Polymerized Liposome/Lipid–Heme as an Oxygen Transporter under Physiological Conditions

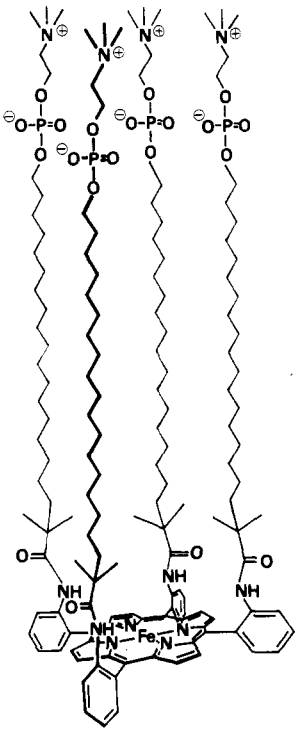
Eishun Tsuchida,* Hiroyuki Nishide, Makoto Yuasa, Etsuo Hasegawa, Kiyoshi Eshima, and Yoh-ichi Matsushita

Department of Polymer Chemistry, Waseda University, Tokyo 160, Japan.
Received August 26, 1988

ABSTRACT: Copolymerized liposome of 1,2-bis(octadeca-2′,4′-dienoyl)-*sn*-glycero-3-phosphocholine and sodium 15-(1′-imidazolyl)pentadeca-*trans*-2,*trans*-4-dienoate is an effective carrier of [5α,10α,15α,20α-tetrakis[*o*-[2′,2′-dimethyl-20′-[2″-(trimethylammonio)ethyl]phosphonatoxyeicosanamido]phenyl]porphinato]iron(II) (lipid–heme): This polymerized liposome/lipid–heme transports molecular oxygen under physiological conditions (in pH 7, at 37 °C). The lipid–heme is embedded and fixed in the bilayer of polymerized liposome based on hydrophilic–hydrophobic balance of the lipid–heme and/or coordination bond between the lipid–heme and the copolymerized imidazole residue. Solution properties of the polymerized liposome/lipid–heme are almost the same as those of human blood, and the solution is able to be stocked for months. Oxygen volume dissolved in the polymerized liposome/lipid–heme solution (100 mL) is determined to be ca. 22 mL by an ^{15}O radiotracer method and by volumetry, which is comparable with that of human blood. Oxygen-binding affinity and rate constant of the polymerized liposome/lipid–heme are ca. 50 mmHg and 10^4 M^{-1} s^{-1}, respectively, which are also similar to those of hemoglobin in blood.

Introduction

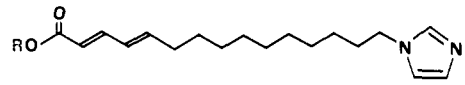
Recently we succeeded in reversible oxygen-binding to (porphinato)iron (heme) under physiological conditions (in pH 7 aqueous solution, at 37 °C):[1] [5α,10α,15α,20α-tetrakis[*o*-(pivalamido)phenyl]porphinato]iron(II) complex of 1-lauryl-2-methylimidazole and [5α,10α,15α,20α-tetrakis[*o*-[2′,2′-dimethyl-20′-[2″-(trimethylammonio)ethyl]-phosphonatoxyeicosanamido]phenyl]porphinato]iron(II) (lipid–heme **1**) embedded in a liposome of phospholipid.[2-5]



1

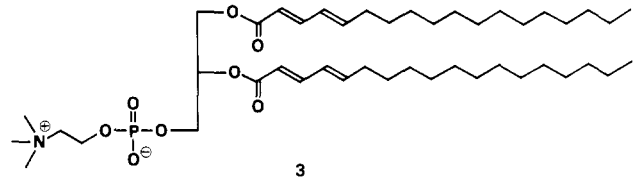
Both stability of the liposome and oxygen-binding ability of the heme are enhanced for the lipid–heme, because stereostructure and hydrophilic–hydrophobic balance of the lipid–heme increase its compatibility with a phospholipid bilayer and because the hydrophobic region of the lipid bilayer surrounds and protects the heme plane (oxygen-binding site) from its degradation during the oxygen-transporting.

