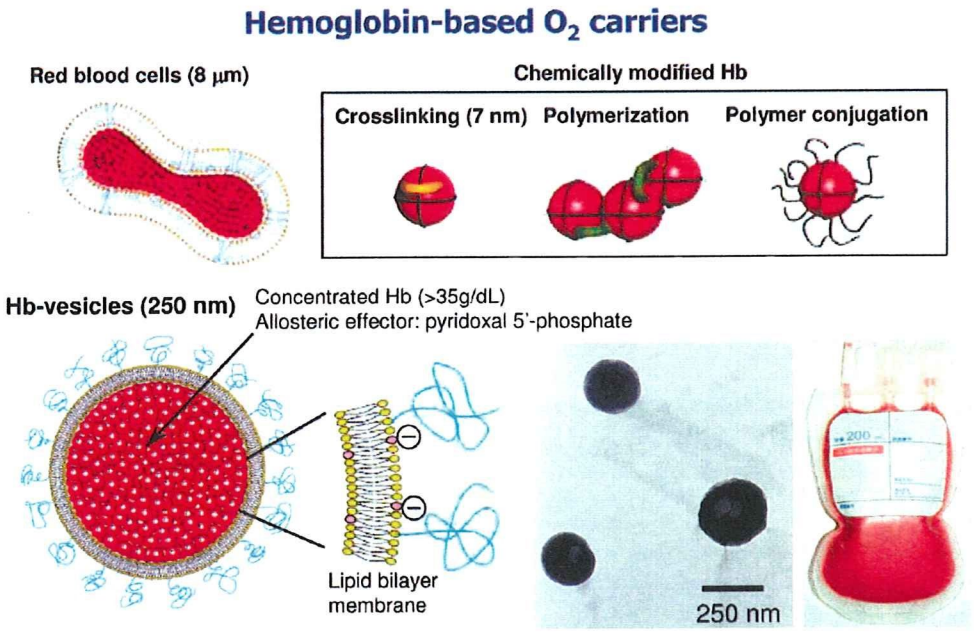


Figure 2. Schematic representation of a series of Hb-based O_2 carriers. Cross-linked Hb, polymerized Hb, and polymer-conjugated Hb are based on the chemical modification of Hb molecules (chemically modified Hbs). In the case of Hb-vesicles, a purified and concentrated Hb solution (35 g/dL) is encapsulated in phospholipid vesicles and the surface is modified using PEG chains. The particle size is well-regulated at 250 nm.

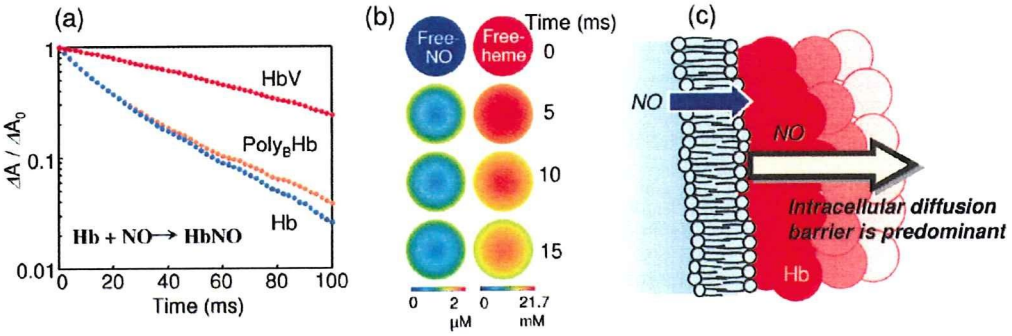


Figure 3. Encapsulation of Hb in vesicles retards NO binding. (a) Time courses of NO binding with HbV, human Hb with PLP (Hb/PLP = 1:2.5 by mol), and polymerized bovine Hb ($Poly_BHb$) observed by stopped-flow rapid scan spectrophotometry. The level of reaction was plotted on a semilogarithmic graph as a ratio of absorption at 430 nm (ΔA) at time t, to the initial absorption (ΔA_0) at time 0. NO-bubbled PBS ([NO] = 3.8 μM) and deoxygenated-Hb-containing solutions in PBS ([heme] = 3.0 μM) were mixed (*31*). (b) Schematic two-dimensional representation of the simulated time courses of distributions of unbound free NO and unbound free heme in one HbV (250 nm). Both free NO and unbound hemes are distributed heterogeneously. The concentration changes gradually from the surface to the core, indicating formation of the intracellular diffusion barrier (*30*). (c) The phospholipid bilayer membrane cannot have any barrier function to gas diffusion. The determinant factor of retardation of NO-binding should be the intracellular diffusion barrier, which was induced by (i) intrinsically larger binding rate constant of NO to a heme in an Hb molecule, (ii) numerous hemes as sites of gas entrapment at a higher Hb concentration, (iii) a slowed gas diffusion in the intracellular viscous Hb solution, and (iv) a longer gas diffusion distance in a larger capsule.

In 1957, Chang performed the pioneering work of Hb encapsulation to mimic the cellular structure of RBCs (*35*); microcapsules (5 μm) were prepared using nylon, collodion, and other materials. Toyoda in 1965 (*36*) and the Kambara–Kimoto group in 1968 (*37*) also investigated encapsulation of Hbs with gelatin, gum arabic, silicone, and so forth. Nevertheless, results emphasized the extreme difficulty of regulating the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 (*38*) that phospholipids assemble to form vesicles in an aqueous medium and encapsulated water-soluble materials in their inner aqueous interior (*39*), it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller in 1977 (*40*) prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, and so forth. The US Naval Research Laboratories and collaborators demonstrated remarkable progress in the use of LEH (*41–43*). Terumo Corp. (Tokyo) developed different LEH, so-called Neo Red Cells (*44, 45*) (Table 1).

However, some intrinsic issues of encapsulated Hbs remained, which were related mainly to the nature of molecular assembly and particle dispersion. What we call Hb-vesicles (HbV), with their high-efficiency production processes and improved properties, were established by our group based on technologies of molecular assembly in concert with precise analyses of their pharmacological and physiological aspects (*46–48*) (Tables 2 and 3). We use stable carbonylhemoglobin (HbCO) for purification with pasteurization at 60 °C for 10 h. The purity of the obtained Hb solution is extremely high (*49, 50*). Use of the stable and purified HbCO enables concentration of the Hb solution to more than 40 g/dL using ultrafiltration and easy handling of Hb encapsulation using the extrusion method,

Table 1. List of Representative Liposome-Encapsulated Hbs (LEH) Extensively Studied Aiming at Industrialization and Other Potential Encapsulated Hbs Using Biodegradable Polymers

product name	group	characteristics	current status
Hb vesicles (HbV)	Waseda Univ. and Keio Univ.	1. Pasteurization of HbCO at 60 °C for virus inactivation 2. Lipid composition to improve blood compatibility 3. PEG modification and deoxygenation for 2 yr storage 4. [Hb] = 10 g/dL	preclinical
Neo Red Cells (NRC)	Terumo Corp.	1. Inositol hexaphosphate to regulate P_{50} (= 40−50 Torr) 2. Lipids: HSPC/cholesterol/fatty acid/PEG-lipid 3. Storage in a refrigerator for 6 months 4. [Hb] = 6 g/dL	preclinical
artificial red cells (ARC)	NOF Corp. and Waseda Univ.	1. Polymerized lipids (DODPC) for stabilization 2. Storage in powdered or frozen state 3. Difficulty in degradation in RES	suspended
liposome-encapsulated Hb (LEH)	US Naval Research Laboratory	1. Freeze-dried powder with trehalose 2. Low Hb encapsulation efficiency 3. Thrombocytopenia, complement activation	suspended
synthetic erythrocytes	Rush-Presbyterian-St. Luke's Medical Center, Univ. Illinois	1. The first attempt of LEH	suspended
Hb-loaded particles (HbP)	East China Univ. of Science and Technology	1. Hb encapsulation by poly(ε-caprolactone) (PCL) and poly(ε-caprolactone-ethylene glycol) (PCL-PEG) 2. Double emulsion and solvent diffusion/evaporation method 3. Biodegradable	basic study
polymersome-encapsulated Hb (PEH)	The Ohio State Univ.	1. Self-assembly of amphiphilic diblock copolymers composed of poly(ethylene oxide) (PEO), poly(caprolactone) (PCL), and poly(lactide) (PLA) 2. Biodegradable and biocompatible	basic study

Table 2. Physicochemical Characteristics of Hb Vesicles

parameter	
particle diameter	250−280 nm
P_{50} $(O_2)^a$	25−28 Torr
[Hb]	10 g/dL
[heme]	6.2 mM
[metHb]	<3%
[HbCO]	<2%
suspending medium	physiologic saline solution (0.9% NaCl)
colloid osmotic pressure	0 Torr
intracellular Hb concentration	ca. 35 g/dL
lipid composition	DPPC/cholesterol/DHSG/DSPE-PEG_{5000}
ζ potential^b	−18.7 mV
weight ratio of Hb to lipids	1.6−1.9 (w/w)
viscosity^c	3.8 cP
lamellarity	nearly 1
stability for storage at room temperature	>2 years, purged with N_2
circulation half-life	35 h (rats)

^{a} Measured with a Hemox Analyzer (pH 7.4, 37 °C). ^{b} Measured with a Zeta-Sizer Nano ZS ([NaCl] = 20 mM, pH 7.4). ^{c} Measured with Anton Parr Rheometer M301 (268 s^{-1}, 25 °C).

