Phosphocholine-substituted 5,10,15,20-Tetraphenylporphyrinatoiron(II): Oxygen Carrier under Physiological Conditions†

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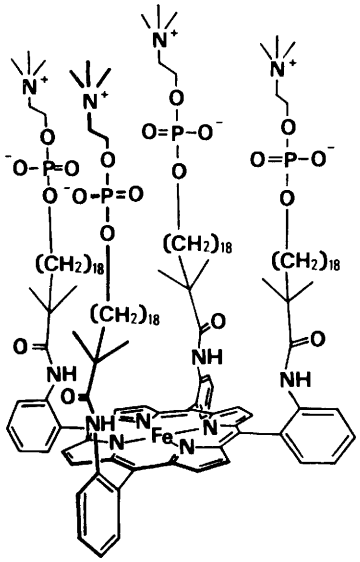
A novel and amphiphilic 5,10,15,20-tetraphenylporphyrinatoiron complex substituted with phosphocholine groups, 5,10,15,20-tetra{α,α,α,α-*o*-[2′,2′-dimethyl-20′-(2″-trimethylammonioethyl phosphonatoxy)eicosanamido]phenyl}porphyrinatoiron(II) (lipid-heme), has been synthesized. The lipid-heme formed a stable liposome with a phospholipid. The liposomal lipid-heme reversibly bound oxygen in neutral aqueous media at 37 °C, and its oxygen-binding rate and affinity were similar to those of red blood cells.

Much research has been directed toward the synthesis of an iron–porphyrin complex which transports molecular oxygen as in the case of hemoglobin (hb) in red blood cells.[1-3] Success was reported in oxygen-binding in aprotic solvents but not in aqueous media.

Recently we found that the *meso*-5,10,15,20-tetra(*o*-pivalamidophenyl)porphyrinatoiron(II) (abbreviated as heme) complex of 1-lauryl-2-methylimidazole (lauryl = dodecyl) incorporated into liposome of phosphatidylcholine (abbreviated as 'liposomal heme') binds molecular oxygen reversibly under physiological conditions (at pH 7.0 in aqueous media at 37 °C).[4] It was considered that the iron–porphyrin complex was embedded in a bilayer of liposome and that the hydrophobic environment of the inner region of liposome protected the oxygen adduct from its proton-driven oxidation. Oxygen-binding and -dissociation proceeded very rapidly, and kinetic constants were comparable to those of hb in red blood cells. The oxygen-binding affinity was also similar to that of hb in red blood cells. We thus demonstrated in these studies that the liposomal heme is a good model of red blood cells.

We recently further synthesized a novel and amphiphilic heme derivative having four phosphocholine groups, 5,10,15,20-tetra{α,α,α,α-*o*-[2′,2′-dimethyl-20′-(2″-trimethylammonioethyl phosphonatoxy)eicosanamido]phenyl}porphyrinatoiron(II) (abbreviated as lipid-heme) (Scheme),[5,6] expecting that compatibility of the heme with a phospholipid and the oxygen-binding ability are much improved. The present paper describes liposome formation of the lipid-heme with a phospholipid and an oxygen-transporting profile of the the liposomal lipid-heme under physiological conditions.



