frequencies of the carbonyl complex [ν(Fe–CO) and ν(CO)] provide crucial information about the Fe–trans ligand bond.[20,21] The high-frequency region of the RR spectra of rHSA1–Fe^{2+}PP(CO) and rHSA1(H146R/K190R)–Fe^{2+}PP(CO) both exhibited an intense peak at 1373 cm^{-1} (λ_{ex}: 413.1 nm), which indicates a deformed pyrrole-ring breathing-like mode (ν_4) and corresponds well to the value of the 6-coordinate low-spin carbonyl complex.[21a] However, while the low-frequency RR spectra of rHSA1–Fe^{2+}PP(CO) exhibited two ν(Fe–CO) bands at 493 and 525 cm^{-1}, rHSA1(H146R/K190R)–Fe^{2+}PP (CO) showed only a single ν(Fe–CO) band at 493 cm^{-1} (Fig. 5).[22] Since it is known that the weaker the Fe-trans ligand coordination, the stronger the Fe–CO bond in the carbonyl complex,[20] we assigned the higher 525 cm^{-1} band of rHSA1–Fe^{2+}PP(CO) to the low CO binding affinity conformer. The 493 cm^{-1} band was therefore assigned to the high affinity conformer, in which the proximal His-185 coordinates to the Fe^{2+}PP without unfavourable strain.

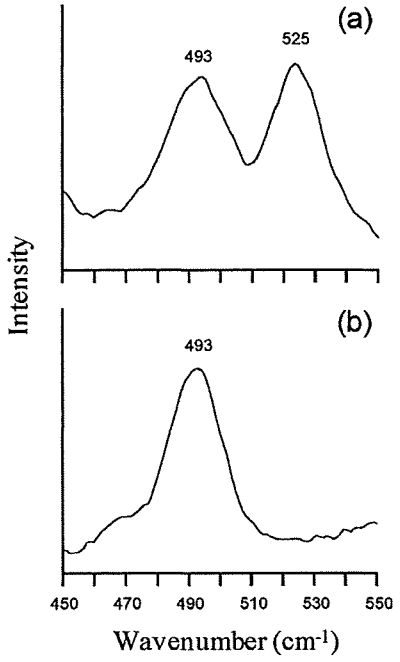

Fig. 5 Resonance Raman spectra of (a) rHSA1–Fe^{2+}PP(CO) and (b) rHSA1(H146R/K190R)–Fe^{2+}PP(CO) in 50 mM potassium phosphate buffered solution (pH 7.0) at 22 °C.

Regarding IR spectra, the ν(CO) vibration appeared at 1963 cm^{-1} for rHSA1–Fe^{2+}PP(CO) and at 1967 cm^{-1} for rHSA1(H146R/K190R)–Fe^{2+}PP(CO). Spiro *et al.* prepared a systematic plot of ν(Fe–CO) *versus* ν(CO) for a large number of carbonyl heme complexes and found a single inverse correlation when imidazole is the axial ligand.[21b,c] This is attributed to back donation of Fe^{2+} dπ electrons to the CO π* orbital. The relationship between ν(Fe–CO) and ν(CO) for rHSA1(H146R/K190R)–Fe^{2+}PP(CO) fits on the line for the imidazole complexes.[21b,c] On the other hand, the low O_2 binding component of rHSA1–Fe^{2+}PP(CO) showed a positive deviation from the line. This result again indicates a very weak electron donation from the proximal His-185 in the low O_2 binding conformer.

Conclusions

We prepared rHSA–Fe^{2+}PP complexes having a single O_2 and CO binding affinity by introducing Arg into the His-146 and/or Lys-190 positions. These artificial hemoproteins have a uniform Fe^{2+}PP orientation and His ligation (His-185 or His-142) geometry to the central ferrous ion without inclination. The key triad of the basic amino acid residues (Arg-114, His-146 and Lys-190) at the entrance of the heme pocket of HSA plays an important role in stabilizing the porphyrin molecule *via* salt-bridge formation and might also discriminate the two sides of the porphyrin ring. In mammals, His-146 is universally conserved, but Lys-190 is present only in primate albumin. The wild-type HSA statistically accommodates the hemin in alternative orientations; the discrimination of the porphyrin plane by serum albumin might be unnecessary for the evolution process. But the engineering of an rHSA–Fe^{2+}PP complex with a single O_2 binding affinity is potentially of tremendous clinical importance for blood substitutes and O_2-transporting therapeutic reagents.

Experimental

Materials and apparatus

All materials were used as purchased without further purification. Iron(III) protoporphyrin IX chloride (Fe^{3+}PP) was purchased from Fluka. Iron(III) protoporphyrin IX dimethyl ester chloride (Fe^{3+}PPDM) was synthesized from protoporphyrin IX dimethyl ester (Sigma). Iron(III) 2,4-dimethyl-deuteroporphyrin chloride (Fe^{3+}DMDP) was synthesized from 2,4-dimethyl-deuteroporphyrin dimethyl ester (Frontier Scientific).[23] UV-vis absorption spectra were obtained on an Agilent 8453 UV-visible spectrophotometer equipped with an Agilent 89090A temperature control unit. Kinetic measurements for the O_2 and CO bindings were carried out on a Unisoku TSP-1000WK time-resolved spectrophotometer with a Spectron Laser Systems SL803G-10 Q-switched Nd:YAG laser, which generated a second-harmonic (532 nm) pulse of 6-ns duration (10 Hz). A 150 W xenon arc lamp was used as the probe light source. The gas mixture with the desired partial pressure of O_2/CO/N_2 was prepared by a Kofloc Gasblender GB-3C. Resonance Raman spectra of the carbonyl rHSA–Fe^{2+}PP complexes were obtained on a JASCO NRS-1000 spectrophotometer using a Kaiser Optical Holographic Notch-Plus filter and a liquid N_2-cooled CCD detector. The excitation source was a Coherent Innova 90C Kr^+ laser. Infrared spectra of the carbonyl rHSA–Fe^{2+}PP complex were obtained on a JASCO FT/IR-4200 spectrophotometer.