Table 3. Characteristics of Hb-Vesicles As a Transfusion Alternative

1. Human derived Hb solution is purified rigorously by pasteurization and ultrafiltration. The blood type antigens and pathogens are eliminated for utmost safety.
2. Encapsulation of concentrated Hb solution (>35 g/dL), like RBCs.
3. Small particle size (250 nm) for homogeneous distribution in the plasma phase of circulating blood.
4. Shielding of the side effects of molecular Hbs by the lipid membrane.
5. PEG modification for dispersion stability for long-term storage and in the bloodstream.
6. Surface properties of HbV (PEG, negative charge, etc.) for blood compatibility.
7. Prompt degradation and excretion of the components after entrapment in RES.
8. Sufficient oxygen transporting capacity ([Hb] = 10 g/dL, *cf*, [Hb] of blood = 12−15 g/dL).
9. Physicochemical properties are easily adjustable because of the nature of the "molecular assembly".

without causing Hb denaturation. It has been confirmed that HbV encapsulates nearly 35 g/dL within a thin bilayer membrane. In final processing, CO of HbCO in HbV is photodissociated by irradiation of visible light under an O_2 atmosphere, and it converts to HbO_2 (*51*).

In fact, Hb autoxidizes to form metHb and loses its O_2-binding ability during storage and during blood circulation (*52*−*56*). For that reason, metHb formation must be prevented. A method exists to preserve deoxygenated Hbs in a liquid state from using intrinsic characteristics of Hb: the Hb oxidation rate in a solution is dependent on the O_2 partial pressure; moreover, deoxyHb is not autoxidized at ambient temperatures (*56*). In the case of HbV, not only the encapsulated Hb but also the capsular structure (liposome) must be physically stabilized to prevent irreversible intervesicular aggregation, fusion, and leakage of the encapsulated Hb.

In addition to HbV, new encapsulated Hbs without liposomes have emerged with the use of recent advanced nanotechnologies, such as polymersome (*57*) and PEG-poly(ε-caprolactone) copolymer nanoparticles (*58*). In vivo evaluation of O_2-carrying capacities of these new materials is anticipated. Encapsulation of Hb can reduce the toxicity of cell-free Hbs. However, numerous hurdles must be surmounted to realize encapsulated Hbs because of the components of the capsules themselves and their structural complexity as a molecular assembly. It is also important to consider the larger dosage requirement of encapsulated Hb for blood substitution than those of conventional drug delivery systems, which require no large dosage.

2.2. Structural Stabilization and Destabilization using Polymerization or PEG Conjugation. Liposomes, as molecular assemblies, have generally been characterized as structurally unstable. The US Naval Research Laboratory tested the addition of cryoprotectants and lyoprotectants such as trehalose to LEH for its preservation as a powder without causing hemolysis after rehydration (*59*, *60*). In addition, many researchers have developed stabilization methods for liposomes that use polymer chains (*61*−*64*). Polymerization of phospholipids that contain two dienoyl groups (1,2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine; DODPC) was studied extensively by our group. 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, resembling rubber balls, which are resistant to freeze−thawing, freeze−drying, and rehydration (*65*, *66*). Actually, the polymerized liposomes were so stable that they were not degraded easily in the macrophages,

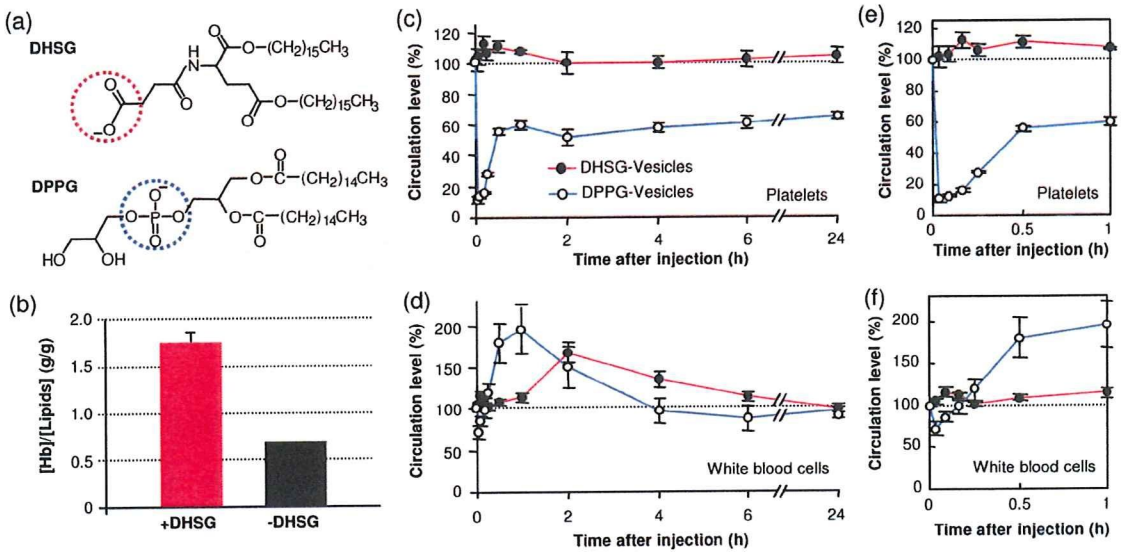


Figure 4. Effect of negatively charged vesicles on encapsulation capability and blood compatibility of HbV. (a) Chemical structures of negatively charged lipids, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG) and 1,2-dipalmitoyl-*sn*-glycelo-phosphatidylglycerol (DPPG). (b) Effect of DHSG on encapsulation efficiency of Hb (35 g/dL). (c–f) Circulation levels of platelets (c) and white blood cells (d) after intravenous infusion of vesicles containing 9 mol % DHSG (DHSG-vesicles) or 9 mol % DPPG (DPPG-vesicles) in rats (lipids; 280 mg/kg body weight). The right side graphs show the initial fluctuations in platelets (e) and white blood cells (f).

even 30 days after injection (*67*). It became widely believed that polymerized lipids are inappropriate for intravenous injection because of the difficulty in excretion. Subsequently, it was clarified that selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-conjugated phospholipid) and that their composition is important to enhance the stability of nonpolymerized liposomes (*45, 68*). Surface modification of liposomes with PEG-conjugated lipids is sufficient for dispersion stability (*69*). In fact, in comparison to RBCs, HbV is highly resistant to hypotonic shock, freeze–thawing, and enzymatic attack by phospholipase A_2 (*70*).

We investigated the possibility of long-term preservation of HbV during storage for two years through a combination of deoxygenation and PEG modification (*71*). As little as 0.3 mol % PEG-conjugated lipid stabilizes the dispersion state and prevents aggregation and fusion for two years through steric hindrance (*71–73*). The original metHb content (ca. 3%) before preservation decreased gradually to less than 1% after 1 month because of the presence of a reductant, such as homocysteine, inside the vesicles that consumed the residual O_2 and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the O_2 partial pressure: a lack of increase in the metHb formation was observed because of the intrinsic stability of the deoxygenated Hb. In fact, the metHb content did not increase for two years. These results suggest the possibility that the HbV suspension can be stored at room temperature for at least two years, which would enable stockpiling of HbV for any emergency.

2.3. Vesicular Surface Modification with PEG and Negative Charges for Blood Compatibility. Liposome is not a solute: it is a particle in a suspension. The particle surface might be recognized, leading to interaction with blood components including complements. The so-called *injection reaction*, or pseudoallergy, results from complement activation, giving rise to anaphylatoxins, which trigger various hypersensitivity reactions. This reaction is observed sometimes not only with liposomal products (*74*), but also with fat emulsions (*75*), and a perfluorocarbon emulsion (*76*). Therefore, examination of blood compatibility of encapsulated Hbs is important for clinical use. Transient thrombocytopenia and pulmonary hypertension in relation to complement activation is an extremely important hematologic effect observed in rodent models after infusion of LEH (containing DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol) developed by the US Naval Research Laboratory (*77, 78*) and other products. In our group, exchange transfusion of prototype HbV (containing DPPG, no PEG modification) in anesthetized rats engendered transient thrombocytopenia and slight hypertension (*79*). Similar effects were also observed for administration of negatively charged liposomes (*80, 81*). The transient reduction in platelet counts caused by complement-bound liposomes was also associated with sequestration of platelets in the lung and liver. Such nonphysiological platelet activation probably engenders initiation and modulation of inflammatory responses because platelets contain several potent proinflammatory substances. A negatively charged lipid is required for encapsulating a large amount of Hb using a minimum amount of lipids (*46–48*). Therefore, we had to overcome the problem of conventional negatively charged vesicles to achieve blood compatibility. In the present formation of HbV, we use a negatively charged lipid (DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate) and confirmed the considerable improvement of Hb encapsulation efficiency (Figure 4a,b). It must be emphasized that the present vesicle formulation for HbV apparently does not induce thrombocytopenia or complement activation in animal experiments (Figure 4c–f) (*82, 83*), probably because the present HbV contains PEG-modification and a different type of negatively charged lipid (DHSG), not DPPG or a fatty acid.