Scheme. Lipid-heme

Experimental

Materials.—5,10,15,20-tetra{α,α,α,α-*o*-[2′,2′-dimethyl-20′-(2″-trimethylammonioethyl phosphonatoxy)eicosanamido]-phenyl}porphyrinatoiron(III) bromide (lipid-hemin) was prepared as reported previously.[5] ω-Benzyloxy-2,2-dimethyleicosanoic acid chloride (10 mmol) was allowed to react with 5,10,-15,20-tetra(α,α,α,α-*o*-aminophenyl)porphyrin (1.5 mmol) in tetrahydrofuran (thf) containing pyridine (16 mmol). The mixture was separated by column chromatography [silica gel, benzene–diethyl ether (10:1)], affording 5,10,15,20-tetra[α,α,α,α-*o*-(20′-benzyloxy-2′,2′-dimethyleicosanamido)phenyl]porphyrin. This porphyrin (0.85 mmol) was debenzylated by aluminium trichloride (2.0 g) and anisole (2 cm^3) in dichloromethane–nitromethane (1:1); the mixture was cooled with ice-water, followed by extraction with dichloromethane. The extract was concentrated and recrystallized from benzene to give 5,10,-15,20-tetra[α,α,α,α-*o*-(20′-hydroxy-2′,2′-dimethyleicosanamido)-phenyl]porphyrin (80%). Reaction with $FeBr_2$ in thf afforded 5,10,15,20-tetra[α,α,α,α-*o*-(20′-hydroxy-2′,2′-dimethyleicosanamido)phenyl]porphyrinatoiron(III) bromide (91%), which was phosphorylated with 2-chloro-2-oxo-1,3,2-dioxaphospholane (3.5 mmol) in dichloromethane, using triethylamine (3.5 mmol) to trap HCl, at room temperature for 12 h. The resultant phosphate triester was cleaved by a large excess of anhydrous trimethylamine in acetonitrile at 60 °C (24 h). The red-brown precipitate was collected by filtration and then purified on a gel column (Sephadex LH-60, methanol), giving a pure lipid-hemin as an amorphous solid (90%).

1-Laurylimidazole was used as an axial ligand of the heme. Dimyristoylphosphatidylcholine (dmpc), dipalmitoylphosphatidylcholine (dppc), egg yolk lecithin (eyl), and cholesterol were purchased from Sigma (special grade).

Preparation of Liposomal Lipid-heme.—The liposomal lipid-heme (deoxy type) was prepared by modifying the previously reported method.[4] The iron(III)–porphyrin bromide complex (1

† *Non-S.I. units employed:* mmHg = (101 325/760) N m^{-2}, cal = 4.184 J.

μmol) was reduced in the presence of 1-laurylimidazole (3 μmol) under hydrogen by mixing the methanol solution with Pd/C catalyst, and then carbon monoxide gas was bubbled through the mixture. The methanol solution was filtered and collected, dried with molecular sieves (4A), and added to the methanol solution of the phospholipid (50 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. The thus prepared deoxy liposomal lipid-heme solution was incubated at room temperature for 2 h. The red transparent solution showed a u.v.–visible absorption spectrum with maxima at 426, 535, and 562 nm.

Physicochemical Measurements.—The structure of the liposomal lipid-heme was estimated by ^1H and ^{31}P n.m.r. spectroscopy (JEOL JNM FX-100), e.s.r. spectroscopy (JEOL FE-3X) on the spin-labelled heme, gel permeation chromatography (g.p.c.) (Sepharose 4B column), ultracentrifugation (Hitachi 65p-7), transmitting electron microscopy (t.e.m.) (Hitachi H-500), particle size distribution measurements (Nicomp model 200), and differential scanning calorimetry (d.s.c.) (Seiko SSC-560U, 5 °C min^{-1}).

The oxygen- and CO-binding reaction was measured using a stopped-flow spectrophotometer equipped with a kinetic data processor (Union Giken RA-401 and RA-601). Oxygen-binding affinity was determined by spectroscopic measurement of the oxygen-binding equilibrium curve as in ref. 7. For reference, the oxygen-binding equilibrium curve of the red blood cell suspension was measured with the above mentioned apparatus; it was consistent with the curve reported in ref. 7, which supports validity of the measurement in the present experiment.

Results and Discussion

The visible absorption spectrum of the deoxy liposomal lipid-heme changed to that assigned to the oxygen adduct[4,8] ($\lambda_{max.}$ 546 nm) on exposure to oxygen, through isosbestic points at 517 and 544 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 the deoxyheme on bubbling through nitrogen. These results show that the oxidation state of the central iron of the lipid-heme remains at +2 during oxygen exposure. The oxy–deoxy cycle could be repeated more than a hundred times.

The oxygen adduct was slowly degraded to the iron(III)–porphyrin complex and this degradation obeyed first-order kinetics. The lifetime (half-life) of the oxygen adduct of the liposomal lipid-heme was 1 d at pH 7.0 in aqueous media at 37 °C. The lifetime was prolonged by the use of lipid-heme in comparison with the previously reported liposomal heme (lifetime *ca.* ½ d)[4].