Preparation of rHSA

The designed rHSAs were prepared according to our previously reported techniques.[9b] The mutations (H146R and/or K190R) were introduced into the rHSA coding region in a plasmid vector encoding the double mutant [rHSA1 or rHSA2] by use of the Stratagene QuikChange mutagenesis kit. All mutations were confirmed by DNA sequencing. The plasmid was then digested by NotI and introduced into yeast (*Pichia pastoris* GS115) by electroporation. The expression protocols and media formulations were as previously described.[9b] The expressed proteins were harvested from the growth medium by precipitation with ammonium sulfate

and purified by a Cibacron Blue column of Blue Sepharose 6 Fast Flow (Amersham Pharmacia Biotech). After concentration using a Vivaspin 20 ultrafilter (10 kDa M_w cutoff), the samples were applied to a Superdex 75 column (Amersham Pharmacia Biotech) using 50 mM potassium phosphate as the running buffer. The purification steps were followed by SDS-PAGE analysis. The purified rHSA was lyophilized and stored in the freezer at −20 °C.

Preparation of rHSA–Fe^{2+}porphyrin

Typically 5 mL of 0.1 mM rHSA in 50 mM potassium phosphate (pH 7.0) was mixed with 0.8 mL of 0.688 mM Fe^{3+}PP in DMSO (Fe^{3+}PP : rHSA was molar ratio of 1:1) and incubated overnight with rotation in the dark at room temperature. The complex was then diluted with 50 mM potassium phosphate (*ca.* 15 mL) and concentrated to the initial volume (5.8 mL) using a Vivaspin 20 ultrafilter (10 kDa M_w cutoff). These dilution and concentration cycles were repeated to reduce the final concentration of DMSO to *ca.* <0.001 vol%. The rHSA–Fe^{2+}PPDM and rHSA–Fe^{2+}DMDP were also prepared in the same manner. The 50 mM phosphate buffered solution (pH 7.0) of rHSA–Fe^{3+}PP ([Fe^{3+}PP]: *ca.* 10 μM) in a 10 mm path length optical quartz cuvette sealed with a rubber septum was purged with N_2 for 30 min. A small excess amount of degassed aqueous sodium dithionite was added by microsyringe to the sample under an N_2 atmosphere to reduce the central ferric ion of the Fe^{3+}PP, generating the deoxy ferrous rHSA–Fe^{2+}PP.

Determination of O_2 and CO binding parameters

The O_2 and CO recombination with rHSA–Fe^{2+}PP after nanosecond laser flash photolysis of the dioxygenated or carbonyl complex occurs according to eqn (1) with the association rate constant (k_{on}^{L}) and dissociation rate constant (k_{off}^{L}) (where L: O_2 or CO).

$$Fe^{2+}PP\text{-}L \xrightarrow{h\nu} Fe^{2+}PP + L \underset{k_{off}^{L}}{\overset{k_{on}^{L}}{\rightleftharpoons}} Fe^{2+}PP\text{-}L \qquad (1)$$

$$[P_{1/2}{}^{L} = (K^{L})^{-1} = (k_{on}{}^{L}/k_{off}{}^{L})^{-1}]$$

The $k_{on}{}^{CO}$ was measured by following the absorption at 436 nm for Fe^{2+}PP(CO) or 411 nm for Fe^{2+}DMDP(CO) after laser pulse irradiation to the carbonyl complex at 22 °C. The $k_{on}{}^{O2}$ and O_2 binding equilibrium constant [$K^{O2} = (P_{1/2}{}^{O2})^{-1}$)] can be determined by a competitive rebinding technique by use of gas mixtures with different partial pressures of $O_2/CO/N_2$ at 22 °C. The relaxation curves that accompanied the O_2 or CO recombination were analyzed by single or double exponential profiles with Unisoku Spectroscopy & Kinetics software. The $k_{off}{}^{O2}$ was calculated from $k_{on}{}^{O2}/K^{O2}$. The $k_{off}{}^{CO}$ was measured by displacement with NO for the carbonyl complex at 22 °C. The time course of the UV-vis absorption change that accompanied the CO-dissociation was fitted to single or double exponential. The CO binding constants [$K^{CO} = (P_{1/2}{}^{CO})^{-1}$] were calculated from $k_{off}{}^{CO}/k_{on}{}^{CO}$.

Raman spectroscopy

Spectra of the carbonyl complexes of rHSA–Fe^{2+}PP ([Fe^{2+}PP]: 2–4 mM in 50 mM phosphate buffered solution (pH 7.0)) were collected using back-scattering geometry at an excitation wavelength of λ_{ex}: 413.1 nm. The laser power for the samples was 1.8 mW. Each spectrum was recorded with 20 s accumulation time at 22 °C, and ten repetitively measured spectra were averaged to improve the signal to noise ratio. Peak frequencies were calibrated relative to indene and CCl_4 as a standard and were accurate to 1 cm^{-1}.

IR spectroscopy

The IR spectra of the carbonyl complexes of rHSA–Fe^{2+}PP ([Fe^{2+}PP]: 2–4 mM in 50 mM phosphate buffered solution (pH 7.0)) were obtained in CaF_2 cells (JASCO, path length: 0.025 mm). The cell containing water was used for the reference. The spectrum was accumulated 64 times to improve its signal-to-noise ratio.

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CHAPTER NINETEEN

HEMOGLOBIN-VESICLES AS AN ARTIFICIAL OXYGEN CARRIER

Hiromi Sakai, Keitaro Sou, *and* Eishun Tsuchida

Contents

Abstract

Hemoglobin-vesicles (HbV) or liposome-encapsulated hemoglobin (LEH) are artificial oxygen carriers that mimic the cellular structure of RBCs. In contrast to other liposomal products containing antifungal or anticancer drugs, one injection of HbV in place of a blood transfusion is estimated as equivalent to a massive dose, such as several hundred milliliters or a few liters of normal blood contents. The fluid must therefore contain a sufficient amount of Hb, the binding site of oxygen, to carry oxygen like blood. Encapsulation of Hb can shield various toxic effects of molecular Hbs. On the other hand, the liposomal structure, surface property, and the balance between the stability for storage and blood circulation and instability for the prompt degradation in the reticulo-endothelial system must be considered to establish an optimal transfusion alternative.