With this lipid–heme we also succeeded in utilizing the bilayer region of polymerized phospholipid liposome of 1-[9′-(*p*-vinylbenzoyl)nonanoyl]-2-*O*-octadecyl-*rac*-glycero-3-phosphocholine as a carrier of heme, which was reported in the previous communication paper.[6] By utilizing the polymerized liposome, we are able to improve stability of the liposome as the carrier of the heme, which brings about a highly concentrated, physically and mechanically stable and storable solution of heme just like or superior to human blood. This paper describes the preparation of a copolymerized liposome of a novel polymerizable and amphiphilic imidazole, sodium 15-(1′-imidazolyl)pentadeca-*trans*-2,*trans*-4-dienoate (**2**), ligated



R = Na : **2**
= C_2H_5 : **5**

with the lipid–heme and a polymerizable phospholipid, 1,2-bis(octadeca-2′,4′-dienoyl)-*sn*-glycero-3-phosphocholine (**3**), which not only transports oxygen under physiological conditions but also satisfies solution properties and high concentration as a blood substitute.



3

Results and Discussion

Preparation of Polymerized Liposome/Lipid–Heme. Lipid–heme (**1**) was synthesized as in the previous paper.[7] Polymerizable and amphiphilic imidazole (**2**), which ligates to the lipid–heme and makes it bind oxygen, was synthesized from 11-(1′-imidazolyl)undecanal, as described in the Experimental Section of this paper. **2** has both a hydrophobic and a hydrophilic part and was dispersed in water of pH 7, while an alkylimidazole such as dodecylimidazole was not able to be solubilized. This suggests that **2** is incorporated in the bilayer of lipids based on its amphiphilic property. Polymerizable phospholipid (**3**) was synthesized as reported in the literature.[8]

The polymerized liposome/lipid–heme was prepared as follows. For example, the liposome of **1**–**3** (molar ratio 1/3/50) was prepared by a normal thin-film method of liposome preparation under nitrogen atmosphere. Then

0024-9297/89/2222-2103$01.50/0

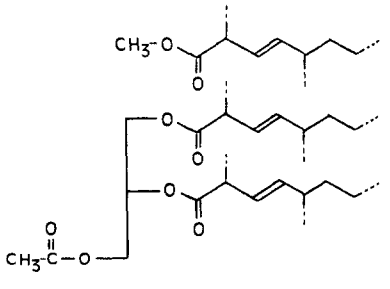
Table I
Molecular Weight of Acetolysis Compounds of the Polymerized Liposome

polymerized liposome	acetolysis M_w (deg polymn)	M_w/M_n
copolymerized 2/3 liposome with 1	5.0×10^3 (8)	2.4
copolymerized 2/3 liposome	1.5×10^4 (23)	2.3
polymerized 3 liposome	1.7×10^4 (26)	2.4

the prepared liposome was allowed to polymerize under ultraviolet (UV) irradiation to polymerize and give the copolymerized liposome/lipid–heme. Reduction of the iron(III) derivative of the lipid–heme to the deoxy lipid–heme (iron(II)) spontaneously occurred during the polymerization. Complete polymerization was confirmed by UV absorption and ^{13}C NMR spectroscopical measurement: disappearance of the UV absorption (λ_{max} = 255 nm) based on the diene group and of characteristic signal (δ_C = 118.4, 128.2, 145.2–146.0 (d) ppm) based on the diene carbons of **2** and **3**. The polymerization completed after 1 h of UV irradiation was 10 times faster than that for the polymerization of **3** with **1** and **2** in a homogeneous chloroform solution under the same conditions. This indicates that the polymerization of the diene groups in a liposome state or in an oriented structure proceeds rapidly as a zipping-up reaction due to a favorably frequency factor for the polymerization. That is, the polymerizable diene groups of **2** and **3** are in alignment in a bilayer state due to their hydrophobic–hydrophilic balance and the stereostructure, and the in situ copolymerization of **2** and **3** occurs rapidly.

The polymerization rate of **1/2/3** in the liposome state was about 3 times larger than that of **3** itself in the liposome state, although the former accompanied the reduction of the iron(III) derivative of **1** or a termination reaction by capturing a propagation radical. It is conjectured that the lipid–heme acts as a photosensitizer for the UV polymerization and accelerates further the polymerization although its reductive reaction retards the polymerization. The polymerized liposome/lipid–heme was concentrated up to ca. 30 wt % solution by an ultrafiltration method.

Structure of Polymerized Liposome/Lipid–Heme. Acetolysis of the copolymerized **2/3** liposome was carried out to improve solubility of the copolymer in organic solvents and to analyze composition and molecular weight of the copolymer directly. The expected structure of acetolysis compound **4** was supported by IR, ^1H NMR, and



4

^{13}C NMR (for details see the Experimental Section). The elemental analysis indicated that the molar ratio of the **2/3** residue in the copolymer agreed with the molar ratio **2/3** in the feed and that **2** and **3** copolymerized completely in a liposome state.