The volume of oxygen bound to the liposomal lipid-heme (oxygen adduct) was measured using the van Slyke method* and with Warburg volumetry. This volume was shown to be 20 cm^3 per 1 mmol of the heme (100 cm^3 of a 10 mmol dm^{-3} liposomal lipid-heme solution) at 37 °C corresponding to *ca.* 80% of that calculated. A heme-free eyl–liposome solution (100 cm^3) physically took up 2.2 cm^3 of oxygen, *i.e.* 22.2 cm^3 of oxygen was dissolved in the liposomal lipid-heme solution at 37 °C, which corresponds to that of blood and is *ca.* 10 times of that physically taken up by water.

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

The incorporation was first confirmed by g.p.c. (Sepharose column) monitored by the absorptions at 300 and 415 nm based on the phosphatidylcholine and the lipid-heme, respectively. The curves coincide with each other, which means that the heme complex is included in the liposome. From t.e.m. the liposomal lipid-heme looks like a unilamellar or single-walled liposome with diameters of *ca.* 300—400 Å. The average particle size of the liposomal lipid-heme was also measured by the particle size counter; the diameters of the particle sizes were distributed in the range 250—350 Å. The liposomal lipid-heme solution was also checked by ultracentrifugation (40 000 r.p.m., 30 min at 40 °C). The solution remained transparent after ultracentrifugation and a precipitate containing the lipid-heme and/or the phospholipid did not develop, thus indicating that particles with a diameter >1 000 Å did not exist. Therefore, the liposomal lipid-heme was prepared as fine particles (diameter < 400 Å) which are expected to pass through small capillaries of the human body.

The formation of liposome (single-walled type) 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. The same shift was observed for the liposomal lipid-heme with the addition of Eu^{3+}, as reported for a liposomal heme solution in a previous paper.[4] The liposome formation was also supported by the sharp resonance in the ^{31}P n.m.r. spectrum [0.8 p.p.m., J(PtH) 12 Hz], which is caused by the spherical geometry of the phosphatidyl group.

The Fourier-transform ^1H n.m.r. spectrum of the liposomal lipid-heme at 37 °C showed a signal at −0.1 p.p.m. assigned to the β-dimethyl groups 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 lipid-heme complex is molecularly dispersed in the bilayer of the dmpc liposome.

The e.s.r. spectra of the lipid-heme complex labelled with nitrogen oxide were also studied. The spin-labelled lipid-heme complex in homogeneous methanol solution gave a triplet signal in the z direction and weak signals in the x and y directions, as reported previously for a labelled heme complex in *NN*-dimethylformamide.[4] On the other hand, the spin-labelled lipid-heme complex solubilized in water showed only the signal in the z direction, which was often observed in the solid state. The spectrum of the spin-labelled lipid-heme complex solubilized in water by the liposome is similar to that in the homogeneous methanol solution. This result also suggests that the lipid-heme complex is molecularly dispersed and well solubilized in the bilayer of the liposome.

A d.s.c. thermogram (5 °C min^{-1}) of the liposomal lipid-heme was measured to estimate the phase transition of the liposomal lipid-heme. The dmpc-liposome showed the endothermic peak at 24 °C, which corresponds to the gel–liquid-crystal phase transition temperature (T_c) of the liposome and agreed with that reported previously.[9] For the liposomal heme, in which the simple heme complex was embedded, the phase transition peak was broadened and shifted to the lower temperature of 22 °C. This suggests that the incorporation of the heme complex into the liposomal bilayer causes partial disorder in the alkyl chain orientation of the phospholipid molecule. On the other hand, the peak of the liposomal lipid-heme was observed at the same temperature of the dmpc-

* The van Slyke method is a conventional technique used to estimate volumes of molecular oxygen gas bound to red blood cells: D. D. van Slyke and J. M. McNeill, *J. Biol. Chem.*, 1924, **61**, 523.

liposome itself at 24 °C. This suggests that the orientation of the phospholipid in the liposome is equivalent for the dmpc-liposome and the dmpc-liposomal lipid-heme and that compatibility of the lipid-heme with the phospholipid is large enough to form a stable liposome.