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1. Introduction: Encapsulated Hemoglobin as an Artificial Oxygen Carrier

Hemoglobin (Hb) (MW 64,500) is the most abundant protein in blood. It contains four subunits with a heme on each, where oxygen binds and dissociates reversibly. About two million Hb molecules are encapsulated in one red blood cell (RBC). The intracellular Hb concentration is as high as 35 g/dL, which makes the Hb concentration of blood as high as 12–15 g/dL. After the discovery of blood type antigens on the outer surface of RBCs by Landsteiner in 1901, the idea was born to use purified Hb as an oxygen-carrying fluid that is free of any blood type. Nevertheless, it was unsuccessful because of various toxic effects. In spite of its abundance in blood, Hb becomes toxic when it is released from RBCs. Dissociation of tetramer Hb subunits into two dimers, $\alpha_2\beta_2 \rightarrow 2\alpha\beta$, induces renal toxicity. Entrapment of gaseous messenger molecules (NO and CO) induces vasoconstriction, hypertension, reduction of blood flow and tissue oxygenation at microcirculatory levels (Sakai *et al.*, 2000c, 2008a,b), neurological disturbances, and malfunction of esophageal motor functions (Murray *et al.*, 1995). Some chemically modified Hb solutions (cell-free Hb-based oxygen carriers) are now confronting difficulties in clinical trials (Chang, 2004; Natanson *et al.*, 2008). These side effects of molecular Hb imply the importance of the cellular structure.

Hb encapsulation was first performed by Chang in the 1950s (Chang, 1991), using a polymer membrane. Some Japanese groups also tested Hb encapsulation with gelatin, gum Arabic, silicone, etc. Nevertheless, it was extremely difficult to regulate the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne (1964) reported that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller (1977) prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, etc. Since then, many groups have tested encapsulated Hbs using liposomes (Djordjevich and Ivankovich, 1988; Gaber *et al.*, 1983; Hayward *et al.*, 1985; Hunt *et al.*, 1985; Jopski *et al.*, 1989; Kato *et al.*, 1984; Liu and Yonetani, 1994) (Table 19.1). Some failed initially, and some are progressing with the aim of clinical usage. The Naval Research Laboratory presented remarkable progress of LEH (Rudolph *et al.*, 1991), but it suspended development about 10 years ago. What we call Hb-vesicles (HbV) with high-efficiency production processes and improved properties have been established by our group, based on nanotechnologies of molecular assembly and precise analyses of pharmacological and physiological aspects (Tsuchida, 1998).

Table 19.1 Trials of liposome encapsulated Hb

References	Lipid composition	Characteristic preparation methods
Djordjevich and Ivankovich (1988)	L-α-Phosphatidylcholine/ cholesterol/palmitic acid	Sonication
Gaber *et al.* (1983), Farmer and Gaber (1987)	EYPC/cholesterol/bovine brain phosphatidylserine; DMPC/cholesterol/ dicetylphosphate	Extrusion
Hunt *et al.* (1985)	EYPC/cholesterol/DPPA/ α-tocopherol	Reverse phase evaporation and extrusion
Beissinger *et al.* (1986)	HSPC/cholesterol/ dicetylphosphate or DMPG	Microfluidizer
Rabinovici *et al.* (1993), Rudolph *et al.* (1988)	HSPC/cholesterol/DMPG/ α-tocopherol; trehalose is added to store LEH as a lyophilized powder	Bovine Hb; thin film hydration and emulsification
Jopski *et al.* (1989)	EYL/PS (EYPA)	Detergent dialysis
Sato *et al.* (1992), Sakai *et al.* (1992), Akama *et al.* (2000)	DODPC/cholesterol/ octadecadienoic acid; gamma-ray polymerization	HbCO, extrusion method
Hayward *et al.* (1985)	Diacetylene phospholipid/ cholesterol; UV-irradiation for polymerization	HbCO, sonication
Kato *et al.* (1984)	EYL/carboxymethyl chitin	Reverse phase evaporation
Yoshioka (1991), Takahashi (1995)	HSPC/cholesterol/myristic acid/α-tocopherol/DPPE-PEG	Microfluidizer
Mobed and Chang (1991)	HSPC/DMPG/ α-tocopherol/ carboxymethyl chitin	Reverse phase evaporation
Liu and Yonetani (1994)	EYL/cholesterol/ dicetylphosphate/ α-tocopherol	Freeze–thaw method
Phillips *et al.* (1999)	DSPC/cholesterl/PEG_{5000}-DSPE/a-tocopherol	α-Cross-linked human Hb; microfluidizer
Sakai *et al.* (1996), Takeoka *et al.* (1996)	DPPC/cholesterol/DPPG or palmitic acid	HbCO, extrusion

(continued)

Table 19.1 *(continued)*

References	Lipid composition	Characteristic preparation methods
Sakai *et al.* (1997)	DPPC/cholesterol/DPPG/ DSPE-PEG_{5000}	HbCO, extrusion
Sou *et al.* (2003), Sakai *et al.* (2002a)	DPPC/cholesterol/DHSG/ DSPE-PEG_{5000}	HbCO, extrusion
Li *et al.* (2005)	DMPC/cholesterol/DMPG/ DSPE-PEG_{2000}/actin	Extrusion
Pape *et al.* (2008)	HSPC/cholesterol/stearic acid/DSPE-PEG_{5000}	Lipid paste rapid dispersion

DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; EYPC: egg yolk phosphatidylcholine; DPPA: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid; HSPC: hydrogenated soy phosphatidylcholine; DMPG: 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol; EYL: egg yolk lecithin; PS: phosphatidylserine; DODPC: 1,2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine; DPPE: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine; DSPE: 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate; HbCO: carbonylhemoglobin.

The major expected advantages of an artificial oxygen carrier are the absence of any pathogen, its stability for long-term storage, and its sufficient oxygen transporting capability. The important points of HbV or LEH to be considered are the following:

i. Source of Hb, virus inactivation, and virus removal procedure
ii. Improvement of encapsulation efficiency without Hb denaturation
iii. Sufficient stability for storage and blood circulation
iv. Blood compatibility and prompt metabolism in the reticuloendothelial system
v. Physicochemical properties comparable with those of blood.

