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Rapid Report

Lipid microsphere containing lipophilic heme: preparation and oxygen transportation under physiological conditions

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Lipophilic heme (1-laurylimidazole-ligated 5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphinatoiron(II) complex) is solubilized in lipid (triglyceride) at high concentrations and emulsified with a phospholipid in physiological salt solution, giving a deeply red-colored suspension of lipid microspheres (approx. 250 nm in diameter). The heme forms an oxygen adduct in a similar manner as oxyhemoglobin and the lipid microspheres take up and release oxygen reversibly at 37°C in the aqueous medium. The oxygen-transporting ability is comparable with that of the red blood cell. Intravenous injection of the heme/lipid microsphere solution to rabbits demonstrates that it transports oxygen even in vivo and that it is cleared from the blood stream with a half-life time of approx. 1 h.

Porphinatoiron (heme) had been extensively converted to chemically modified hemes or hemoglobin (Hb) models to mimic Hb's oxygen-binding and transportation capability in physiological aqueous solution [1]. However, these hemes themselves could not bind oxygen reversibly under physiological conditions (pH 7.4, 37°C), since they are oxidized immediately and irreversibly to the corresponding ferric hemes by the reaction of iron-bound dioxygen with a proton of water. It is necessary that a heme derivative is immobilized into a specific hydrophobic microenvironment, such as the 'heme pocket' of Hb, to bind oxygen reversibly in an aqueous medium [2]. Until now, the only successful examples of reversible oxygen-binding under physiological conditions are our liposome embedded hemes [1]: We have synthesized phospholipid and/or sterole derivatives of heme, e.g., 5,10,15,20-tetrakis(α,α,α,α-*o*-(2',2'-dimethyl-20'-(2''-triethylammonioethyl)phosphonatoxyicosanamido)phenyl)porphinatoiron, and embedded them in the bilayer of phospholipid liposomes [3]. Good compatibility of the superstructured hemes with a phospholipid molecule enhances not only trapping efficiency of the hemes in the liposome, but also reversible oxygen-binding ability of the hemes. They transport oxygen under physiological conditions.

It is well known that oil-in-water (O/W) lecithin emulsions (lipid microspheres) have been used for clinical nutrition and as carriers for a variety of lipophilic drugs [4–6]. Lipid microspheres have certain similarities to liposomes, however, there are several advantages over phospholipid vesicles, such as a high colloidal stability, which makes it possible to store the emulsions for a long period (several months) at room temperature without any change in their physicochemical properties. The oil phase acts as a solubilizer of large amounts of lipophilic substances and can also serve to stabilize drugs that are unstable in an aqueous medium. Furthermore, lipid microspheres are well tolerated by the body since they resemble chylomicrons and a lower incidence of side effects has been observed.

In the present report, we have prepared a new type of finely dispersed and stable O/W lipid microsphere, of which the lipid phase contains the lipophilic imidazole-ligated heme complex (5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphinatoiron(II)-1-laurylimidazole) at high concentrations, as an oxygen transporter under physiological conditions or as an Hb model system (Fig. 1). One of the merits of the lipid microsphere system is that the solubilized amount of heme or the oxygen solubility in the medium is much

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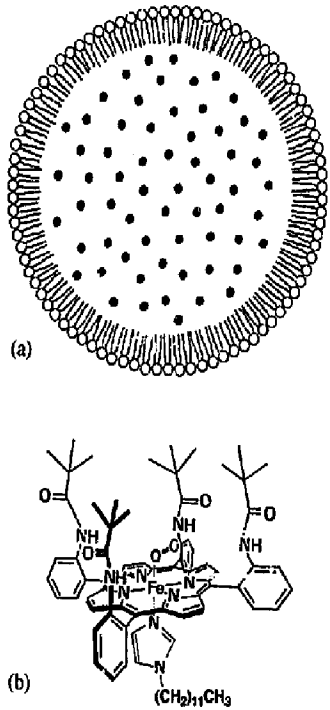

Fig. 1. (a) Heme/lipid microsphere, •, Heme-LIm complex; shaded area, Triglyceride (b) Heme-LIm-O_2 complex.

enhanced in comparison to previously reported homogeneous heme solutions and our liposome embedded heme dispersions [7]. Oxygen-binding ability of the heme/lipid microsphere under physiological conditions and in the blood stream of rabbits will be described. The present combination of a lipophilic heme with a lipid provides a new opportunity for oxygen transportation in vivo using a totally new microsphere/emulsion scheme.