The above results lead to the following conclusion. The lipid-heme complex is included and dispersed in the phospholipid bilayer of a liposome which protects the oxygen adduct of the heme from irreversible oxidation. The geometry of the lipid-heme is assumed to emphasize the incorporation of the lipid-heme into the phospholipid bilayer of the liposome.

The kinetic and equilibrium profile of oxygen-transport by the liposomal lipid-heme was studied by the stopped-flow method and by oxygen-binding and -dissociation curve measurement [equations (1) and (2); L = axial ligand of the heme (1-laurylimidazole)].

$$\mathrm{Fe^{II}L} + \mathrm{O_2} \underset{k(\mathrm{O_2})_{off}}{\overset{k(\mathrm{O_2})_{on}}{\rightleftharpoons}} \mathrm{Fe^{II}L{-}O_2},\ K(\mathrm{O_2}) = k(\mathrm{O_2})_{on}/k(\mathrm{O_2})_{off} \quad (1)$$

$$\mathrm{Fe^{II}L} + \mathrm{CO} \underset{k(\mathrm{CO})_{off}}{\overset{k(\mathrm{CO})_{on}}{\rightleftharpoons}} \mathrm{Fe^{II}L{-}CO},\quad K(\mathrm{CO}) = k(\mathrm{CO})_{on}/k(\mathrm{CO})_{off} \quad (2)$$

After the rapid mixing of the liposomal lipid-heme with a phosphate buffer solution (KH_2PO_4–Na_2HPO_4, 0.067 mol dm^{-3}, pH 7.0) saturated with oxygen, the spectrum of the deoxy-heme immediately changed to that assigned to the oxygen adduct. To the contrary, after the rapid mixing of the oxygen adduct with the same buffer solution saturated with argon, the spectrum returned to that of the deoxy-heme. These results supported rapid and reversible oxygen binding to the liposomal lipid-heme.

Oxygen-binding and -dissociation rate constants [$k(O_2)_{on}$ and $k(O_2)_{off}$ respectively] are summarized in Table 1. The values of k_{on} for the liposomal lipid-hemes were a little larger than or similar to those of the red blood cell suspension. The oxygen-binding rate constants for the homogeneous systems[10–13] (cited in Table 1) were *ca.* 10^2 times larger than those of the liposomal lipid-hemes and the red blood cell suspension, thus showing that the oxygen-binding reactions for the liposomal lipid-heme and the red blood cell suspension are comparable. The oxygen-binding reaction is assumed to be largely retarded by the diffusion process of oxygen in and through the phospholipid membrane.

The oxygen-binding rate was larger for the eyl- and for the dppc-liposomal lipid-hemes. This may be explained as follows. The dppc-liposome is below its T_c temperature (41 °C)[9] and the eyl-liposome is above (−15 to −7 °C)[9] under these experimental conditions (at 25 °C), so that membrane fluidity and probably also oxygen permeability of the eyl-liposome is larger than that of dppc. The largest oxygen-binding rate was observed for the burst eyl-liposomal lipid-heme. It is assumed that this liposome has a lamellar structure or a non-closed (non-vesicle) bilayer structure, and this is advantageous for the supply of oxygen to the lipid-heme.

The CO-binding and -dissociation rate constants [$k(CO)_{on}$ and $k(CO)_{off}$ respectively] are summarized in Table 2. The value of $k(CO)_{on}$ for the eyl-liposomal lipid-heme was similar to that of the red blood cell suspension and considerably different from those given previously[10,12,14] for homogeneous systems. It seems that the CO-binding reaction is also retarded by the diffusion process of CO in and through the phospholipid membrane. The CO-binding rate was larger for the burst eyl-liposomal lipid-heme. This further supports the suggestion that the CO-binding rate is influenced by the gas diffusion.