The last point differs completely from other liposomal products. It is necessary to design not only a particle but also its suspension because we intend to prepare a substitute for blood, which has 12–15 g/dL hemoglobin, 40–50% hematocrit, and 5–7 g/dL plasma proteins, and unique rheological and osmotic properties.

2. Encapsulation of Concentrated Hb in Liposomes

The performance of LEH depends on the weight ratio of Hb to lipid ([Hb]/[Lipids]): to carry more Hb with fewer vehicles made of lipids. This value is improved by lowering the number of bilayer membranes

(lamellarity) of the vesicle, and raising the concentration of Hb in the interior of the vesicle ([Hb] < 40 g/dL). Table 19.1 shows that many groups add cholesterol and negatively charged lipids to phospholipids, which is plausible because cholesterol not only improves membrane stability but it also reduces the curvature for LUV. Addition of a small amount of negatively charged lipid increases the repulsive force between the lipid membranes and reduces the lamellarity in addition to controlling the zeta potential for blood compatibility (Sou and Tsuchida, 2008). Saturated phospholipids (e.g., HSPC, DSPC, and DPPC) are preferred to unsaturated lipids (e.g., EYL and soy phosphatidylcholines) because of the synergistic, facilitated oxidation of both unsaturated lipids and Hb and physical instability (Szebeni *et al.*, 1984, 1985), but cholesterol lowers such Hb denaturation to some degree.

To encapsulate Hb solution in liposomes, some groups have used detergent dialysis and reverse phase evaporation methods. However, it is difficult to guarantee the absence of a detergent and an organic solvent in the final product that would result in a massive dosage. A simple mixing of a concentrated Hb solution and lipids (as a powder, paste, or thin film on the wall of a flask) produces multilamellar vesicles with a wide particle size distribution, necessitating size regulation by a homogenizer (microfluidizer) (Beissinger *et al.*, 1986; Vidal-Naquet *et al.*, 1989; Vivier *et al.*, 1992), or an extrusion method, which would be more practical. In the case of a microfluidizer, the solution temperature increases by a strong shear force. Denaturation of the components and wide particle distribution must be prevented. Use of an extrusion method can avoid such difficulties; but conditions for efficient filter permeation must be established.

We studied optimal conditions for Hb encapsulation using the extrusion method, and considered the behavior of the Hb and lipid assemblies as a kind of polymer electrolyte (Takeoka *et al.*, 1993, 1994a,b, 1996). The maximum [Hb]/[Lipid] ratio is obtainable at ca. pH 7, which would relate to the isoelectric point (p*I*) of Hb. The Hb molecule is negatively charged when the pH is greater than 7.0, and the electrostatic repulsion between Hb and the negatively charged bilayer membrane results in lower encapsulation efficiency. Lower pH, however, is expected to enhance Hb denaturation through strong interaction with the lipid bilayer membrane and metHb formation. Therefore, a physiological pH, 7.0–7.4, would be optimal. In addition, the higher ionic strength shields repulsion between the negatively charged lipid bilayer membranes and thereby increases the lamellarity.

The lamellarity decreases concomitantly with increased microviscosity of the lipid bilayer membrane (decreased lipid mobility). Multilamellar vesicles are converted to smaller vesicles with a smaller lamellarity during the extrusion procedure. When the membrane fluidity is high, deformation of the vesicles during extrusion occurs more easily even for multilamellar vesicles, resulting in larger lamellarity in the final vesicles. Therefore, a

phospholipid with a higher phase transition temperature that typically forms a membrane with lower membrane fluidity is preferred. However, these lipids would make extrusion more difficult, because a higher shear rate (high extrusion pressure) is required. Based on this reasoning, mixed lipids contain 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) as the main component. The encapsulation efficiency of the Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method (Sakai *et al.*, 1996; Sou *et al.*, 2003). Mixed lipids—DPPC, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG), and DSPE-$PEG_{5,000}$ at a molar ratio of 5:5:1:0.033—are hydrated using a NaOH solution (7.6 m*M*) to obtain a polydispersed multilamellar vesicle dispersion (50 nm–30 μm diameter). The polydispersed vesicles are converted to smaller vesicles with average diameter of ca. 500 nm by repeating two cycles of freezing by liquid nitrogen and thawing at 40 °C, and finally the suspension is freeze-dried to obtain a lyophilized powder. It is then rehydrated into a concentrated Hb solution (40 g/dL), retaining the size and size distribution of the original vesicles. The resulting vesicle dispersion permeates smoothly through the membrane filters during extrusion (Extruder, Northern Lipids Inc.; Filters, Fuji Film Microfilter, Filter pore size: 3.0, 0.45, 0.3, and 0.22 μm). The average permeation rate of the frozen–thawed vesicles is ca. 30 times higher than that of the simple hydrated vesicles. During extrusion, the Hb solution is encapsulated into reconstructed vesicles with 250 ± 20 nm diameter. The [Hb]/[lipid] ratio reaches 1.7–1.8. This improvement of the Hb encapsulation procedure is a breakthrough for HbV scalability. The main physicochemical characteristics of HbV and their analytical methods are presented in Table 19.2.

3. Source of Hb and Its Purification

Hb is the most abundant protein in blood. It is easily obtained from human or animal blood by washing RBCs to remove plasma proteins, hemolysis by adding pure water, removal of the cell membrane components by ultrafiltration, and dialysis, or ion chromatography (DeLoach *et al.*, 1986; Palmer *et al.*, 2009; Sheffield *et al.*, 1987; Winslow and Chapman, 1994). The significantly high dosage as a transfusion alternative requires high purity, absence of pathogen (sterility assurance, virus-free), and the abundance of Hb as a starting material (Sakai *et al.*, 2004d). Possible sources of Hb would be (i) outdated human donated blood, (ii) bovine Hb, because blood is abundant in the cattle industry (Sakai *et al.*, 2002b), and (iii) recombinant Hb. Because of some patients' religious beliefs, human-derived or cow-derived Hb would be unacceptable. The absence of prion contamination must be verified in the case of bovine Hb. The influence of

Table 19.2 Physicochemical characteristics of Hb vesicles (HbV) developed at Waseda University