The acetolysis copolymers were soluble in chloroform and tetrahydrofuran, and their molecular weight could be determined by GPC (Table I). The reference datum for the **3** homopolymer prepared under the same conditions is also listed in Table I. Although **2** does not influence the degree of polymerization, **3** reduces the degree of polymerization about one-third of that without **1**. This indicates that **1** retards the (co)polymerization of **1** (and **2**) through radical capturing by the iron(III) of **1**, as has been mentioned above in the discussion on the polymerization rate with and without **1**.

Table II
Oxygen Volume Dissolved in Various Media at 37 °C under Oxygen Atmosphere

media	heme concn, mM	oxygen volume (mL of O_2/100 mL of soln) volumetry	tracer method
polymerized 2/3 liposome/lipid–heme	5.0	12	
	10	22	26
	15	33	
polymerized 3 liposome/LIm[a]/lipid–heme	5.0	13	
polymerized 2/3 liposome		2.2	
water		2.1	2.2
human blood	9.2	23	22

[a] LIm, 1-laurylimidazole

Incorporation of **1** in the polymerized **2/3** liposome was first confirmed by ultracentrifugation; the supernatant did not contain **1–3**. This indicates that **1** is included in the liposome. The solution was also checked by gel permeation chromatography (GPC) monitored by the absorption at 300 and 415 nm based on **3** and **1**, respectively. The elution curves coincided with each other, which means that **1** is included in the polymerized **2/3** liposome. Transmission electron micrography of the polymerized liposome/lipid–heme indicated that it is prepared as a single-walled liposome with the diameter of ca. 400 Å. The average particle size of the polymerized **1/2/3** liposome was also measured by a dynamic light scattering method; the average diameter determined by a laser particle analyzer was 380 ± 20 Å based on the light-scattering intensities.

ESR spectrum of the **1** labeled with nitrogen oxide embedded in the **2/3** liposome was similar to that of the labeled **1** in a homogeneous solution and did no change before and after the polymerization. Fluorescence intensity of the zinc derivative of **1** at 666 nm was enhanced in the polymerized **2/3** liposome in comparison with those in alcohol solutions. These results suggest that **1** is molecularly dispersed and well solubilized in a hydrophobic region of the bilayer of the polymerized **2/3** liposome.

Oxygen-Binding Ability of Polymerized Liposome/Lipid–Heme. The deoxy solution (λ_{max} = 429, 535, and 562 (sh) nm) of the polymerized **2/3** liposome/lipid–heme was changed to its oxygen adduct solution on exposure to oxygen (λ_{max} = 422 and 544 nm). The oxygen adduct formation was rapid and reversible, and the oxy–deoxy cycle could be repeated more than 10^6 times under the physiological conditions, even at remarkably high concentration of the polymerized liposome/lipid–heme such as 20 wt %. The life time (half-life time) for the reversible oxygen adduct formation was ca. 1 day under the physiological conditions.

Volume of the oxygen gas bound to the polymerized liposome/lipid–heme was measured volumetrically and listed in Table II. A 100 mL solution of the polymerized liposome/lipid–heme (lipid–heme concentration 10 mM) uptakes 22 mL of oxygen, which is corresponding to ca. 80% of the saturated value and comparable with that of blood. The heme-free polymerized liposome solution (100 mL) physically uptakes 2.2 mL of oxygen; i.e. the oxygen-transporting capability of the polymerized lipo-

Table III
Oxygen- and CO-Binding and Oxygen- and CO-Dissociation Equilibrium Affinities and Thermodynamic Parameters of the Polymerized Liposome/Lipid-Heme in pH 7 Aqueous Solution at 37 °C

	O_2			CO			
heme	p_{50}, mmHg	ΔH, kcal/mol	ΔS, eu	p_{50}, mmHg	ΔH, kcal/mol	ΔS, eu	ref
polymerized 2/3 liposome/lipid-heme	43	−13	−39	0.026	−16	−30	this work
polymerized 3 liposome/LIm[d]/lipid-heme	40	−13	−38	0.018	−16	−32	this work
red blood cell suspension	27	−14	−42	0.10	−17	−38	this work[a]
stripped hemoglobin	0.9–13.4	−13		0.035	−17		*a–c*
myoglobin	0.6–2.7	−13, −21	−32, −37	0.018–0.034			*a*

[a] Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*; North-Holland Publishing: Amsterdam, 1970. [b] Gaud, H. T.; Barisas, Gill, S. *J. Biochem. Biophys. Res. Commun.* **1974**, *59*, 1389. [c] Wang, M. R.; Hoffman, B. M.; Shire, S. J.; Gurd, F. R. N. *J. Am. Chem. Soc.* **1979**, *101*, 7394. [d] LIm, 1-laurylimidazole.