Heme and 1-laurylimidazole (LIm) were synthesized as described in the literature [7,8]. Trioctanoylglyceride (TG) was a special grade material gifted from Nippon Oil & Fats. Its acid value and iodine number are less than 0.1 and 1.0, respectively. Egg yolk phosphocholine (PC; egg hydrogenated)) was purchased from Nippon Fine Chemical. Pronon 204 and propyleneglycol (PG) were purchased from Kanto Chemicals (special grade).

The heme/lipid microsphere was prepared as follows. Heme (0.5 μmol), LIm (1.0 μmol) and PC (2.4 or 3.0 mg) were dissolved in TG (25.0 mg). Ligation of LIm to heme and homogeneous solubilization of heme-LIm were confirmed by its visible absorption spectrum. Then 0.03 M phosphate buffer (pH 7.4, 10 ml) was added to the mixture. The solution was homogenized by ultrasonic generator (Nihon Seiki US-600) under nitrogen to give a red-colored O/W emulsion. The sample for the in vivo test was dispersed in physiological saline solution.

The microsphere was also prepared by D phase emulsification. Heme (5.0 μmol), LIm (10.0 μmol), Pronon 204 (7.6 mg), PG (75.8 mg) and TG (0.1 g) were dispersed by homogenizer (Nihon Seiki AM-3, 10000 rpm, 15 min) at 70°C. Oxygen-free phosphate buffer (pH 7.4, 100 ml) was added to the mixture and the solution was further homogenized (10000 rpm, 15 min) at 30°C to give an O/W emulsion.

The concentration of TG could be enhanced up to 40 wt% and the emulsion was stable without phase separation.

Particle size of the heme/lipid microsphere with different compositions was estimated by the dynamic light scattering method using a submicron particle analyzer (Coulter Electronics N4SD). The diameter for the heme/lipid microsphere ([heme] = 5 mM, [TG] = 20 wt%, TG/PC = 10 (weight ratio)) was 260 ± 80 nm, which decreased with the PC content (140 ± 50 nm for TG/PC = 5 (weight ratio)). On the other hand, the most frequent diameters of the heme/lipid microspheres homogenized by D phase emulsification were 590 ± 200 nm. The particle size was affected by the composition, surfactant species and emulsification procedure. Transmission electron microscopy of the heme/lipid microsphere (TG/PC = 10 (weight ratio)) indicated also the diameter of approx. 250 nm.

Incorporation of the heme in the lipid microsphere was confirmed by elution curves in gel permeation chromatography (Pharmacia Fine Chemical, Separose CL-4B, 2.2 cm (i.d.) × 70 cm), monitored by the absorption at 420 and 255 nm, attributed to the heme and the phospholipid, respectively (A small amount of phospholipid, which contains an unsaturated fatty acid residue, 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine, was added for the UV probe.). The elution curves coincided with each other, indicating that the lipophilic heme was completely included in the lipid microsphere and was not eluted to the aqueous phase.

The heme/lipid microsphere dispersion was also checked by centrifugation (3000 rpm, 2 h at 25°C). The solution remained homogeneous after centrifugation and no precipitate containing the heme and/or the TG developed. Therefore, the heme/lipid microsphere was prepared as a stable and suitable particle with a diameter of approx. 250 nm which is expected to be stable for the moment even in vivo and to pass through small capillaries of animals.