Ikeda and his co-workers (*83–86*) thoroughly examined blood compatibility of HbV to human blood in vitro. The present PEG-modified HbV containing DHSG did not affect the extrinsic or intrinsic coagulation activities of human plasma, although HbV containing DPPG and no PEG modification tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the 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 present HbV (up to 40%) in vitro did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by platelet agonists. These results imply that HbV, at concentrations of up to 40%, has aberrant interactions with neither unstimulated

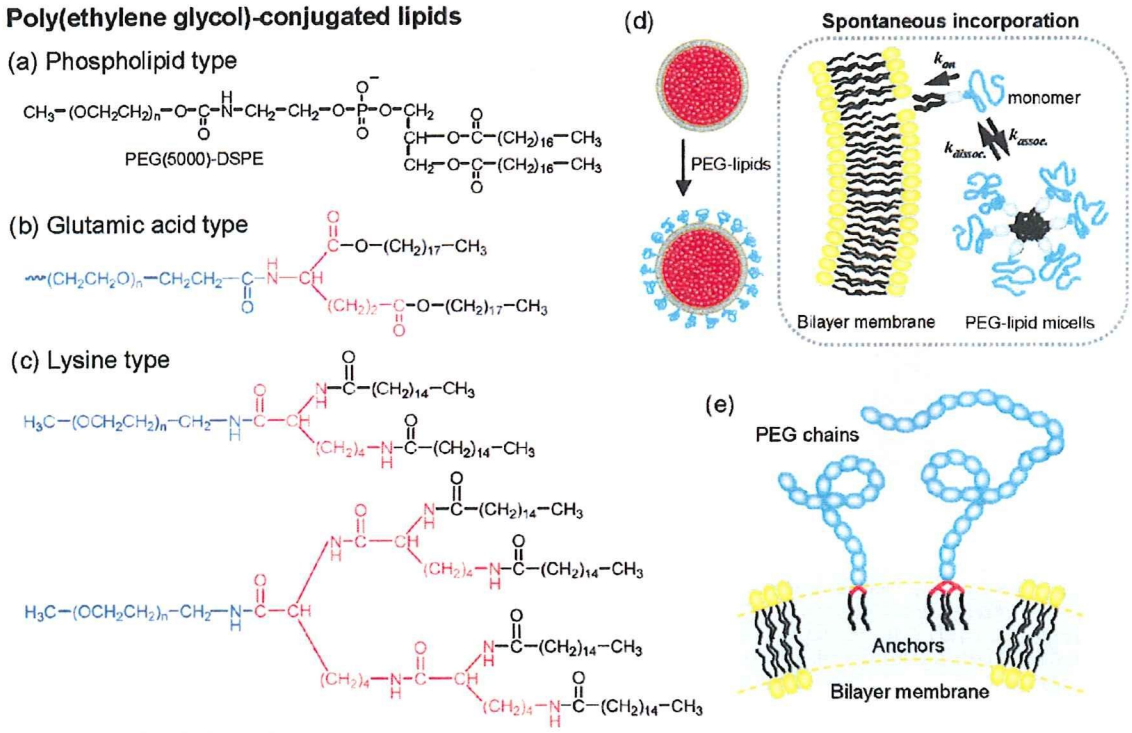


Figure 5. Surface modification of HbV with PEG chains. (a–c) Chemical structures of PEG-conjugated lipids. (a) PEG(5000)-DSPE is commercially available and widely used for surface modification of lipid vesicles. (b) Glutamic acid-type and (c) lysine-type PEG-conjugated lipids were reported previously (*87, 88*). (d) PEG-modification of the HbV using the spontaneous incorporation of a PEG-lipid into the bilayer membrane (*72*). (e) Immobilization of the large water-soluble polymers on the bilayer membranes (*87, 89*).

nor agonist-induced platelets. It can be concluded that the present PEG-DHSG-HbV has higher blood compatibility.

For surface modification of HbV with PEG chains, several PEG-conjugated lipids having amino acid backbone have been synthesized, as presented in Figure 5b,c (*87, 88*). For other applications, these PEG-conjugated lipids are available to modify the surfaces of not only HbV but also lipid vesicles. Because the PEG-lipids having a large hydrophilic group form thermodynamically unstable self-assemblies, called micelles (*73*), they easily dissociate to monomers and spontaneously incorporate only to the outer surface of preformed HbV, as illustrated in Figure 5d (*45, 72*). Using this method, the required amount of PEG-conjugated lipid can be reduced by half or less because only the outer surface of HbV requires PEG chains. In addition, the PEG chains extending from the inner surface are expected to reduce the interior volume for encapsulation because of their exclusion volume effect. Therefore, modification only to the outer surface is important to encapsulate large amounts of Hb in lipid vesicles. To immobilize the hydrophilic macromolecules stably, such as PEG chains and proteins on lipid vesicles, a large hydrophobic anchor having a tetraacyl structure has been synthesized (Figure 5c,e). It has been confirmed that this hydrophobic anchor can stably immobilize PEG chains of high molecular weight (Mw 12 500) or water-soluble proteins (*87, 89*). These surface modification technologies and lipid chemistry have been applied to the development of other functional biomaterials (*87–93*).

2.4. Circulation Time, Biodistribution, and Metabolism. The dosage of blood substitutes is expected to be considerably larger than those of other drugs, while their circulation time is considerably shorter than that of RBCs. Therefore, their biodistribution, metabolism, excretion, and side effects must be characterized in detail, especially in relation to the reticuloendothelial system (RES, alternatively, the mononuclear phagocytic system, MPS). Normally, free Hb released from RBCs is bound rapidly to haptoglobin and is consequently removed from circulation by hepatocytes. However, when the Hb concentration is greater than the haptoglobin binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. Hemoglobinuria and eventual renal failure occur when the kidney reabsorption capacity is exceeded. The encapsulation of Hb in vesicles completely suppresses renal excretion. However, HbV in the bloodstream is ultimately captured by phagocytes in the RES in much the same manner as senescent RBCs are, as confirmed by radioisotope ^{99m}Tc-labeled HbV injection (*42, 94*). The circulation half-life is dose-dependent: for the dosage of 14 mL/kg body weight, the circulation half-life was 34.8 h in rats and 62.6 h in rabbits (Figure 6a). The HbV are finally distributed mainly in the liver, spleen, and bone marrow (Figure 6b). The species-dependent circulation time is inferred to be dependent upon the species-specific weight balance of phagocyte organs, particularly the liver, spleen, and bone marrow, against body weight (*94*). The circulation half-life in the case of the human body can be estimated as about 2–3 days at the same dosage.

It is generally accepted that the liposome clearance by RES at a small dosage is accelerated by opsonization (absorption of plasma proteins such as antibodies and complements on the liposomal surface). In fact, PEG-modification prevents opsonization for prolonged circulation times (*95*). However, considering a condition in which the dosage of HbV is extremely high and requires a considerable amount of opsonins, and in which HbV does not induce complementary activation (*82, 83*), then opsonin-dependent phagocytosis would not be a major component in the case of HbV with a large dosage. Actually, opsonin-independent phagocytosis, a direct recognition by macrophages, has been clarified in some studies (*96, 97*).

Analysis of the spleen by transmission electron microscopy (TEM) 1 day after infusion of HbV revealed the presence of HbV particles in the phagosomes of macrophages (*98*) (Figure

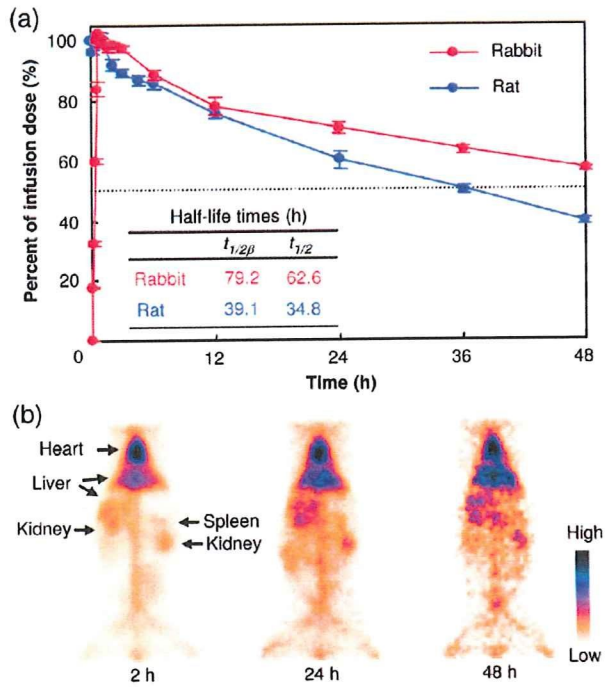


Figure 6. Circulation kinetics and organ distribution of HbV labeled with technetium-99m after top-loading intravenous infusion (14 mL/kg) in rats and rabbits (*94*). (a) Elimination profiles of HbV from blood. (b) Static gamma camera images of a rabbit acquired at 2, 24, and 48 h after HbV infusion.

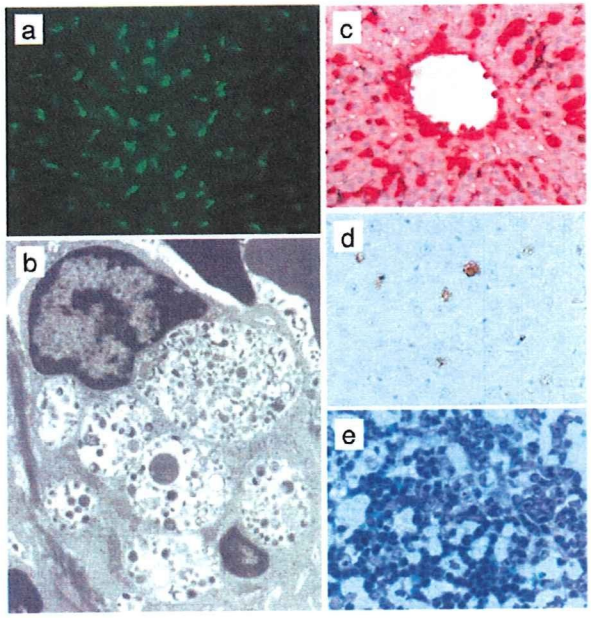


Figure 7. Histopathological examination of livers and spleens of rodents after injection of HbV. (a) Hamster liver 1 h after injection of fluorescence-labeled HbV, observed by a confocal laser scanning microscopy. The strong fluorescence indicates that HbV particles accumulate in Kupffer cells. (b) TEM of rat spleen macrophage 1 day after injection of HbV. The small black dots are HbV particles in phagosomes. The particles disappeared within a week (data not shown) (*98*). (c) Rat liver 1 day after injection of HbV. It was immunohistochemically stained with anti-human Hb antibody. The red parts indicate the presence of human Hb in HbV. It disappeared within a week (data not shown) (*98*). (d) Rat liver 7 day after injection of HbV. It was immunochemically stained with anti-methoxy-PEG antibody. The brown parts indicate the presence of PEG derived from PEG-lipid of HbV during the degradation. Fourteen days after injection, PEG was not detectable (data not shown) (*102*). (e) Rat spleen 3 days after 40% exchange transfusion with HbV. It was stained with Giemsa method. A large amount of blue cells, erythroblasts, are seen, indicating the enhanced hematopoiesis for the complete recovery of hematocrit within one week, while HbV are degraded in RES (*101*).