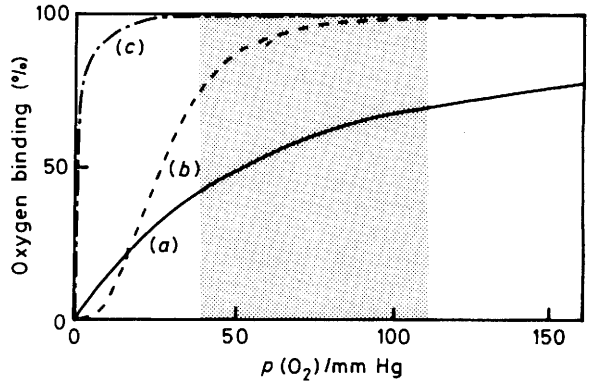


Figure Oxygen-binding and -dissociation equilibrium curves of liposomal lipid-hemes at pH 7.0 in aqueous media at 37 °C: (*a*) liposomal lipid-heme, (*b*) blood, and (*c*) myoglobin. The shaded area corresponds to the oxygen pressure at the lung and muscle tissue

Table 1. Oxygen-binding rate constants for the liposomal lipid-hemes at 25 °C

Heme	Solvent (pH)	Measurement method[a]	$k(O_2)_{on}$/dm^3 mol^{-1} s^{-1}	$k(O_2)_{off}$/s^{-1}	$K(O_2)$[b]/dm^3 mol^{-1}
Liposomal lipid-heme[c]					
eyl	Phosphate buffer (7.0)	s.f.	3.7×10^4	2.2	1.6×10^4
dmpc	Phosphate buffer (7.0)	s.f.	3.5×10^4	2.3	1.7×10^4
dppc	Phosphate buffer (7.0)	s.f.	2.2×10^4	0.86	2.6×10^4
Burst eyl[d]	Water (1:5) (7.0)[d]	s.f.	4.4×10^4	2.5	1.7×10^4
Liposomal heme[c]					
dmpc[e]	Phosphate buffer (7.0)	s.f.	7.9×10^3	0.32	2.5×10^4
Red blood cell suspension[f]	(7.4)[f]		1.1×10^4	0.16	6.8×10^4
Heme–1,2Me_2-im[g]	Toluene	f.p.	1.1×10^8	46 000	2.3×10^3
Chelated heme[h]	(7.3)	f.p.	2.6×10^7	47	5.5×10^5
Myoglobin[i]	(7.0—7.4)	f.p., s.f.	$(1.0—2.0) \times 10^7$	10—30	$(0.67—1.0) \times 10^6$
Stripped hemoglobin[j]	(7.0—7.4)	f.p., s.f.	3.3×10^7	12—13	$(2.5—2.7) \times 10^6$

[a] Measurement method; s.f. = stopped flow, f.p. = flash photolysis. [b] $K(O_2) = k(O_2)_{on}/k(O_2)_{off}$. [c] The liposomal lipid-hemes and the liposomal hemes composed of eyl, dmpc, or dppc. [d] The liposomal lipid-heme in higher concentration (1:5) was poured into water and diluted. The liposome is expected burst by osmotic pressure and to have a lamellar structure. [e] E. Tsuchida, H. Nishide, and M. Yuasa, *J. Chem. Soc., Chem. Commun.*, 1984, 96; E. Tsuchida, M. Yuasa, and H. Nishide, *J. Chem. Soc., Dalton Trans.*, 1985,65. [f] The preparation of the red blood cell suspension was as in ref. 9; see footnote *e*. [g] From ref. 10, heme–1,2Me_2-im = *meso*-tetra[α,α,α,α-(*o*-pivalamidophenyl)]porphyrinatoiron(II) complex of 1,2-dimethylimidazole. [h] From ref. 11, chelated heme (pH 7.3) = protoheme *N*-(3-imidazol-1-ylpropyl)amide methyl ester solubilized by myristyltrimethylammonium bromide at 20 °C. [i] From ref. 12. [j] From refs. 12 and 13.