The iron (III) derivatives of heme in the lipid microsphere ([heme] = 50 μM, [TG] = 0.25 wt%, TG/PC = 10 (weight ratio), 260 ± 80 nm ϕ) was reduced to the deoxy (iron(II)) form as described in our previous pa-

TABLE I

Infrared spectral data of the heme / lipid microsphere in aqueous medium

Heme/lipid microsphere was dispersed in phosphate buffer (pH 7.4, 0.03 M). [Heme] = 5 mM, [TG] = 20 wt%. TG/PC = 10 (wt ratio). IR spectral data were measured at 25°C.

	Ligand	Solvent	$\nu_{O\text{-}O}(\nu_{1/2})$ (cm^{-1})	$\nu_{CO}(\nu_{1/2})$ (cm^{-1})
Heme/lipid microsphere	Lim	H_2O	1160(13)	1967(15)
Lipid heme/liposome [a]	Lim	H_2O	1161(13)	1966(15)
Heme [b]	MeIm [b]	Benzene	1159	1968(14)
Hb		H_2O	1107(9 ± 1) [c]	1951(12) [c]
Mb		H_2O	1103(9 ± 1) [c]	1945(12) [c]
O_2 or CO gas [c]			1556	2143
O_2^- [d]			1145	

[a] From Ref. 10. [b] MeIm, 1-methylimidazole. From Ref. 11. [c] From Ref. 12. [d] From Ref. 13.

per [9]. The visible absorption spectrum of the deoxy heme/lipid microsphere (λ_{max} 535 and 561 nm) changed to that assigned to the oxygen-heme adduct (λ_{max} 541 nm) upon exposure to dioxygen. The deoxy-oxy cycle was able to be repeated more than 100 times at 37°C by bubbling nitrogen and oxygen gas through the dispersion. The spectrum of the oxygen adduct changed to that of the CO-heme adduct (λ_{max} 539 nm) when CO was bubbled through the dispersion; it returned to the deoxy heme upon continuously bubbling nitrogen.

Infrared spectra on the oxygen-heme and the CO-heme adduct were measured for the heme/lipid microsphere dispersion ([heme] = 5 mM) using the differential spectroscopic method in $^{16}O_2$ vs. $^{12}C^{16}O$ and $^{12}C^{16}O$ vs. nitrogen atmospheres. The IR cells used were precisely matched in terms of path-length (50 μm) and CaF_2 window thickness [10]. The CO stretching frequency (ν_{CO}) of the bound CO was 1967 cm^{-1}, similar to that of the CO adduct of heme-1-methylimidazole in benzene (ν_{CO} = 1969 cm^{-1} [11]) (Table I). The differential spectrum of the $^{16}O_2$ adduct vs. the CO adduct of the heme/lipid microsphere involved an intense band at 1160 cm^{-1} with band-width of 13 cm^{-1} at half-height, which also agreed with the derivatives' reported band for the corresponding heme in benzene. The $\nu(O_2)$ value of the bound oxygen differs from that of gaseous molecular oxygen ($\nu_{O\text{-}O}$ = 1556 cm^{-1}), but is similar to those of oxy-Hb, oxy-Mb and superoxide (O_2^-: 1145 cm^{-1}). Dioxygen bonding to the iron of the heme in the heme/lipid microsphere is a bent end-on type which had been described for oxy-Hb and oxy-Mb [12,13].

Oxygen-binding affinity, the O_2 pressure of at half-maximum oxygen-binding for the heme(II) ($= P_{1/2}(O_2)$) was determined by the spectral changes in response to oxygen partial pressures [7]. The $P_{1/2}(O_2)$ value of the heme/lipid microsphere was 50 torr, close to that of Hb in the red blood cell, but considerably different to that of myoglobin (Mb) [14–17] (Table II). This indicates that the heme/lipid microsphere has the potential to act as an oxygen transporter under physiological

TABLE II

Oxygen-binding affinities of the heme / lipid microsphere under the physiological condition

Heme/lipid microsphere was dispersed in phosphate buffer (pH 7.4, 0.03 M). [Heme] = 50 μM. (a) Liposome composed of dimyristoylphosphocholine. In phosphate buffer (pH 7.0, 0.03 M). LMIm, 1-lauryl-2-methylimidazole. From Ref. 7. (b) At 25°C. Me_2Im, 1,2-dimethylimidazole. From Ref. 8. (c) At 20°C, pH 7.0–7.4 From Refs. 15–17.