Parameter		Analytical method (references)
Particle diameter	250–280 nm	Light scattering, Beckman Coulter submicron particle analyzer (Sakai *et al.*, 1996; Sato *et al.*, 2009)
P_{50}	25–28 Torr (pH 7.4)	Hemox Analyer (37 °C, pH 7.4) (Sakai *et al.*, 2000a)
[Hb]	10 g/dL	A modified cyanomet Hb method
[heme] (iron)	6.2 m*M*	Atomic absorption, Inductively coupled plasma spectrometry
[metHb]	<3%	Soret band absorption (Sakai *et al.*, 1996)
[HbCO]	<2%	Soret band absorption (Sakai *et al.*, 1996)
[Lipids]	5.3–6.3 g/dL	Choline oxidase DAOS method, molybdenum blue method, cholesterol oxidase method (Sakai *et al.*, 1996; Sou *et al.*, 2003)
Occupied volume of particles	40%	Ultracentrifugation (50,000×*g*, 30 min) and supernatant volume measurement
Colloid osmotic pressure	20 Torr in 5% HSA	Colloid Osmometer; Wescor Model 4420 (Sakai *et al.*, 2000a)
Lipid composition	DPPC/cholesterol/ DHSG/DSPE-PEG_{5000}	(Sakai *et al.*, 2002a; Sou *et al.*, 2003)
ζ potential	−18.7	Zetasizer ([NaCl] = 20 m*M*, pH 7.4) (Sou and Tsuchida, 2008)
Viscosity	3.8 cP	Anton Parr Rheometer M301 (268 s^{-1}, 25 °C) (Sakai *et al.*, 2007)
Lamellarity	Nearly 1	Theoretical calculation (Takeoka *et al.*, 1996); small angled X-ray scattering (Sato *et al.*, 2009)
Sterility, [LPS]	<0.2 EU/mL	A modified limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. (Sakai *et al.*, 2004d)
Stability for storage at room temperature	>2 years, purged with N_2	Measurements of particle size distribution, turbidity, P_{50}, hemolysis, [metHb], lipid decomposition (Sakai *et al.*, 2000b)
Circulation half-life	35 h (rats)	Biodistribution of ^{99m}Tc-, ^{3}H-, and ^{125}I-labeled HbV (Sou *et al.*, 2005; Taguchi *et al.*, 2009a,b)

immunoglobulin G antibovine Hb has to be examined carefully (Hamilton and Kickler, 2007). A commercially available powdered Hb reagent for laboratory use is not recommended for *in vivo* use, because some molecules are already oxidized and a regeneration procedure would be required. Sterility (absence of lipopolysaccharide contamination) also presents a problem.

In Japan, the research and development of blood substitutes was initiated because of the beneficial use of outdated blood. The primary advantage of artificial O_2 carriers is expected to present no fear of infectious disease derived from human blood. The donated blood is inspected strictly by the nucleic acid amplification test (NAT) for possible detection of human immunodeficiency virus, hepatitis B virus, and hepatitis C virus. It is necessary, however, to introduce procedures to inactivate and remove viruses in the process of Hb purification from outdated RBCs to guarantee sterility, in light of the unforgettable tragedy of the HIV transmission caused by the distribution of nonpasteurized, plasma-derived products. In our purification process, virus inactivation was performed by pasteurization at 60 °C for 10 h, which is the same condition for the pasteurization of human serum albumin (HSA) (Fukutomi *et al.*, 2002; Sakai *et al.*, 1993). This process can be used because carbonylhemoglobin (HbCO) is stable under these conditions. Thermograms of HbCO indicate a denaturation temperature at 78 °C, which is much higher than that for oxyhemoglobin (64 °C) (Sakai *et al.*, 2002b).

The virus inactivation efficiency must be evaluated using spike tests (Abe *et al.*, 2001; Huang *et al.*, 2002). The Hb solution spiked with vesicular stomatitis virus (VSV) is treated at 60 °C for 1 h under a CO atmosphere. VSV is inactivated by >6.0 $\log_{10}$ without metHb formation and denaturation. Some protein bands other than Hb disappear on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing after heat treatment. During pasteurization, all other proteins are denatured and precipitated. Consequently, we obtain an ultrapure Hb solution. This high purity is crucial for preventing membrane plugging during subsequent ultrafiltration to remove viruses. The US FDA requires two orthogonal steps, not only of virus inactivation but also of virus removal.

We tested ultrafiltration of the HbCO solution to remove viruses using PLANOVA^{TM}-35N and -15N (P35N, P15N, Bemberg Microporous Membrane, BMM; Asahi Kasei Medical Co. Ltd.) (Naito *et al.*, 2002). The virus removal mechanism is size exclusion through the capillary pores, and by depth filtration. The unit membrane, which has a network structure of capillaries and voids, is accumulated to form 150 layers. P35N and P15N have respective mean pore sizes of 35 and 15 nm. P35N is suitable for removing lipid-enveloped viruses of 40–100 nm such as HIV and HCV. P15N is useful to remove naked capsid viruses smaller than

40 nm, such as parvoviruses. The permeation flux is sufficiently high because of the absence of denaturation of HbCO. Denatured and insoluble proteins cause plugging of filter pores. A high removal efficiency of a bacteriophage, $\phi \times 174$, (>7.7 log) was confirmed (Naito *et al.*, 2002). In addition, P15N is effective for the process of virus removal from the Hb solution. We also confirmed the effectiveness of other virus removal ultrafiltration systems such as Viresolve (Millipore Corp.).

The obtained purified HbCO solution can be concentrated to greater than 40 g/dL using a tangential flow ultrafiltration process (e.g., Millipore Biomax V screen, cut off Mw: 8 kDa). After regulation of the electrolyte concentrations, this is supplied for the encapsulation procedure. The ligand of the resulting HbV, CO, is photodissociated and converted to O_2 by illuminating the liquid membrane of the HbV suspension with visible light under flowing O_2 (Chung *et al.*, 1995). A recent new idea is intravenous injection of CO-bound Hb-based O_2 carriers. Actually, CO is dissociated unexpectedly rapidly in the bloodstream while showing some cytoprotective effects (Sakai *et al.*, 2009c; Vandegriff *et al.*, 2008).