Table 2. Carbon monoxide-binding rate constants for the liposomal lipid-hemes at 25 °C

Heme	Solvent (pH)	Measurement method[a]	$k(CO)_{on}$/dm^3 mol^{-1} s^{-1}	$k(CO)_{off}$/s^{-1}	$K(CO)^b$/dm^3 mol^{-1}
Liposomal lipid-heme[c]					
eyl	Phosphate buffer (7.0)	s.f.	2.5×10^4	3.1	8.1×10^3
dppc	Phosphate buffer (7.0)	s.f.	2.5×10^4	0.85	3.1×10^4
Burst eyl[d]	Water (1:5) (7.0)[d]	s.f.	3.3×10^4	2.6	1.3×10^4
Liposomal heme[c]					
dmpc[e]	Phosphate buffer (7.0)	s.f.	2.8×10^5		
Red blood cell suspension[f]	(7.4)[f]	s.f.	1.1×10^4	0.01	1.4×10^6
Heme–1,2Me_2-im[g]	Toluene	f.p.	1.4×10^6	0.14	1.0×10^7
Chelated heme[h]	(7.3)	f.p.	3.6×10^6	0.0089	4.0×10^8
Myoglobin[i]	(7.0—7.4)	f.p., s.f.	$(3.0—5.0) \times 10^5$	0.0015—0.040	$(1.3—20) \times 10^7$
Stripped hemoglobin[j]	(7.0—7.4)	f.p., s.f.	4.6×10^6	0.009	5.1×10^8

[a] See *a* of Table 1. [b] $K(CO) = k(CO)_{on}/k(CO)_{off}$. [c] See *c* of Table 1. [d–i] See *d*—*i* of Table 1 respectively. [j] From refs. 12 and 14.

Table 3. Oxygen-binding affinity at 37 °C and thermodynamic parameters

Heme	Solvent (pH)	$p_{\frac{1}{2}}(O_2)$/mmHg	ΔH/kcal mol^{-1}	ΔS/cal K^{-1} mol^{-1}
Liposomal lipid-heme[a]				
eyl	Phosphate buffer (7.0)	53	−15	−40
dmpc	Phosphate buffer (7.0)	50		
Liposomal heme[a]				
eyl[b]	Phosphate buffer (7.0)	51	−16	−46
Blood[c]		27		
Stripped hemoglobin (R)[d]	(7.0—7.4)	0.22—0.36	−14 to −15	*ca.* −40
Myoglobin[e]	(7.0—7.4)	0.37—1.0	−14 to −21	*ca.* −40
Heme–1,2Me_2-im[f]	Toluene	38[g]	−14	−41
Chelated-heme[h]	(7.3)		−14	−35

[a] See *c* of Table 1. [b] E. Tsuchida, H. Nishide, M. Yuasa, and M. Sekine, *Bull. Chem. Soc. Jpn.*, 1984, **57**, 776. [c] From ref. 15. [d] From refs. 12 and 16; R denotes the 'relaxed' state. [e] From ref. 12. [f] J. P. Collman, J. I. Brauman, K. M. Doxsee, T. R. Halbert, and K. S. Suslick, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 564. [g] At 25 °C. [h] From ref. 11.

The oxygen-binding affinity ($p_{\frac{1}{2}}$: oxygen pressure at half oxygen-binding for the heme) of the liposomal lipid-heme was determined by the oxygen-binding equilibrium curve measurement (Figure) and values are listed in Table 3. For the dmpc- and eyl-liposomal lipid-hemes, $p_{\frac{1}{2}} = 50$ and 53 mmHg at 37 °C respectively, which are close to that of hb in red blood cells,[15] but considerably different to that of myoglobin.[12] This suggests that the liposomal lipid-heme has the potential to act as the oxygen carrier under physiological conditions which transports oxygen from the lungs [$p(O_2) = ca.$ 110 mmHg] to myoglobin in muscle tissue [$p(O_2) = ca.$ 40 mmHg] as does hb. Table 3 also shows the thermodynamic parameters for the oxygen-binding. The enthalpy (ΔH) and entropy changes (ΔS) for the oxygen-binding of the liposomal lipid-heme were estimated to be *ca.* −15 kcal mol^{-1} and *ca.* −40 cal K^{-1} mol^{-1}, respectively. These values are comparable to those of hb[12,16] and myoglobin.[12] This result indicates that oxygen-binding by the liposomal lipid-heme proceeds in the same way as does the binding by hb and myoglobin. We would like to emphasize from the Figure that the oxygen-transporting efficiency of the liposomal lipid-heme between the oxygen pressures at the lungs and muscle tissue is *ca.* 30%, which corresponds to that of blood.

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