7). However, after 7 days, the HbV structure cannot be observed. We confirmed transient splenomegaly with no irreversible damage to the organs and complete metabolism within a week. The phagocytic activity transiently, but not completely, decreased 1 day after injection, and it turned to increasing at 3 days. The influence on the defense function and its mechanism has been carefully examined in the ongoing research. Immunochemical staining with a polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV. Results clarified that HbV had almost disappeared in both the spleen and liver after 7 days.

Bilirubin and iron are believed to be released during metabolism of Hb, but our animal experiments of topload infusion, daily repeated infusions, and 40% blood exchange showed that neither of those products increased in the plasma within 14 days (*99*−*101*). Bilirubin would normally be excreted in the bile as a normal pathway; no obstruction or stasis of the bile is expected to occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen after injection of stored RBCs, partly because of the accumulation and degradation of stored RBCs with lowered membrane deformability and shortened circulation half-life (*101*).

As for membrane components of Hb-vesicles, the plasma cholesterol level elevated transiently 3 days after injection: cholesterol was released from macrophages after degradation of HbV in phagosomes (*99, 101*). It was recently clarified using ^{3}H-cholesterol that cholesterol of HbV is released from macrophages to blood; it is ultimately excreted in feces. The PEG chain is widely used for surface modification of liposomal products. The chemical cross-linker succinic acid ester of PEG-conjugated phospholipid is susceptible to hydrolysis to release PEG chains during metabolism. Actually, immunohistochemistry using an anti-methoxy-PEG antibody clarified that PEG had disappeared from the liver and spleen in one week (*102*). The released PEG chains, which are known as inert macromolecules, are expected to be excreted in urine through the kidneys (*103*).

To determine the physiological capacity of RES for degradation of HbV, we tested massive intravenous doses by daily repeated infusion of 10 mL/kg body weight/day into Wistar rats for 14 days. The cumulative dosage was 140 mL/kg body weight (Hb and lipids, 20 689 mg/kg body weight). The total volume was equal to 2.5 times the total blood volume (56 mL/kg body weight) (*100*). Although splenohepatomegaly was considerable, all rats tolerated the infusions; their body weight increased during the succeeding 14 days until their intended sacrifice. The phagocyted HbV had disappeared, though the considerable hemosiderin deposition was confirmed in the spleen, liver, kidney, adrenal grand, and bone marrow. We were unable to define a lethal dose of HbV in this experiment.

The profile of liposome clearance is species-dependent. More precise data are necessary to extrapolate the phenomena observed in animal experiments to humans. However, these results imply that the metabolism of HbV and excretion are within the physiological capacity that has been well-characterized for the metabolism of senescent RBCs and conventional liposomal products.

2.5. Regulation of O_2 Transporting Capability (MetHb Reduction, $P_{50}(O_2)$). Actually, Hb encapsulation provides a unique opportunity to add new functions to particles by coencapsulation or embedding of functional molecules (*52*−*56, 104*−*106*). MetHb formation of HbV during the

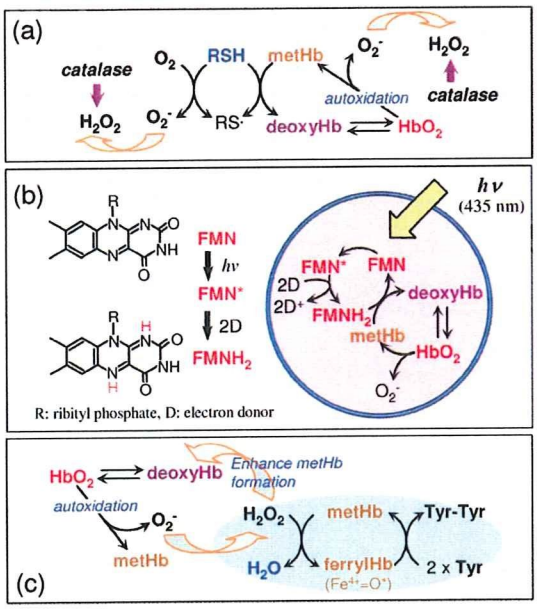


Figure 8. Artificial metHb reduction systems in HbV. (a) Coencapsulation of thiols (RSH), such as glutathione and homocyteine, reduces metHb. However, thiols react with O_2 to generate O_2^- and H_2O_2. A more reactive thiol such as cysteine shows extremely rapid reaction with O_2 and adversely facilitates metHb formation. Coencapsulation of catalase effectively eliminates H_2O_2 and prevent such reactions (*52, 53, 56*). (b) Photoreduction system by coencapsulation of flavin monomucleotide (FMN) and an electron donor such as EDTA and methionine. Visible light irradiation (435 nm) primarily converts FMN to the photoexcited triplet FMN*, and this reacts with two electron donors (D) to generate $FMNH_2$. MetHb is rapidly reduced once $FMNH_2$ is produced (*54*). (c) During autoxidation of Hb and in blood circulation, H_2O_2 is a potent metHb enhancer. A combination of L-tyrosine (Tyr) and metHb effectively eliminates H_2O_2 and prolongs the functional lifetime of HbV (*55*).

preservation is completely prevented simply by deoxygenation. However, autoxidation of HbO_2 is initiated once HbV is administered into the bloodstream because the entire metHb enzymatic system is eliminated during the Hb purification for the utmost safety from infection. An artificial metHb reducing system is required to prolong the O_2-carrying capacity of HbV. We have tested coencapsulation of reductants to directly reduce metHb and a photoreduction system using a photosensitizer such as flavin mononucleotide (FMN). A more practical method is to create an artificial enzymatic system using L-tyrosine (Tyr) and metHb that eliminates H_2O_2 as does catalase (Figure 8).

Without a chemical modification of Hb, O_2 binding affinity (expressed as $P_{50}(O_2)$, O_2 tension at which Hb is half-saturated with O_2) of HbV can be regulated by coencapsulation of an allosteric effector (*105, 106*) (Figure 9a). The $P_{50}(O_2)$ of purified Hb in a saline solution (in the presence of Cl^-) is about 14 Torr; 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 is expected to be regulated similarly to that of RBC, namely, about 25−30 Torr, using an allosteric effector or by a direct chemical modification of the Hb molecules. This enables sufficient O_2 unloading during blood microcirculation, as evaluated by the arterio-venous difference in the levels of O_2 saturation in accordance with an O_2 equilibrium curve. Pyridoxal 5′-phosphate (PLP) is coencapsulated in HbV as an allosteric effector to regulate $P_{50}(O_2)$ (*105, 106*). The main binding site of PLP is the N-terminal of the α-chain and β-chain 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 the 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 does. Therefore, the O_2 affinity of Hb decreases in the presence of PLP. The $P_{50}(O_2)$ of HbV can be regulated to 8−150 Torr by coencapsulating the appropriate amount of PLP or inositol hexaphosphate as an allosteric effector. Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was coencapsulated, and $P_{50}(O_2)$ was regulated to 18 Torr. Furthermore, $P_{50}(O_2)$ was regulated to 32 Torr when the molar ratio PLP/Hb was 3/1. The O_2 affinities of HbV can be regulated easily without changing other physical parameters, whereas in the case of the other modified Hb solutions, their chemical structures determine their O_2 binding affinities. Consequently, regulation is difficult. The present HbV contains PLP at PLP/Hb = 2.5 by mol; the resulting $P_{50}(O_2)$ is about 25−28 Torr, which shows sufficient O_2 transporting capacity as a transfusion alternative. Actually, HbV has been shown to provide O_2-transport capacity that is both sufficient and comparable to that of RBCs in experiments related to extreme blood exchange (*68, 69, 79, 105, 107, 108*) and fluid resuscitation from hemorrhagic shock (*102, 109−112*) (Figure 10). A recent experiment of HbV as a priming solution for cardiopulmonary bypass (CPB) in a rat model showed that HbV protects neurocognitive function by transporting O_2 to brain tissue even when the hematocrit is reduced markedly (*113*).

The appropriate O_2 binding affinities for O_2 carriers have not yet been decided completely. However, the easy regulation of the O_2 binding affinity might be useful to meet the requirement of clinical indications such as oxygenation of ischemic tissues. The $P_{50}(O_2)$ of HbV without PLP and Cl^- is 8−9 Torr. This formulation is effective for targeted O_2 delivery to anoxic tissues caused by reduced blood flow (*107, 114, 115*).

2.6. Rheological Properties and Their Physiological Implications for Tissue Oxygenation. The extremely high concentration of the HbV suspension ([Hb] = 10 g/dL; [lipids] = 6 g/dL, volume fraction, ca. 40 vol %) imparts an O_2 carrying capacity that is comparable to that of blood. The HbV suspension does not possess a colloid osmotic pressure (COP), 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. Therefore, HbV must be suspended in or coinjected with an aqueous solution of a plasma substitutes. This requirement is identical to that for emulsified perfluorocarbon, which does not possess COP (*116, 117*); it contrasts to characteristics of other Hb-based O_2 carriers, intramolecular cross-linked Hbs, polymerized Hbs, and polymer-conjugated Hbs, which all possess very high COP as protein solutions (*15, 118*) (Figure 9b).