An alternative purification method would be to preserve the well-organized, but unstable, enzymatic systems that are originally present in RBCs, aiming at the prolonged stability of the ferrous state of Hb (Ogata *et al.*, 1997). The enzymatic system can be preserved in part with the compensation of insufficient virus removal or inactivation, but this cannot guarantee the safety of the resulting Hb-based oxygen carriers. One advantage of HbV is that any reagent can be coencapsulated in the vesicles. It has been confirmed that coencapsulation of the appropriate amount of a reductant, such as glutathione or homocysteine, and active oxygen scavengers, such as catalase, retards metHb formation (Sakai *et al.*, 2000b, 2004c; Takeoka *et al.*, 1997, 2002; Teramura *et al.*, 2003). It is also interesting to coencapsulate L-tyrosine to establish an artificial catalase system by the combination of metHb (Atoji *et al.*, 2006). Our recent interpretation, however, is that metHb formation might not present a serious problem in an emergency situation, because HbV would be infused as an interim measure until blood transfusion in a clinical setting.

4. Regulation of Oxygen Affinity

The O_2 affinity of purified Hb (expressed as P_{50}, O_2 tension at which Hb is half-saturated with O_2) is about 14 Torr in phosphate buffered saline. Hb strongly binds O_2 and does not release O_2 at 40 Torr (partial pressure of mixed venous blood). Historically, it has been regarded that the O_2 affinity should be regulated similar to that of RBC—about 25–30 Torr—using an allosteric effector or by direct chemical modification of the Hb molecules.

Theoretically, this allows sufficient O_2 unloading during blood microcirculation, as might be evaluated according to the arteriovenous difference in the levels of O_2 saturation, in accordance to an O_2 equilibrium curve. The P_{50} of Hb in HbV is expected to be equivalent to that of human RBCs, that is, 28 Torr, or higher if this theory is correct. Pyridoxal 5′-phosphate (PLP) is coencapsulated in HbV as an allosteric effector to regulate P_{50} (Sakai *et al.*, 2000a; Wang *et al.*, 1992). The main binding site of PLP is the N-termini of the α- and β-chains, and β-82 lysine within the β-cleft, which is part of the binding site of the natural allosteric effector, 2,3-diphosphoglyceric acid (2,3-DPG). The bound PLP retards dissociation of the ionic linkage between the β-chains of Hb during conversion of deoxy to oxyHb in the same manner as 2,3-DPG. Consequently, the O_2 affinity of Hb decreases in the presence of PLP. The P_{50} of HbV can be regulated to 5–150 Torr by coencapsulating the appropriate amount of PLP or inositol hexaphosphate (IHP) as an allosteric effector (Takahashi, 1995; Wang *et al.*, 1992). PLP equimolar to Hb (PLP/Hb = 1:1 by mol) is coencapsulated, and P_{50} is regulated to 18 Torr. When the molar ratio PLP/Hb is 3:1, P_{50} is regulated to 32 Torr. Furthermore, IHP strongly binds Hb; the resulting oxygen dissociation curve is distorted, and the Hill number becomes nearly 1. When bovine Hb is encapsulated in liposomes, Cl^- can regulate P_{50} (Sakai *et al.*, 2002b). It is 16 Torr without Cl^-, but it becomes 28 Torr with 100 m*M* Cl^-.

The O_2 affinities of HbV can be regulated easily without changing other physical parameters, but chemical structures determine O_2 affinities in the case of the other modified Hb solutions. For this reason, regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not yet been decided completely. Easy regulation of the O_2 affinity, however, might be useful to meet the requirements of clinical indications such as oxygenation of ischemic tissues (Cabrales *et al.*, 2005; Contaldo *et al.*, 2003; Sakai and Tsuchida, 2007).

5. Structural Stabilization of Liposome-Encapsulated Hb

Hb autoxidizes to form metHb and loses its O_2-binding ability during storage, as well as during blood circulation. Therefore, prevention of metHb formation is necessary. Deoxygenated Hb and HbCO are stable, and can be stored in a liquid state (Kerwin *et al.*, 1999). Hb oxidation requires bound O_2 and depends on the O_2 partial pressure. Therefore, complete depletion of O_2 can prevent metHb formation. For LEH or HbV, not only the encapsulated Hb, but also the membrane structure must be physically stabilized, because liposomes, as molecular assemblies, have generally been characterized as structurally unstable. The US Naval

Research Laboratory tested the addition of disaccharides such as trehalose and maltose as cryoprotectants and lyoprotectants to LEH for its preservation as a powder, without causing hemolysis after rehydration (Rudolph, 1988). Furthermore, stabilization methods using polymer chains have been suggested, such as carboxymethylchitin, poly(ethylene glycol) (PEG), and actin (Kato *et al.*, 1984; Li *et al.*, 2005; Mobed and Chang, 1991; Yoshioka, 1991). Polymerization of phospholipids containing diacetylene groups was studied by Hayward *et al.* (1985). Liposomes encapsulating HbCO were irradiated by UV rays to obtain polymerized liposomes. The absorption spectrum of the resulting suspension differs from that of Hb, indicating that the color would not be the same as that of blood. Our group extensively studied the polymerization of phospholipids containing two dienoyl groups (1,2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine; DODPC) (Hosoi *et al.*, 1997; Sakai *et al.*, 1992; Sato *et al.*, 1992). For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in DODPC. This method produces remarkably stable liposomes encapsulating Hb, which are resistant to freeze-thawing, freeze-drying, and rehydration. A salient disadvantage, however, is that the polymerized liposomes are so stable that they are not degraded easily in macrophages, even 30 days after injection (Akama *et al.*, 2000). Polymerized lipids would be inappropriate for intravenous injection because of the difficulty in excretion. Subsequently, selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-conjugated phospholipid) and the composition are important to enhance the stability of nonpolymerized liposomes.