	Heme	Ligand	Diameter (nm)	$P_{1/2}(O_2)$ (torr)	
				25°C	37°C
Lipid microsphere	Heme	Lim	140 ± 50	34	56
		Lim	260 ± 80	26	50
		Lim	590 ± 200	36	–
Lipid liposome(a)	Heme	LMeIm	80	22	49
	Lipid heme	Lim	40	–	50
Toluene(b)	Heme	Me_2Im		38	–
RBC suspension				–	27 [a]
Hb(R-state)a(c)				0.22	
Mb(c)				0.37–1.0	

[a] From Ref. 14.

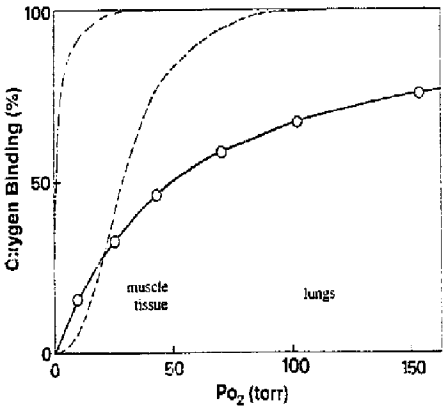

Fig. 2. Oxygen equilibrium curve of the heme/lipid microsphere in 0.03 M phosphate buffer (pH 7.4) at 37°C, heme concn. = 50 μM, TG concn. = 0.25 wt%, molar ratio; heme/LIm = 1:2, PC/TG = 10 (wt/wt). Hb (— — —) and Mb (- - - -), pH 7.4 at 37°C.

conditions, which binds oxygen at the lungs ($P(O_2) \approx$ 110 torr) and delivers the oxygen at terminal tissue ($P(O_2) \approx 40$ torr) as Hb does (Fig. 2).

An important feature of the heme/lipid microsphere dispersion is that the microsphere contains the heme at high concentrations in the medium. The microsphere dispersion ([heme]: 10 mM, [TG]: 40 wt%) took up and held oxygen gas to the amount of approx. 20 ml/100 ml medium, which is comparable to that of human blood.

Oxygen transportation and life-time in the blood stream of animals were tested for the heme/lipid microsphere dispersion (260 ± 80 nm ϕ, [heme] = 5 mM, [TG] = 20 wt%). The experiments were performed with five male rabbits weighing approx. 3.0 kg. A volume of 20 ml/kg blood from rabbits was shed, and then the same amount of the heme/lipid microsphere solution was intravenously injected through the rostral or caudal auricular veins. After the injection, blood was drawn through the rostral or caudal auricular artery and the withdrawn blood was centrifuged (3000 rpm, 20 min). The supernatant contains only the heme/lipid microsphere. The visible absorption spectrum of the supernatant showed the deoxy and oxy form of the heme/lipid microsphere on bubbling nitrogen and oxygen gas, respectively. This indicates that the heme/lipid microsphere delivers oxygen in blood stream.

Oxidation of the heme, that is, met-formation in the blood stream was approx. 20% at 1 h after the injection.

The apparent half-life time (50% disappearance time of the heme/lipid microsphere) in the blood stream was estimated by measuring the heme concentration in the drawn blood. The quantitative analysis of heme concentration was carried out by the cyanomet-heme method using Hb test-wako (Wako Pure Chem. Industries) [18]. The apparent half-life time in the blood stream was determined to be 1 h, indicating that the heme/lipid microsphere remains and transports oxygen in the blood stream at least for 1 h after the injection.