Animal tests of HbV suspended in plasma-derived HSA or rHSA showed an O_2 transporting capacity that is comparable to that of blood (*110, 113*). We reported previously that HbV suspended in plasma-derived HSA or rHSA was almost Newtonian: no aggregation was detected microscopically (*68, 69*). In Japan, rHSA was very recently approved for clinical use, in May 2008 (*119*), but various plasma substitutes are used worldwide, such as hydroxylethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG). The selection among these plasma substitutes is best determined not only according to their safety and efficacy, but also according to their associated price, experience of clinicians, and customs of respective countries. Water-soluble polymers generally interact with particles such as polystyrene beads, liposomes, and RBCs to induce aggregation or flocculation (*120, 121*). For that reason, it is important to determine the compatibility of HbV with these plasma substitutes. With that background, we studied rheological properties of HbV suspended in these plasma substitute solutions using a complex rheometer and a microchannel array (*122*). The rheological property of an Hb-based O_2 carrier is important

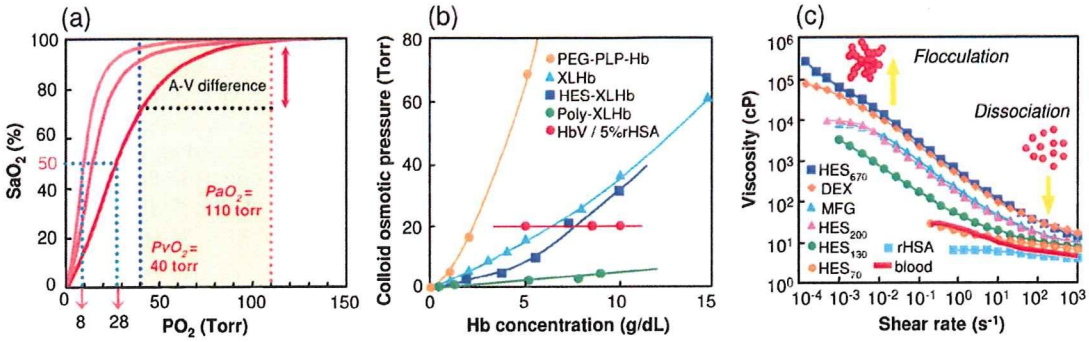


Figure 9. Regulation of physicochemical properties of HbV for versatile applications. (a) Oxygen dissociation curves of HbVs. Oxygen affinity (P_{50}, partial pressure of oxygen at which Hb is half-saturated with oxygen) is regulated by coencapsulation of PLP (*105, 106*). (b) Colloid osmotic pressure (COP) of chemically modified Hb solutions increase with the Hb concentration (*15*). In contrast, HbV particles have no oncotic effect. The figure shows 20 Torr when HbV is suspended in 5% rHSA. XLHb, intramolecularly cross-linked Hb. (c) Rheological properties of HbV suspended in various plasma substitute solutions (*122*). [Hb] = 10 g/dL, 25 °C.

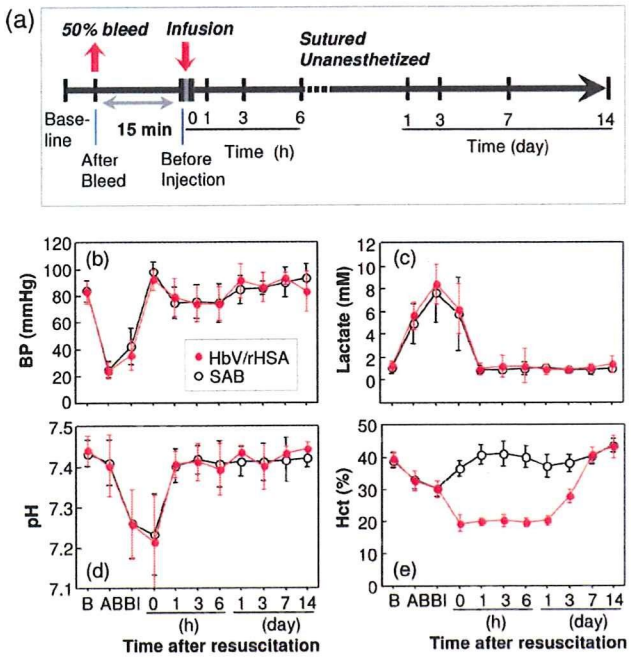


Figure 10. (a) Scheme depicting the experimental protocol of hemorrhagic shock and resuscitation (*102*). Shock was induced by withdrawing 50% of circulating blood volume from Wistar rats. After 15 min, they were resuscitated with either HbV suspended in recombinant human serum albumin (HbV/rHSA) or shed autologous blood (SAB). (b) blood pressure, (c) lactate, (d) pH, and (e) hematocrit (Hct). Mean ± SD. B, Baseline; AB, after bleeding; BI, before injection (B-6 h, n = 24; 1−14 days, n = 5).

because the infusion amount is expected to be considerably large, which might affect the blood viscosity and hemodynamics.

The HbV suspended in rHSA was nearly Newtonian (Figure 9c). Its viscosity was similar to that of blood, and the mixtures with RBCs at various mixing ratios showed viscosities of 3−4 cP. Other polymers, HES, DEX, and MFG, induced flocculation of HbV, possibly by depletion interaction, and rendered the suspensions as non-Newtonian with the *shear-thinning* profile (*122*). These HbV suspensions showed high viscosity and a high storage modulus (G') because of the presence of flocculated HbV. On the other hand, HbV suspended in rHSA exhibited a very low G'. The viscosities of HbV suspended in DEX, MFG, and high-molecular-weight HES solutions responded 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, 20 cmH_2O applied pressure) showed no plugging. Furthermore, the time required for passage was directly related to the viscosity.

Table 4. Publications of Preclinical Studies Aiming at Applications of Hb-Vesicles for a Transfusion Alternative and for Oxygen Therapeutics

indication	ref
1. Resuscitative fluid for hemorrhagic shock	102, 109−112
2. Hemodilution	68, 69, 79, 101, 105, 107, 108
3. Priming fluid for extracorporeal membrane oxygenator (ECMO) for cardiopulmonary bypass	113
4. Perfusate for resected organs (transplantation)	24, 129
5. Oxygenation of ischemic brain (stroke)	130
6. Oxygenation of ischemic skin flap (plastic surgery)	115, 127, 128
7. Tumor oxygenation for sensitization to irradiation	131
8. CO carrier for cytoprotection at reperfusion	132

It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower "plasma viscosity" decreases shear stress on the vascular wall, causing vasoconstriction and reducing the functional capillary density (*123*). Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. The large molecular dimension of HbV can result in a transfusion fluid with high viscosity. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors (*24, 25, 30, 31*) (see Figure 3). These new concepts suggest reconsideration of the design of artificial O_2 carriers (*124*). Actually, new products are appearing, although they are in the preclinical stage, not only HbV but also zero-link polymerized Hb (*125*) and others with larger molecular dimensions and higher O_2 affinities (*126*). Erni et al. clarified that HbV with a high O_2 binding affinity (low $P_{50}(O_2)$, such as 8−15 Torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution was effective for oxygenation of an ischemic skin flap (*115, 127, 128*). That study showed that 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 an Hb-based O_2 carrier (*114*). A high O_2 binding affinity (low $P_{50}(O_2)$) would also be effective to improve the O_2 saturation of Hb in pulmonary capillaries when exposed to a hypoxic atmo-

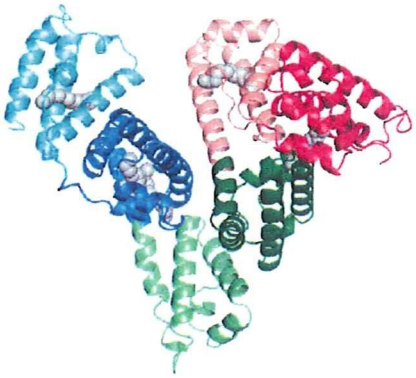


Figure 11. Crystal structure of HSA with myristate (PDB ID: 1BJ5) from ref (*136*).

sphere or with an impaired lung function. Some plasma substitutes cause flocculation of HbV and hyperviscosity. However, reports show that hyperviscosity would not necessarily be deteriorative and might be, in some cases, advantageous in the body (*32*). HbV provides a unique opportunity to manipulate the suspension rheology, $P_{50}(O_2)$, and other physicochemical properties, not only as a transfusion alternative, but also for other clinical applications such as oxygenation of ischemic tissues and ex vivo perfusion systems (*129*−*132*) (Table 4).

3. ALBUMIN-HEMES AS O_2 CARRYING PLASMA PROTEINS

3.1. HSA Incorporating Synthetic Fe^{2+} Porphyrin (HSA-FeP). HSA (Mw: 66 500) has a remarkable ability to bind a wide variety of exogenous compounds in the human circulatory system (*133*). This carrier protein comprises 3 homologue helical domains (I−III) with 9 loops formed by 17 disulfide linkages; each domain contains 2 subdomains (A and B) (Figure 11) (*134*−*137*). It is known that many common drugs such as warfarin, diazepam, and ibuprofen bind to one of the two primary sites (site 1 in subdomain IIA, site 2 in subdomain IIIA) (*138*). In fact, HSA helps to solubilize these compounds to achieve high concentration in the bloodstream; otherwise, they would easily aggregate and be poorly distributed.