Table IV
Oxygen- and CO-Binding and Oxygen- and CO-Dissociation Rate Parameters of the Polymerized Liposome/Lipid-Heme in pH 7 Aqueous Solution at 37 °C

	O_2			CO			
heme	$10^{-4}k_{on}$, M^{-1} s^{-1}	k_{off}, s^{-1}	$10^{-4}K$, M^{-1}	$10^{-4}k_{on}$, M^{-1} s^{-1}	k_{off} s^{-1}	$10^{-4}K$, M^{-1}	ref
polymerized 2/3 liposome/lipid-heme	2.1	0.7	1.6	1.0			this work
polymerized 3 liposome/LIm[d]/lipid-heme	2.3	0.74	1.7	1.1			this work
red blood cell suspension	1.1–4.7	0.16	6.8	1.1–3.7	0.01	1.0	this work[a]
stripped hemoglobin	290–3300	12–180	0.47–490	10–460	0.009–0.09	110–6700	*a–c*
myoglobin	110–190	10–70	22–130	30–50	0.017–0.04	250–750	*a*

[a] Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*, North-Holland Publishing: Amsterdam, 1970. [b] Gibson, Q. H. *J. Biol. Chem.* **1970**, *245*, 3285. [c] Sharma, V. S.; Schmidt, M. R.; Ranney, H. M. *J. Biol. Chem.* **1976**, *251*, 4267. [d] LIm, 1-laurylimidazole.

some/lipid-heme solution is about 10 times that of the physical one. The polymerized liposome/lipid-heme (heme concentration 15 mM) uptakes 33 mL of oxygen/100 mL of solution, which is superior to that of blood.

An ^{15}O radiotracer method was applied to the precise determination of chemically bound oxygen to the polymerized liposome/lipid-heme. The ^{15}O–O gas produced with a cyclotron was passed through the polymerized liposome/lipid-heme solution (see the Experimental Section), and the volume of oxygen bound to the heme was evaluated with the annihilation radiation intensity within ca. 10% error of the value given above (listed also in Table II).

An oxygen-binding and -dissociation equilibrium curve (Figure 1) shows that the polymerized liposome/lipid-heme binds oxygen in response to the oxygen pressure. The oxygen-binding affinity ($p_{50}(O_2)$: oxygen pressure at half oxygen binding for the heme) of the polymerized liposome/lipid-heme was determined by this equilibrium curve and listed in Table III. The $p_{50}(O_2)$ values of the polymerized liposome/lipid-hemes are ca. 50 mmHg at 37 °C and are close to that of hemoglobin in human blood but are significantly different from that of myoglobin. This suggests that the polymerized liposome/lipid-heme has a potential to act as an oxygen carrier under physiological conditions, which transports oxygen from lungs (oxygen pressure ($p(O_2)$; ca. 110 mmHg) to myoglobin in tissue ($p(O_2)$; ca. 40 mmHg), as hemoglobin does.

Table III also shows thermodynamic parameters for the oxygen-binding. Enthalpy and entropy change for the oxygen-binding to the polymerized liposome/lipid-heme are comparable to those of hemoglobin and myoglobin. This result indicates that the oxygen-binding to the polymerized liposome/lipid-heme proceeds in the same way as the binding to hemoglobin and myoglobin. Carbon monoxide binding affinity and its thermodynamic parameters also corresponds to those of hemoglobin in human blood.