Surface modification of phospholipid vesicles with PEG-conjugated lipid is a well-known means to prolong the circulation time of the vesicles *in vivo* for drug delivery systems (Klibanov *et al.*, 1990; Papahadjopoulos *et al.*, 1991). The surface of HbV can be modified with PEG chains to improve the dispersion state of the vesicles when mixed with blood components (Yoshioka, 1991). PEG-modified HbV has shown improved blood circulation and tissue oxygenation attributable to the prevention of HbV aggregate formation and viscosity elevation (Sakai *et al.*, 1997, 1998), and prolonged circulation *in vivo* (Phillips *et al.*, 1999; Sou *et al.*, 2005). We studied the possibility of long-term preservation of HbV through the combination of two technologies: surface modification of HbV with PEG chains, and complete deoxygenation during storage for 2 years (Sakai *et al.*, 2000b). Samples stored at 4 and 23 °C show a stable dispersion state for 2 years, but the sample stored at 40 °C exhibits precipitation and decomposition of the vesicular components, decreased pH, and 4% leakage of the total Hb after 1 year. The PEG chains on the vesicle surface stabilize the dispersion, and prevent aggregation and fusion because of their stearic hindrance. The original metHb content (ca. 3%) before the preservation decreases gradually to less than 1% in all samples after 1 month because of

the presence of homocysteine inside the vesicles that consume residual O_2 (thiol groups in homocysteines react with oxygen to generate disulfide and active oxygen species), and gradually reduce the trace amount of metHb. The rate of metHb formation is strongly dependent on the O_2 partial pressure; no increase in the metHb formation is observed because of the intrinsic stability of the deoxygenated Hb. These observations suggest that the HbV suspension can be stored at room temperature for at least 2 years, which would enable stockpiling of HbV for any emergency. Hemolysis (rupture of liposomes and release of Hb) must be prevented both *in vitro* and *in vivo*. In comparison to RBCs, HbV are highly resistant to hypotonic shock, freeze-thawing, and enzymatic attack by phospholipase A_2 (Sakai *et al.*, 2009b) because of the larger curvature of liposomes (i.e., larger surface volume ratio, or larger amount of lipids compared to RBCs), and the PEG chains that prevent intervesicular access and enzymatic attack.

Biodistribution of HbV can be examined using ^{99m}Tc-conjugated homocysteine or glutathione containing HbV (Phillips *et al.*, 1999; Sou *et al.*, 2005) and HbV containing ^{125}I-labeled Hb (Taguchi *et al.*, 2009a). These experiments show that HbV are finally captured by macrophages, mainly in the spleen and liver. Electron microscopic observation can detect the presence of Hb-encapsulating particles in the phagosomes of macrophages because of the high densities of protein and electrons (derived from Fe) in the particles such as RBCs. The HbV particles disappear in 1 week (Sakai *et al.*, 2001). This rapid disappearance is in contrast to the polymerized liposomes using DODPC (Akama *et al.*, 2000), which remained in the liver and spleen even after 30 days. Immunohistochemical staining with antihuman Hb antibody and antimethoxy-PEG indicates that Hb and PEG of HbV disappear in 2 weeks (Sakai *et al.*, 2001, 2004b, 2009a). It was shown recently that ^{125}I-labeled Hb and ^{3}H-labeled cholesterol in HbV have identical blood clearance, indicating that HbV retains its integrity in the bloodstream, and distributes to the reticuloendothelial system together. However, ^{125}I mainly appears in urine, and ^{3}H in feces, showing different metabolic routes in the macrophages (Taguchi *et al.*, 2009b).

Taking the points described above into consideration, it is important to consider the instability of particles after their capture by macrophages and their prompt excretion, in addition to sufficient stability during storage for years, and during blood circulation for a week.

6. Blood Compatibility of LEH and HbV

A so-called injection reaction, or pseudo-allergy, resulting from complement activation after injection of a small amount of liposome is well known, giving rise to anaphylatoxins, which trigger various hypersensitivity

reactions (Chonn *et al.*, 1991; Loughrey *et al.*, 1990; Szebeni *et al.*, 2005). Transient thrombocytopenia and pulmonary hypertension in relation to complement activation is an extremely important hematologic effect observed in rodent and porcine models after infusion of LEH (containing DPPG) developed by the US Naval Research Laboratory (Phillips *et al.*, 1997; Rabinovici *et al.*, 1992; Szebeni *et al.*, 1999). Neo red cells (Terumo Corp.) containing stearic acid showed pulmonary hypertension in beagle and porcine models (Pape *et al.*, 2008), but not in monkeys. In our group, exchange transfusion of prototype HbV (containing DPPG, no PEG modification) in anesthetized rats engendered transient thrombocytopenia and slight hypertension (Izumi *et al.*, 1997). The transient reduction in platelet counts and increase of thromboxane B_2 caused by complement-bound liposomes was also associated with sequestration of platelets in the lung and liver (Phillips *et al.*, 1997). In the present formation of HbV, we use a negatively charged lipid (DHSG) instead of DPPG. It does not induce thrombocytopenia or complement activation in animal experiments (Abe *et al.*, 2007; Sou and Tsuchida, 2008), probably because it contains PEGylated lipids and a different type of negatively charged lipid (DHSG), instead of DPPG or a fatty acid.

The *in vitro* human blood compatibility of HbV has been extensively studied (Abe *et al.*, 2006, 2007; Wakamoto *et al.*, 2001, 2005). The present PEG-modified HbV containing DHSG does not affect the extrinsic or intrinsic coagulation activities of human plasma, although HbV-containing DPPG and no PEG modification tends to shorten the intrinsic coagulation time. The kallikrein–kinin cascade of plasma was activated slightly by the prototype DPPG-HbV, but not by the present PEG–DHSG–HbV. The exposure of human platelets to high concentrations of the this HbV (up to 40%) *in vitro* do not cause platelet activation and do not affect adversely the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by platelet agonists (Ito *et al.*, 2001). These results imply that HbV, at concentrations of up to 40%, do not have aberrant interactions with either unstimulated or agonist-induced platelets. It can be concluded that the PEG–DHSG–HbV described here have higher blood compatibility.