In 1995, we found that tetrakis(*o*-pivalamido)phenylporphinatoiron bearing a covalently linked proximal imidazole (FeP**1**, Figure 12) was incorporated into HSA, yielding a red HSA-FeP**1** hybrid (*11*). This synthetic hemoprotein can reversibly bind and release O_2 under physiological conditions (pH 7.4, 37 °C) in much the same way as Hb. The HSA host adsorbs a maximal eight FeP**1** molecules. Their stepwise binding constants (K_1−K_8) range from 1.2×10^6 to 1.2×10^4 (M^{-1}) (Table 3) (*139, 140*). Solution properties of the HSA-FeP**1** solution ([rHSA] = 5 wt %, FeP/HSA=1−8, mol/mol) are almost identical to those of HSA itself: the specific gravity, 1.013; viscosity, 1.1 cP; and COP, 20 Torr. Circular dichroism (CD) spectroscopy and isoelectric focusing measurement revealed that the second-order structure and surface charge distribution of HSA were unaltered after binding of FeP**1**. The obtained solution showed a long shelf-life of over two years at room temperature (*141*). Furthermore, HSA-FeP**1** has no effect on the morphology of blood cell components (*142*) and does not engender immunological reaction and platelet activation (*143*). Upon addition of O_2 gas through this solution, the visible absorption spectrum immediately changed to that of the O_2 adduct complex. After exposure to CO gas, a stable carbonyl complex of HSA-FeP**1** was formed (*139, 140*). The coordination structure of FeP**1** and spin-state of the central ferrous ion was characterized by IR, resonance Raman, and magnetic circular dichroism (MCD) spectroscopy (*139, 140, 144*). The carbonyl HSA-FeP**1** moved to the NO adduct complex after bubbling NO gas (*145*). Subsequent ESR spectroscopy revealed that FeP**1** in albumin formed a six-coordinate nitrosyl complex. The proximal imidazole moiety does not dissociate from the central ferrous ion when NO binds to the trans side (*146*).

The $P_{50}(O_2)$ value of HSA-FeP**1** is always constant (33 Torr, 37 °C) independent of the binding number of FeP**1** (eq 1, Table 5)

$$\text{HSA-FeP} + \text{L} \underset{k_{off}(\text{L})}{\overset{k_{on}(\text{L})}{\rightleftarrows}} \text{HSA-FeP(L)} \quad [\text{L}: O_2 \text{ or CO}]$$

$$K(\text{L}) = [P_{50}(\text{L})]^{-1} = k_{on}(\text{L})/k_{off}(\text{L}) \quad (1)$$

The O_2 binding equilibrium curve shows no cooperativity. However, the O_2 transporting efficiency between the lungs [$P(O_2)$: ca. 110 Torr] and muscle tissue [$P(O_2)$: ca. 40 Torr] is 22%, which is identical to that for RBC.

The O_2 association and dissociation rate constants [$k_{on}(O_2)$ and $k_{off}(O_2)$] can be measured using laser flash photolysis (*147, 148*). Interestingly, the rebinding process of O_2 to HSA-FeP**1** included two phases (fast and slow phase), perhaps because of the different environment around each FeP**1** in the protein (*149*). The $P_{50}(O_2)$ value can be controlled by tuning the chemical structure of FeP**1**. We have synthesized quantities of Fe(II)porphyrins (Figure 12) and evaluated the O_2 binding parameters of their HSA-FeP hybrids (Table 6).

Actually, FeP**2** has a bulky 1-methylcyclohexanamide group on the porphyrin ring plane (*150*). The O_2 binding affinity of HSA-FeP**2** [$P_{50}(O_2)$: 35 Torr] was almost identical to that of HSA-FeP**1**. However, the stability of the oxygenated complex increased to 4.5 times its usual value [half-life $\tau_{1/2}(O_2)$, 9 h; pH 7.3; 37 °C] (*150*).

In general, the basicity and structure of the proximal base greatly influences the O_2 binding property of Fe^{2+}porphyrin. Both FeP**3** and FeP**4**, similar analogues having an His ligand, showed high O_2 binding affinities [$P_{50}(O_2)$: 3 Torr] (Figure 13, Table 6). Kinetically, substitution of the 2-methylimidazole to His reduces the O_2 dissociation rate constant (*150*). Although one might think that high O_2 binding affinity is not useful as a blood substitute, it can be efficient for oxygenation of hypoxic regions in tumors. Furthermore, the HSA-FeP**4** showed long $\tau_{1/2}(O_2)$ of 25 h (37 °C), which is 13-fold longer than that of HSA-FeP**1**.

Another HSA-FeP**5**, in which the active porphyrin has 3-methyl-L-hisitidine as a proximal base, exhibits an extraordinarily high O_2 binding affinity [$P_{50}(O_2)$: 1 Torr] that approaches those of relaxed-state Hb and Mb (*151*). It is remarkable that replacement of the 3-methyl-L-histidine moiety by 1-methyl-L-histidine isomer (HSA-FeP**6**) reduced O_2 binding affinity to 1/35th of its former level. The low O_2 affinity of FeP**6** is predominantly reflected by the high $k_{off}(O_2)$ value. The axial Fe−N(1-methyl-L-histidine) coordination might be restrained by steric interaction between the 4-methylene group of the His ring and the porphyrin plane (*151*).

The proximal histidyl side chain can be introduced easily into the β-pyrrolic position of the porphyrin via an acyl bond in two steps, FeP**7** (*152*). Although an electron-withdrawing acyl group is bound at the porphyrin periphery, the O_2 binding affinity of HSA-FeP**7** is slightly higher than that of HSA-FeP**4**. The rigid His-Gly(carboxy)butanoyl spacer of FeP**7** probably produces a favorable geometry to fix the imidazole onto the central Fe^{2+} of the porphyrin.

Double-sided porphyrins (FeP**8**, FeP**9**, and FeP**10**) were also incorporated into HSA (*153*). We expected that steric encumbrances on both sides of the porphyrin enable the HSA-FePs to form a stable O_2 adduct complex. Actually, the $\tau_{1/2}(O_2)$ of HSA-

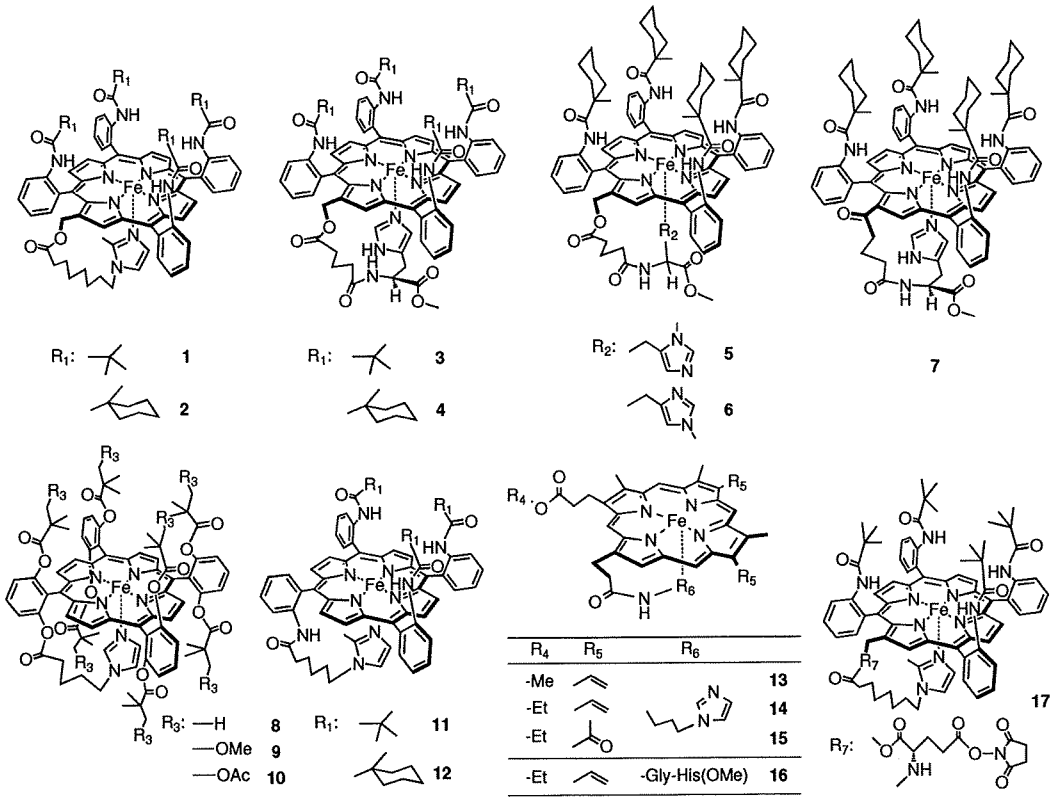


Figure 12. Structure of FePs in HSA-FePs.

Table 5. Solution Properties and Characteristics of HSA-FeP1

binding number of FeP1 (n)	1−8
binding constant of FeP1	$1.2 \times 10^6 - 1.2 \times 10^4$ M^{-1}
M_w	(66.5 + 1.3n) kDa
pI	4.8
viscosity[a,b]	1.1 cP
COP[a,b,c]	20 Torr
shelf life[a,d]	>2 years

[a] In phosphate buffered solution (pH 7.3). [HSA]: 5 g/dL. [b] At 37 °C. [c] A membrane filter with a cutoff (M_w, 30 × 10^3) was used. [d] At 25 °C.

FeP**8** was 5 h, which is 2.5-fold longer than that of HSA-FeP**1**. In addition, HSA-FeP**8** showed high stability against hydrogen peroxide. The HSA incorporating double-sided porphyrins would be useful for the synthetic analogue of the oxidation enzyme.

Tailed porphyrins having an α,α,α,β-conformer, FeP**11** and FeP**12**, were synthesized easily via four steps from atropisomers of tetrakis(*o*-aminophenyl)porphyrin relative to eight steps of FeP**1** (*154*). Although HSA-FeP**12** binds O_2 reversibly, HSA-FeP**11** was quickly oxidized by O_2. We concluded that the 1-methylcyclohexanamide groups are necessary for the tailed porphyrin to form an O_2 adduct complex under physiological conditions.

