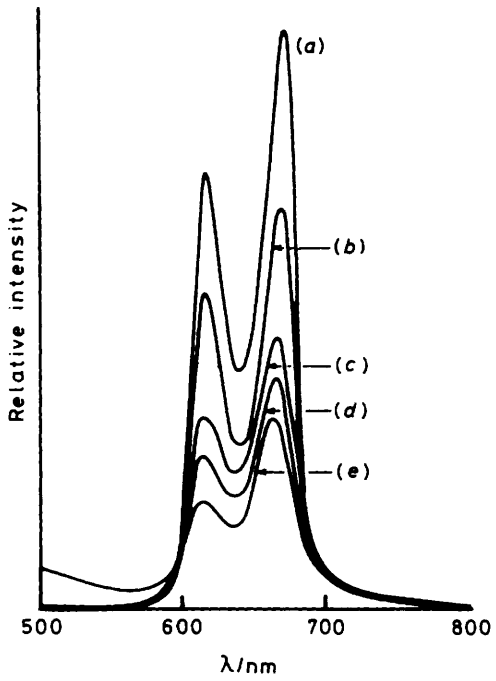

Figure 7. Fluorescence spectra of the Zn porphyrin–Imi complex: (*a*) in benzene; (*b*) in dichloromethane; (*c*) incorporated into liposome in aqueous media; (*d*) in ethanol; (*e*) in methanol

organic solvents (Figure 7). The fluorescence intensity for the liposomal heme is intermediate between those in dichloromethane and ethanol. This result indicates that the heme complex is molecularly dispersed in the liposome and surrounded by an environment similar to that in an aprotic, organic solvent.

The above results lead to the following conclusion. The heme complex is included and dispersed in the hydrophobic environment of liposome which protects the oxygen adduct of heme from irreversible oxidation. The imidazole ligand, substituted with a long-chain alkyl group, positions the heme in the inner region of liposome.

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