Kinetics of the oxygen-binding to the polymerized liposome/lipid-heme were studied by flash photolysis and

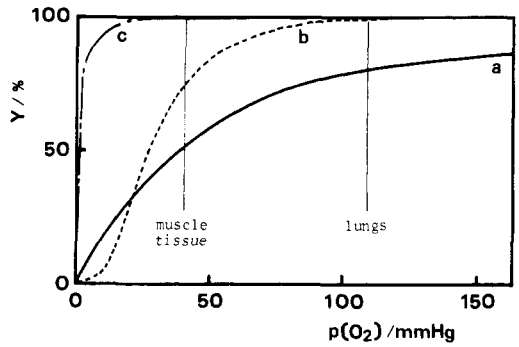


Figure 1. Oxygen-binding and -dissociation equilibrium curve of the polymerized liposome/lipid-heme in pH 7 at 37 °C: (a) polymerized liposome/lipid-heme, (b) hemoglobin in blood, (c) myoglobin.

stopped flow spectroscopies. Oxygen-binding and -dissociation of the polymerized liposome/lipid-heme occurred rapidly and were completed within 1 ms; they are rapid enough to act as an oxygen transporter. Oxygen- and CO-binding and -dissociation rate constants ($k_{on}(O_2)$, $k_{off}(O_2)$, $k_{on}(CO)$, and $k_{off}(CO)$) are summarized in Table IV with references. The $k_{on}(O_2)$ and $k_{on}(CO)$ values of the polymerized liposome/lipid-heme are similar to those of red blood cell suspension and smaller than those of stripped hemoglobin and myoglobin.

Solution Properties of the Polymerized Liposome/Lipid-Heme. Solution properties of the polymerized liposome/lipid-heme were comparable with those of human blood as summarized in Table V. The solution possesses satisfactory rheological properties, osmotic pressure, and colloidal osmotic pressure (in the presence of a small amount of dextran) compatible with physiological needs.

The solution of the polymerized liposome/lipid-heme was stable and could be stocked for 1 year without precipitation and change of particle size, i.e. without aggregation and fusion of liposome at ambient temperature. Long-term storage stability is achieved by the combination

Table V
Solution Properties of the Polymerized Liposome/Lipid-Heme

solution	specific gravity	viscosity, cP	osmotic pressure, m Osm	colloidal osmotic pressure, cmH_2O
polymerized 2/3 liposome/lipid-heme[a]	1.010	3.2–3.6	285–300	43.0[b]
polymerized 3 liposome/LIm*/lipid-heme[a]	1.012	3.1–3.5	285–305	42.5[b]
human blood	1.050–1.063	4.5–5.0	280–290	42.0

[a] Content 20 wt/vol % in lipid, physiological salt solution. [b] 3 wt/vol % dextran was added. Colloidal osmotic pressure for the solution of the polymerized liposome/lipid-heme was 15 cmHg and for the 1/3 (v/v) mixture of the polymerized liposome/lipid-heme and human blood was 41.3 mmHg.

of the lipid-heme with the polymerized liposome. The polymerized liposome guarantees not only the oxygen-transporting ability of the lipid-heme but also a highly concentrated, reproducible, and easily stored heme suspension.

Experimental Section

Materials. Ethyl 15-(1′-imidazolyl)pentadeca-*trans*-2,*trans*-4-dienoate (**5**) was synthesized as follows.

Imidazole (50 mmol, 3.4 g) was refluxed in tetrahydrofuran containing sodium hydride (50 mmol, 1.2 g) under a nitrogen atmosphere and added dropwise to a tetrahydrofuran solution of 11-bromoundecanoic acid methyl ester (50 mmol, 14 g). This mixture was refluxed for 6 h before being poured into ice–water. The aqueous mixture was extracted with ether. After drying over sodium sulfate, the mixture was evaporated and separated on a column chromatography (silica gel, chloroform/methanol, 20/1 (v/v)) to give a yellowish oil. The oily crude product was dissolved in ether and crystallized at −78 °C to give 11-(1′-imidazolyl)undecanoic acid methyl ester (10 g, 75%; mp 35–36 °C).

Dichloromethane solution of the ester (36 mmol, 9.6 g) was added dropwise to toluene solution containing diisobutylaluminum hydride (49 mmol) at −78 °C under a nitrogen atmosphere. Methanol/water (5/1 (v/v)) was added to the mixture and stirred at room temperature before byproducts were filtered. After the filtrate was dried over sodium sulfate, the mixture was evaporated and separated by column chromatography (silica gel, chloroform/methanol, 95/5 (v/v)) to give 11-(1′-imidazolyl)undecanal (3 g, 35%, m/e = 235 $(M - 1)^+$): IR (NaCl) ν_{CHO} = 1720 cm^{-1}; ^1H NMR (in $CDCl_3$, TMS standard) δ_H 9.76 (1 H, t, $-CHO$).