Investigations have also revealed that heme [Fe^{2+}protoporphyrin IX; protoheme] derivatives having a proximal base at the propionate side chain (FeP**13**−FeP**16**) were incorporated into HSA (*155, 156*). The oxidation process of HSA-FeP**13**(O_2) to the inactive ferric state obeyed first-order kinetics, suggesting that the μ-oxo dimer formation was prevented by the immobilization of FeP**13** into albumin. In fact, HSA-FeP**15** showed lower O_2 binding affinity [high $P_{50}(O_2)$] than the others did. The acetyl groups at the 3,8-positions of FeP**15** decrease the electron density of the porphyrin macrocycle, thereby reducing the O_2 binding affinity. Actually, HSA-FeP**16**, in which the His-Gly tail coordinates to the Fe^{2+} center, showed the most stable O_2 adduct complexes [$\tau_{1/2}(O_2)$, 90 min; pH 7.3; 25 °C] of any of these heme compounds.

3.2. Surface-Modified HSA-FeP with PEG. A remaining defect of HSA-FeP is that the active Fe^{2+} porphyrin sites dissociate slowly from HSA when infused into animals because FeP is bound noncovalently to albumin. One possible solution is to bind the FeP molecule covalently to the protein. We have synthesized FeP**17** having a succinimide side chain; it can react with the Lys amino group of HSA (*157*). The O_2 binding property of HSA-FeP**17** is almost identical to that of HSA-FeP**1**.

Another approach is surface modification with PEG. Actually, PEG decollations of proteins and liposomes are well-known to enhance their plasma half-life, thermostability, nonimmunogenicity, and solubility in organic solvents (*158−163*). We surmised that surface modification of HSA-FeP**2** by PEG might help to prolong the circulation lifetime of FeP**2** and retain its O_2 transporting ability in vivo for a long period. Consequently, HSA-FeP**2** (FeP**2**/HSA = 4/1, mol/mol) was modified with maleimide-PEG, and the solution properties, O_2 binding behavior, and circulatory persistence of the PEG-modified HSA-FeP**2** [PEG(HSA-FeP**2**)] were examined (*164*). A thiolation reagent, iminothiolane, first reacted with the Lys amino groups of HSA to create active thiols that bind to α-maleimide-ω-methoxy PEG (Figure 14a). Mass spectroscopy measurements and quantification of the mercapro group of PEG(HSA-FeP**2**) revealed the conjugation of six PEG chains on the HSA-FeP**2** surface. The initial FeP**2**/HSA ratio 4/1 (mol/mol) was unchanged after the PEG binding. The adjustment of viscosity is important to design an artificial O_2 carrier. Maintenance of viscosity is necessary to preserve shear stress on the vascular wall that prevents loss of the functional capillary density (*123, 165*). The viscosity and COP of PEG(HSA-FeP**2**) were modulated to some degree by changing the molecular weight of PEG [M_w: 2 × 10^3 (PEG_{M2}) and 5 × 10^3 (PEG_{M5})] (Figure 14b). In fact, PEG_{M2}(HSA-FeP**2**) showed almost identical values of viscosity

Table 6. O_2 Binding Properties of HSA-FePs in Phosphate Buffered Solution (pH 7.3, 25 °C)

FeP	$k_{on}(O_2)$ (μM^{-1} s^{-1})		$k_{off}(O_2)$ (ms^{-1})		$P_{50}(O_2)^a$ (Torr)	refs
	fast	slow	fast	slow		
1	34	9.5	0.75	0.20	13 (33)	139, 149, 150
2	46	7.3	0.98	0.16	13 (35)	150
3	36	6.1	0.059	0.010	1 (3)	150
4	54	8.8	0.089	0.014	1 (3)	150
5	54	6.8	0.02	0.0024	0.2 (1)	151
6	54	8.1	0.62	0.093	7 (22)	151
7	34	4.5	0.045	0.0059	0.8 (2)	152
8	11	1.5	0.50	0.069	28	153
9	11	2.0	0.41	0.076	23	153
10	8.9	2.3	0.34	0.088	23	153
12	29	4.4	1.10	0.16	22 (45)	154
13	–	–	–	–	0.1	155, 156
14	–	–	–	–	0.1	155, 156
15	–	–	–	–	0.4	156
16	–	–	–	–	0.1	155, 156
17	28	–	0.33	–	9 (27)	157

[a] At 37 °C in parentheses.

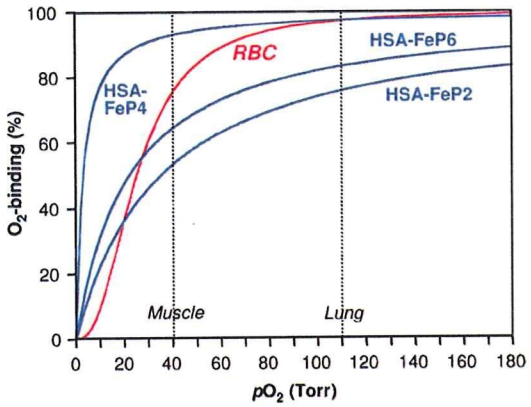


Figure 13. O_2 binding equilibrium curve of HSA-FePs under physiological conditions (pH 7.3, 37 °C).

and COP to those of the nonmodified HSA-FeP**2**. In contrast, PEG_{M5}(HSA-FeP**2**) showed a higher viscosity and more pronounced hyperoncotic property relative to those of HSA-FeP**2**. Nevertheless, PEG_{M5} conjugate may be useful as an efficient plasma expander (*118, 166*).

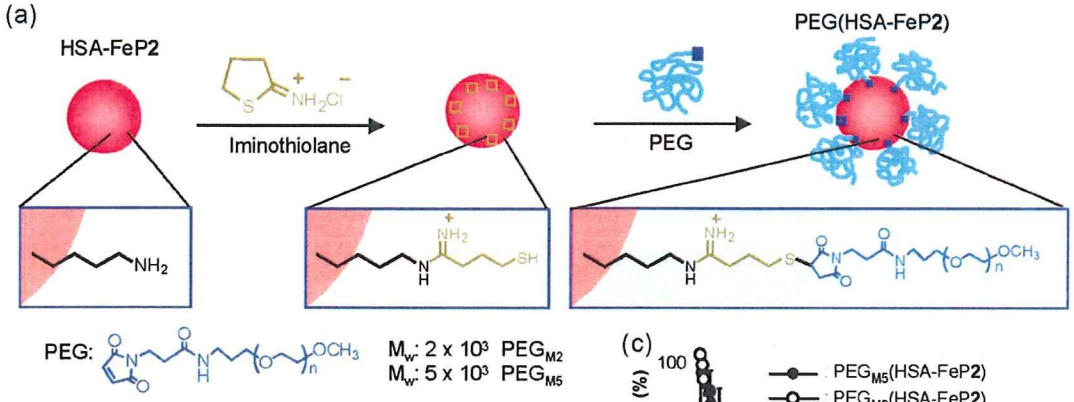
Under physiological conditions, PEG_{Mn}(HSA-FeP**2**) binds and releases O_2. The $P_{50}(O_2)$ values were almost identical to those of the original HSA-FeP**2**, indicating that the O_2 binding equilibrium was not influenced by the presence of the PEG chains (Figure 14b). Surface modification by PEG delays proton-driven oxidation of the O_2 adduct complex, giving HSA-FeP**2** the $\tau_{1/2}(O_2)$ of 12 h, which is almost equal to that of a natural hemoprotein, Mb [$\tau_{1/2}(O_2)$, 12 h; pH 7, 35 °C] (*167*). The conjugated PEG might change the local proton concentration of the HSA interior compared to the outer aqueous solution.

The circulation persistence of FeP**2** in the bloodstream was measured after administration of PEG_{Mn}(HSA-FeP**2**) to anesthetized rats (*164*). The PEG_{Mn}(HSA-FeP**2**) solution (20% volume of the circulatory blood) was injected intravenously into rats from the tail vein. The concentration decays of PEG_{Mn}(HSA-FeP**2**) in the blood showed single exponentials with half-life [$\tau_{1/2}$(FeP**2**)] of 13−16 h (Figure 14c). These values are considerably longer than those of the corresponding nonmodified HSA-FeP**1** (*168*). Surface modification of HSA-FeP**2** by PEG prevented the rapid clearance of the incorporated FeP**2**. On the basis of these findings, we can conclude that surface modification of HSA-FeP**2** by PEG comprehensively improved its O_2 transporting ability.

We then proceeded to evaluate physiological responses to an exchange transfusion with PEG_{M2}(HSA-FeP**2**) in an acute anemia rat model (*169*) (Figure 15). The animals were first placed in a 65 vol % hemodilution with 5 g/dL HSA. They subsequently underwent a 30 vol % blood replacement with the PEG_{M2}(HSA-FeP**2**) solution. As negative and positive control groups, a 5 g/dL HSA solution (HSA group) and washed RBC suspension (RBC group) were infused, respectively, to similarly operated rats in hemorrhage. The isovolemic 65% hemodilution with HSA reduced the Hb concentration, thereby decreasing the O_2 supply to the tissue. Consequently, the mean arterial pressure (MAP), renal cortical O_2 partial pressure [PtO_2(R)], and O_2 partial pressure of muscle tissue [PtO_2(M)] were decreased. During hemorrhagic shock by 30% bleeding, significant decreases in the MAP, venous O_2 pressure (PvO_2), PtO_2(R), and PtO_2(M) were observed by the loss of the circulation blood volume. The heart rate (HR) and respiration rate were also decreased. In contrast, arterial O_2 pressure (PaO_2) increased to about 160% of the basal value (b.v.). The arterial CO_2 pressure ($PaCO_2$) decreased to about 62% of the b.v.; the pH increased to 7.55.