7. Regulation of Osmotic Pressure and Suspension Rheology to Mimic and Overwhelm the Function of Blood

The osmotic pressure of blood comprises crystalloid osmotic pressure and colloid osmotic pressure (COP) components. The former is derived mainly from Na^+, Cl^-, other electrolytes, and small molecules; the latter is derived from plasma proteins, mainly albumin, which is dissolved at about 5 g/dL in blood. Albumin regulates the fluid balance across a semipermeable biomembrane between the interstitial tissue and blood. A solution of Hb has

COP, while the HbV suspension does not, because one HbV particle (ca. 250 nm diameter) contains about 30,000 Hb molecules. In fact, HbV acts as a particle, not as a solute, as do RBCs. Therefore, HbV must be suspended in or coinjected with an aqueous solution of a plasma substitute, because injection of a blood substitute results in substitution of a large amount of blood. This contrasts with the characteristics of other Hb-based O_2 carriers, intramolecular cross-linked Hbs, polymerized Hbs, and polymer conjugated Hbs, which all possess high COP as protein solutions (Sakai *et al.*, 2000a).

Animal tests of HbV suspended in 5% plasma-derived HSA or rHSA showed an O_2 transporting capacity that is comparable to that of blood in extreme hemodilution and resuscitation from hemorrhagic shock studies (Izumi *et al.*, 1997; Sakai *et al.*, 1997, 1998, 1999, 2002a, 2004a, 2009a). As a primer fluid for extracorporeal circulation, HbV suspended in rHSA is effective (Yamazaki *et al.*, 2006). We previously reported that HbV suspended in plasma-derived HSA or rHSA was almost Newtonian: no aggregation was detected microscopically (Sakai *et al.*, 1997, 1998, 2009d,e). In Japan, rHSA was approved for clinical use in 2008, but various plasma substitutes are used worldwide, such as hydroxylethyl starch (HES, MW 70, 130, 200, 670 kDa), dextran (DEX, 70 kDa), and modified fluid gelatin (MFG, 32 kDa). The selection among these plasma substitutes is best determined not only according to their safety and efficacy but also according to their associated cost, experience of clinicians, and customs in different countries. Water-soluble polymers generally interact with particles such as polystyrene beads, liposomes, and RBCs to induce aggregation or flocculation by depletion interaction (Meyuhas *et al.*, 1996; Sakai *et al.*, 2009d; Sato *et al.*, 2009). A depletion layer develops near a particle surface that is in contact with a polymer solution if the loss of the configurational entropy of the coil of the polymer is not balanced by adsorption energy. Within this layer, the polymer concentration is lower than in the bulk phase. Consequently, as particles approach the osmotic pressure difference between the interparticle polymer-poor depletion layer and the bulk phase engenders solvent displacement into the bulk phase and consequent depletion interaction. Because of this interaction, the attractive force of particles tends to minimize the polymer-poor space between the particles, thereby inducing flocculation. Consequently, it is important to determine the compatibility of HbV with these plasma substitutes as water-soluble polymers. With that background, we studied rheological properties of HbV suspended in these plasma substitute solutions using a complex rheometer and a microchannel array (Sakai *et al.*, 2007). The rheological property of an Hb-based O_2 carrier is important because the infusion amount is expected to be considerably large, which might affect the blood viscosity and hemodynamics.

HbV suspended in rHSA is nearly Newtonian. Its viscosity resembles that of blood, and the mixtures with RBCs at various mixing ratios has viscosities of 3–4 cP. Other polymers, HES, DEX, and MFG, induce flocculation of

HbV, possibly by depletion interaction, and render the suspensions as non-Newtonian with the shear-thinning profile. These HbV suspensions have high viscosities and high storage moduli (G') because of the presence of flocculated HbV. On the other hand, HbV suspended in rHSA exhibits a very low G'. The viscosities of HbV suspended in DEX, MFG, and high-molecular-weight HES solutions respond quickly to rapid step changes of shear rates of 0.1–100 s^{-1} and a return to 0.1 s^{-1}, indicating that flocculation formation is both rapid and reversible. Microscopically, the flow pattern of the flocculated HbV perfused through microchannels (4.5 μm deep, 7 μm wide, and 20 cm H_2O applied pressure) shows no plugging. Furthermore, the time required for passage is related directly to the viscosity.

Lower blood viscosity after hemodilution has been regarded as being effective for tissue perfusion. Microcirculatory observations indicate, however, that in some cases lower "plasma viscosity" decreases shear stress on the vascular wall, causing vasoconstriction and reduction of the functional capillary density (Tsai *et al.*, 1998). Therefore, an appropriate viscosity that maintains the normal tissue perfusion level might exist. The large molecular dimension of HbV can yield a transfusion fluid with high viscosity. Liposomes with a large molecular dimension are also effective in reducing vascular permeability and minimizing the reaction with NO and CO as vasorelaxation factors (Sakai *et al.*, 2000c, 2008a,b). Erni and collaborators have shown that HbV with a high O_2-binding affinity (low P_{50}, such as 8–15 Torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution is effective for oxygenation of an ischemic skin flap (Contaldo *et al.*, 2003, 2005; Plock *et al.*, 2005, 2007). HbV retains O_2 in the upper arterioles, then perfuses through collateral arteries, and delivers O_2 to the targeted ischemic tissues: a concept of targeted O_2 delivery by a Hb-based O_2 carrier. A high O_2-binding affinity (low P_{50}) is also effective for improving the O_2 saturation of Hb in pulmonary capillaries when exposed to a hypoxic atmosphere or with an impaired lung function. Some plasma substitutes cause flocculation of HbV and hyperviscosity. Hyperviscosity, however, would not necessarily be deleterious in the body and might even be advantageous in some cases (Martini *et al.*, 2006).

The use of HbV provides a unique opportunity to manipulate the suspension rheology, P_{50}, and other physicochemical properties, not only as a transfusion alternative, but also for other clinical applications, including oxygenation of ischemic tissues and *ex vivo* perfusion systems.

8. Concluding Remarks

In spite of the long history of blood substitute development, no material using Hb is clinically available yet, because many investigators have specifically addressed cell-free Hb-based oxygen carriers, which are

much easier to produce. Now that the physiological significance of the cellular structure of RBCs has been widely recognized, cellular type HbV or LEH is becoming the next promising blood substitute. The use of HbV presents both advantages and disadvantages in comparison to sophisticated RBC functions. We hope this chapter can provide various useful alternatives to investigators for modification or manipulation of the components, and production of new formulations of LEH.

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