Triethyl 4-phosphonocrotonate (13 mmol, 3.2 g) was added to tetrahydrofuran solution of sodium hydride (13 mmol, 0.3 g) at 0 °C. To this solution was added tetrahydrofuran solution of 11-(1′-imidazolyl)undecanal (13 mmol, 3 g) at 0 °C. The mixture was stirred at room temperature and diluted with water to be extracted with ether. After drying over sodium sulfate, the residue was purified on a column chromatography (silica gel, chloroform/ethanol, 95/5 (v/v)) to give ethyl 15-(1′-imidazolyl)pentadeca-*trans*-2,*trans*-4-dienoate (**5**; 2.4 g, 54%, m/e = 332 (M^+)): IR (NaCl) $\nu_{C=O}$ = 1700 (ester), $\nu_{C=C}$ = 1635, 1610 (diene), and ν_{CO} = 1250 (ester) cm^{-1}; ^1H NMR (in $CDCl_3$, TMS standard) δ_H 1.27 (14 H, br s, $-CH_2-$), 1.28 (3 H, t, $-CH_2CH_3$), 1.70 (2 H, p, $-CH_2-$), 2.20 (2 H, m, $-CH_2-$), 3.90 (2 H, t, $>NCH_2-$), 4.20 (2 H, tetra (te), $-CH_2CH_3$), 5.70 (1 H, d, $-CH{=}CHCOO-$), 5.90–6.40 (2 H, m, $-CH{=}CHCH_2-$), 7.00–7.40 (1 H, m, $-CH{=}CHCOO-$), 6.90, 7.00, 7.40 (3 H, s (each), imidazole H). Elemental analysis. Calcd for $C_{20}H_{32}N_2O_2$: C, 72.0; H, 9.8; N, 8.2. Found: C, 72.3; H, 9.7; N, 8.4.

5 was hydrolyzed with aqueous sodium hydroxide solution to yield **2**.

Lipid-heme (**1**) was synthesized as in the literature.[7] 1,2-Bis(octadeca-2′,4′-dienoyl)-*sn*-glycero-3-phosphocholine (**3**) was synthesized according to the procedure in the literature.[8]

Preparation of Polymerized Liposome/Lipid-Heme. The polymerized liposome/lipid-heme was prepared by modifying the normal methods[9,10] of liposome preparation and photopolymerization.[6,11] **1** and **2** (molar ratio 1/3) were dissolved in methanol (0.5 mL) to be added to benzene solution (5 mL) of **3** (1/3 = 1/50 (molar ratio)). The solution was freeze-dried to give a brownish powder. The powder was added to oxygen-free and pH 7 isotonic buffered saline,[14] and the mixture was then shaken by a Vortex mixer. It was ultrasonicated and homogenized in an ice–water bath under a nitrogen atmosphere. The prepared liposome/lipid-heme solution was incubated at room temperature for a few hours in a quartz cell (path length 10 mm) and then was allowed under nitrogen atmosphere to polymerize under ultraviolet irradiation for 2 h at 50 °C with a low-vacuum UV lamp (Riko Kagaku Sangyou Co., Chiba, Japan, UVL-32 type; distance between the cell and the lamp was kept at 3 cm) to give the polymerized liposome/lipid-heme.

The reduction to the deoxy lipid-heme spontaneously occurred during the polymerization. Complete reduction was confirmed by UV/vis spectra (λ_{max} = 429, 535, and 562 (sh) nm). The polymerized liposome/lipid-heme solution was concentrated by ultrafiltration (Millipore Corp., MA, U.S.A., Pericon Labocassette, XX42-OLC-KO; a filter with excluded molecular weight 10000).

The polymerized liposome/lipid-heme was derived to the organic solvents soluble compound (**4**) through acetolysis to determine its molecular weight, as follows. The freeze-dried sample (200 mg) was dissolved in a mixture of acetic anhydride (2 mL) and acetic acid (3 mL) and heated at 150 °C in a sealed tube for 12 h. The precipitate was collected, washed with water and absolute methanol, and dried in vacuo. (IR (KBr) $\nu_{C=O}$ = 1730 (ester) cm^{-1}; IR absorptions attributed to phosphocholine residue (3400, 1250, 1060, 970 cm^{-1}) completely disappeared.) The molecular weight was estimated by a GPC column (Toyo Soda, TSK GMH_{XL}, eluent; tetrahydrofuran) with polystyrene standards.