References

1 Tsuchida, E. and Nishide, H. (1986) Top. Curr. Chem. 132, 63.
2 Lavalette, D., Tetreau, C., Mispelter, J., Momenteau, M. and Lhoste, J.-M. (1984) Eur. J. Biochem., 145, 555; Gerothanassis, I.P., Momenteau, M. and Loock, B. (1989) J. Am. Chem. Soc. 111, 7006.
3 Tsuchida, E., Nishide, H., Yuasa, M., Hasegawa, E., Matsushita, Y. and Eshima, K. (1985) J. Chem. Soc. Dalton Trans., 275; Tsuchida, E., Komatsu, T., Babe, T., Nakata, T., Nishide, H. and Inoue, H. (1990) Bull. Chem. Soc. Jpn. 63, 2323; Tsuchida, E. and Nishide, H. (1987) in Liposome as drug Carriers (Gregoriades, G., ed.), p. 569, John Wiley, New York.
4 Davis, S.S., Washington, C., West, P. Illum, L., Liversidge, G., Stenson, L. and Kirsh, R. (1987) Ann. N.Y. Acad. Sci. 507, 75.
5 Lenzo, N.P., Martins, I., Mortimer, B.C. and Redgrave, T.G., (1988) Biochim. Biophys. Acta, 960, 111; Redgrave, T.G. and Maranhao, R.C. (1985) Biochim. Biophys. Acta 835, 104.
6 Mizushima, Y., Hamano, T. and Yokoyama, K., (1982) Ann. Rheum. Dis. 41, 263.
7 Tsuchida, E., Nishide, H., Yuasa, M., Hasegawa, E. and Matsushita, Y. (1984) J. Chem. Soc. Dalton Trans., 1147; Tsuchida, E., Yuasa, M. and Nishide, H. (1985) J. Chem. Soc. Dalton Trans., 65.
8 Collman J.P., Gagne, R.R., Reed, C.A., Halbert, T.R., Lang, G. and Robinson, W.T. (1975) J. Am. Chem. Soc. 97, 1427; Collman, J.P., Brauman, J.I., Iverson, B.L., Sessler, J.L., Morris, J.L. and Gibson, Q.H. (1983) J. Am. Chem. Soc. 105, 3052.
9 Tsuchida, E., Nishide, H., Yuasa, M. and Sekine, M. (1984) Bull. Chem. Soc. Jpn. 57, 776.
10 Yuasa, M., Yamamato, K., Nishide, H. and Tsuchida, E. (1988) Bull. Chem. Soc. Jpn. 61, 313.
11 Collman, J.P., Brauman, J.I., Halbert, T.R. and Suslick, K.S. (1976) Proc. Natl. Acad. Sci. USA 73, 3333.
12 Bulow, C.H., Maxwell, J.C. Wallace, W.J. and Caughey, W.S. (1973) Biochem. Biophys. Res. Commun. 55, 91; Caughey, W.S. (1970) Ann. N.Y. Acad. Sci. 174, 148; Maxwell, J.G., Volpe, J.A., Barlow, C.H. and Caughey, W.S. (1974) Biochem. Biophys. Res. Commun. 58, 166.
13 Herzberg, G. (1950) Molecular Spectra and Molecular Structure, I. Spectra of Diatomic Molecules, p. 560, Van Nostrand Co., New York.
14 Imai, K., Morimoto, H., Kotani, M., Watari, H. and Kuroda, M. (1970) Biochim. Biophys. Acta 200, 189.
15 Sharma, V.S., Schmidt, M.R., Ranney, H.M. (1976) J. Biol. Chem., 251, 4267.
16 Steinmeier, R.C. and Parkhuyst, L.J. (1975) Biochemistry 14, 1564.
17 Burunori, M. and Schuster, T.M. (1969) J. Biol. Chem. 244, 4046; Antonini, F. and Burunori, M. (1971) Hemoglobin and Myoglobin and their Reactions with ligands, Elsevier, New York.
18 Rahbar, S. (1968) Clin. Chim. Acta, 22, 296.