Physicochemical Measurements. The GPC of the polymerized liposome/lipid-heme solution was measured with Sepharose 4B column (Pharmacia Fine Chemical, water). The transmission electron micrography (Hitachi H-500) was carried out by the negative staining method using uranyl acetate. The particle size was measured by a dynamic light-scattering method (Coulter Electronics, nanosizer N4D). The ESR spectrum was measured at −196 °C, using a JEOL FE-3X spectrophotometer, on the NO-labeled lipid-heme prepared by bubbling nitrogen oxide gas through the polymerized liposome/lipid-heme solution. The fluorescence spectra were measured with a fluorescence spectrophotometer (Jasco FP-550).

Volumetrical Measurement of Dissolved Oxygen. When the van Slyke method[12] was modified, oxygen volume bound to the polymerized liposome/lipid-heme was measured. The apparatus was equipped with a tonometer having three cells (main, subinner, and subouter cells), a manometer and a mercury tank (100 mL). The main mercury tank was first filled with mercury, and the oxypolymerized liposome/lipid-heme solution (5 mL) and the $K_3Fe(CN)_6$ solution (1 mL) were added in the main cell and the subouter cell, respectively. After being kept in a closed system, mercury in the main cell was moved to mix the $K_3Fe(CN)_6$ solution with the heme solution to measure pressure (p_1) in the tonometer. A sodium hydroxide solution (carbon dioxide absorbing reagent) was added in the subouter cell. By moving mercury in the main cell, the mixed solution was added to the sodium hydroxide solution to determine pressure in the tonometer (p_2). Then a sodium dithionite solution (oxygen-absorbing reagent) was set in the subouter cell. The mixed solution was moved to the sodium dithionite solution to measure pressure in the tonometer (p_3). The oxygen volume (v) bound to the polymerized liposome/lipid-heme was calculated according to the equation $v = A(p_3 - p_2)\rho_{Hg}(273/(273 + T)$ [A, constant of apparatus; ρ_{Hg}, specific gravity of mercury; T, temperature (°C)].

The volumetry using ^{15}O radiotracer (radioactive ^{15}O–O with a half-life ca. 120 s) was carried out as in the previous paper.[13]

Measurements of Oxygen-Binding Equilibrium and Kinetics. UV/vis spectrum and oxygen concentration of the polymerized liposome/lipid-heme solution were measured at the same time to draw the oxygen-binding and -dissociation equi-

librium curve as in the previous paper.[3] Kinetic parameters for the oxygen-binding was determined with stopped-flow and flash-photolysis spectrophotometers (Unisoku Co.) equipped with a kinetic data processor.

Solution Property Measurements. The viscosity was measured with an Ubellohde-type viscometer (JIS standard 0.03 and 0.005) at 37 °C. Osmotic pressure and colloidal osmotic pressure were measured with a membrane-type osmotic pressure apparatus (Knauer).

Acknowledgment. This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Area of Macromolecular Complexes (No. 62612005 and 63612005) from the Ministry of Education, Science and Culture, Japan.

Registry No. 1, 92786-54-4; 2, 119480-89-6; (2)(3) copolymer, 119503-05-8; 3, 95721-44-1; 4, 119528-78-8; 5, 116462-06-7; imidazole, 288-32-4; 11-bromoundecanoic acid methyl ester, 6287-90-7; 11-(1′-imidazolyl)undecanoic acid methyl ester, 72338-54-6; 11-(1′-imidazolyl)undecanal, 116679-84-6; triethyl 4-phosphomocrotonate, 10236-14-3; oxygen, 7782-44-7.

References and Notes

(1) *Chem. Eng. News*, 1985, *62* (Jan 14), p. 42.
(2) Tsuchida, E.; Nishide, H.; Yuasa, M.; Hasegawa, E.; Matsushita, Y. *J. Chem. Soc., Dalton Trans.* 1984, 1147.
(3) Tsuchida, E.; Nishide, H.; Yuasa, M.; Sekine, M. *Bull. Chem. Soc. Jpn.* 1984, *57*, 776.
(4) Tsuchida, E.; Yuasa, M.; Nishide, H. *J. Chem. Soc., Dalton Trans.* 1985, 65.
(5) Tsuchida, E.; Nishide, H.; Yuasa, M.; Hasegawa, E.; Matsushita, Y.; Eshima, K. *J. Chem. Soc., Dalton Trans.* 1985, 275.
(6) Tsuchida, E.; Hasegawa, E.; Matsushita, Y.; Eshima, K.; Yuasa, M.; Nishide, H. *Chem. Lett.* 1985, 969.
(7) Matsushita, Y.; Hasegawa, E.; Eshima, K.; Tsuchida, E. *Chem. Lett.* 1983, 1387.
(8) (a) Hupfer, B.; Ringsdorf, H.; Schupp, H. *Makromol. Chem.* 1981, *182*, 247. (b) Ohno, H.; Ogata, Y.; Tsuchida, E. *Macromolecules* 1987, *20*, 929.
(9) Papahajopoulos, D.; Watkins, J. C. *Biochim. Biophys. Acta* 1967, *135*, 639.
(10) Gregoriadis, G. *Liposome Technology*; CRC Press: Boca Raton, FL, 1984; Vol. 1.
(11) (a) Tundo, P.; Kippenberger, D. J.; Klahn, P. L.; Prieto, N. E.; Jao, T. C.; Fendler, J. H. *J. Am. Chem. Soc.* 1982, *104*, 456. (b) Reed, W.; Guterman, L.; Tundo, P.; Fendler, J. H. *Ibid.* 1984, *106*, 1897.
(12) van Slyke, D. D. *J. Biol. Chem.* 1924, *61*, 523, and references therein.
(13) Yuasa, M.; Ogata, Y.; Nishide, H.; Tsuchida, E.; Iwamoto, M.; Nozaki, T. *Chem. Lett.* 1984, 1889.
(14) Hasegawa, E.; Eshima, K.; Matsushita, Y.; Nishide, H.; Tsuchida, E. *Biochim. Biophys. Acta* 1986, *862*, 235.

Sequencing Bacterial Poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) by Partial Methanolysis, High-Performance Liquid Chromatography Fractionation, and Fast Atom Bombardment Mass Spectrometry Analysis

Alberto Ballistreri,† Domenico Garozzo,‡ Mario Giuffrida,‡ Giuseppe Impallomeni,‡ and Giorgio Montaudo*,†

Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy, and Istituto per la Chimica e la Tecnologia dei Materiali Polimerici, Consiglio Nazionale delle Ricerche, Viale A. Doria 6, 95125 Catania, Italy. *Received July 7, 1988*

ABSTRACT: The partial methanolysis of poly(β-hydroxybutyrate) (PHB) and of poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) (P(HB-Co-HV)) has been performed, and reaction kinetics has been optimized in order to produce oligomers with molecular masses below the detection limit (about 2000 daltons) of the mass spectrometer used for the subsequent analysis. The fractionation of the methanolysis products was achieved by HPLC, and the fractions collected were analyzed by fast atom bombardment mass spectrometry (FAB-MS), in the presence of NaCl. FAB spectra of oligomers from PHB and P(HB-Co-HV) consist only of pseudomolecular ions MH^+ and MNa^+, allowing for the identification of the methanolysis products, the estimate of the copolymer composition, and the determination of the sequence distribution of monomeric units.

Introduction

The structural characterization of poly(β-hydroxybutyrate) (PHB) and poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) (P(HB-Co-HV)) has attracted much attention recently[1-3] because these materials are important members of a family of poly(β-hydroxyalkanoates) of microbial origin, which are potential sources of thermoplastic materials.[1-5]

Since the mechanical, physical, and processing properties of the P(HB-Co-HV) copolymers vary systematically with composition,[1,2] it is important to have accurate estimates of this parameter.

The composition of P(HB-Co-HV) copolyester samples can be determined by ^1H NMR spectra,[1-3] and the sequence distribution of monomeric units in P(HB-Co-HV) has been deduced analyzing the diad and triad sequences from their 125-MHz ^{13}C NMR spectra.[2,3]

However, since NMR can hardly discern beyond the triad level, the characterization of more complex sequence arrangements (such as partial blocks or higher order regularities) cannot be easily achieved without using complementary techniques.

Mass spectrometry is able to look at the mass of individual molecules in a mixture, and it is therefore an interesting alternative to NMR. Recently,[6] a pyrolysis MS method was used to obtain the sequence of P(HB-Co-HV).

The recent development of fast atom bombardment mass spectrometry (FAB-MS) has shown that a such technique is of considerable importance for the structural characterization of biopolymers. In the case of proteins, for instance, it allows us to perform direct analysis of the mixtures of peptides produced by the enzymatic degra-

† Università di Catania.
‡ Istituto per la Chimica e la Tecnologia dei Materiali Polimerici.

0024-9297/89/2222-2107$01.50/0