The injection of the sample solutions increased the blood volume and improved the circulatory flow. Lactate was washed out from the tissues and into the circulatory system, which decreased the pH to the initial level of 7.43 in all groups. The administration of HSA restored no parameters: death occurred within 41 min. In contrast, the infusion of PEG_{M2}(HSA-FeP**2**) or RBC kept all the rats alive until the end of measurements. After injection of PEG_{M2}(HSA-FeP**2**), the animals showed marked and rapid recovery in MAP, HR, PaO_2, PvO_2, $PaCO_2$, and pH, resembling that shown in the RBC group. These results demonstrate the O_2 transporting capability of the PEG_{M2}(HSA-FeP**2**) solution as a resuscitative fluid. We observed that albumin-based oxygen carrier does not induce hypertensive action, because of its low permeability through the vascular endothelium in comparison with that of Hb molecules. The heart rate responses after the injection were also negligibly small. Visualization of the intestinal microcirculatory changes clearly revealed the widths of the venule and arteriole to be fairly constant (*170*).

Reversible oxygenation of PEG_{M2}(HSA-FeP**2**) was observed even in the solid state (*171*). The aqueous solution of PEG_{M2}(HSA-FeP**2**(CO)) complex was spread on the glass plate and dried overnight at room temperature, producing a red transparent solid membrane (Figure 16a). In contrast, HSA-FeP**2** without PEG decollation yielded a brittle membrane with many cracks. Scanning electron microscopy (SEM) observations of the PEG_{M2}(HSA-FeP**2**) membrane showed a uniform thickness of 15 μm and a smooth surface (Figure 16b). The $\tau_{1/2}(O_2)$ was 40 h, which is three times longer than the value in water. The



(b)

Solution and O_2 binding properties of PEG_{Mn}(HSA–FeP**2**)[a]

Samples	M_w of PEG	Viscosity (cP)	COP (Torr)	P_{50} (Torr)
PEG_{M2}(HSA–FeP**2**)	2 × 10^3	1.14	27	32
PEG_{M5}(HSA–FeP**2**)	5 × 10^3	2.34	65	31
HSA–FeP**2**	-	1.05	21	35

a) In phosphate buffered solution ([HSA]: 5g/dL, PEG_{Mn}/HSA–FeP**2**= 6, mol/mol) in average, pH 7.3, 37°C.

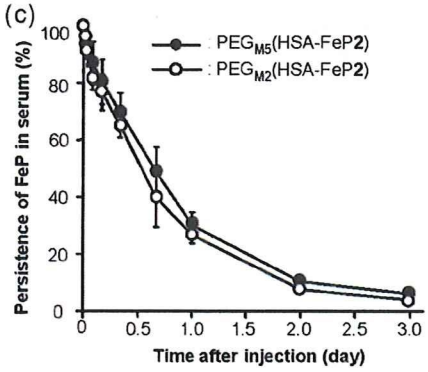


Figure 14. Surface modification of HSA-FeP with poly(ethylene glycol). (a) Synthetic scheme of PEG_{Mn}(HSA-FeP**2**). (b) Solution and O_2 binding properties. (c) Persistence of FeP**2** in serum after administration of PEG_{Mn}(HSA-FeP**2**) into Wistar rats. Each value represents the mean ± SD of four rats.

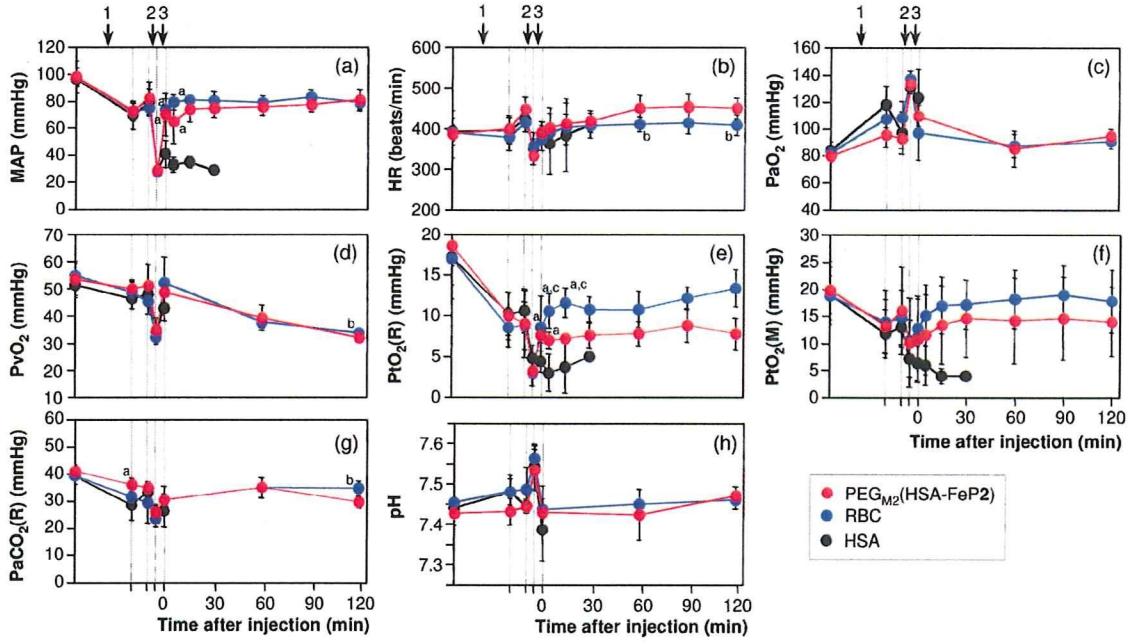


Figure 15. Effect of PEG_{M2}(HSA-FeP**2**) solutions on (a) MAP, (b) HR, (c) PaO_2, (d) PvO_2 (e) PtO_2(R), (f) PtO_2(M), (g) $PaCO_2$, and (h) pH in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SD of five rats [red, PEG_{M2}(HSA-FeP**2**) group; blue, washed RBC group; and black, HSA group]. Arrows (1), (2), and (3), respectively, indicate the periods of 65% hemodilution, 30% bleeding, and sample infusion. [a]$p < 0.05$ versus HSA group (Tukey-Kramer test), [b]$p < 0.05$ versus PEG_{M2}(HSA-Fe**2**) group (unpaired t-test), and [c]$p < 0.05$ versus PEG_{M2}(HSA-FeP**2**) group (Tukey-Kramer test).

O_2 binding affinity was about a half that of the monomeric PEG_{M2}(HSA-FeP**2**).

We subsequently added hyaluronic acid (HA) as a supporting polymer to the protein solution and prepared the solid membrane on a poly(styrene) dish. Actually, HA is known as a glycosaminoglycan component of connective tissues, hyaline bodies, and extracellular matrix (*172*). Water evaporation of the PEG_{M2}(HSA-FeP**2**)/HA mixture ([HSA]: 2.5 wt % and [HA]: 0.2 wt %) produced a uniform red solid membrane that was easily peeled from the dish, yielding a free-standing homogeneous thin film of the PEG(HSA-FeP**2**)/HA hybrid (Figure 16c,d).

The PEG_{M2}(HSA-FeP**2**) solution is useful as a valuable O_2-carrying plasma. Membranes of PEG_{M2}(HSA-FeP**2**) with micrometer thickness can serve as a RBC substitute that can be preserved anywhere and reproduced as a saline solution at any time.

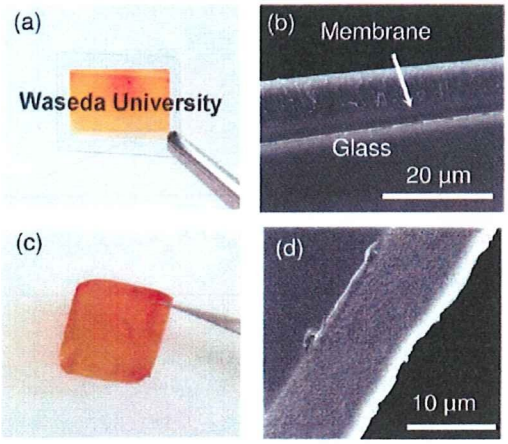


Figure 16. The solid membrane of PEG_{M2}(HSA-FeP). (a) Photograph of the membrane on the glass, (b) SEM of the membrane section, (c) photograph of the flexible film peeled from the poly(styrene) dish, and (d) SEM of the isolated film.

3.3. Recombinant HSA-Heme (rHSA-Heme) Prepared Using Site-Directed Mutagenesis. Hemin [Fe^{3+}protoporphyrin IX] released from metHb during enucleation of RBC or through hemolysis is captured by HSA with a high binding constant ($K \approx 10^8\ M^{-1}$) (*173*). Crystallographic studies have revealed that hemin is bound within a narrow D-shaped hydrophobic cavity in subdomain IB with axial coordination of Tyr-161 to the central ferric ion and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (Arg-114, His-146, and Lys-190) (Figure 17) (*174, 175*). In terms of the general hydrophobicity of this α-helical heme pocket, the subdomain IB of HSA potentially has similar features to the heme binding site of Hb or Mb. However, when one reduces HSA-hemin to obtain the ferrous complex, it is autoxidized rapidly by O_2, even at low temperature (~0 °C), because HSA lacks the proximal His, which, in Hb and Mb, enables the prosthetic heme group to bind O_2. Knowledge of the detailed architecture of the heme binding site in HSA enables us to design mutagenesis experiments to construct a tailor-made heme pocket for stable O_2 binding. Therefore, we used site-directed mutagenesis to introduce an His into the heme binding site that was expected to provide axial coordination to the central Fe^{2+} atom of the heme and thereby promote O_2 binding.

Results of our modeling experiments suggested that a favorable position for the axial imidazole insertion would be Ile-142 (Figure 17). The N_ϵ(His)-Fe distances were estimated as 2.31 Å for H142 (compared to 2.18 Å for Mb). We therefore designed and produced two single mutants I142H and a double mutant I142H/Y161L (HL) (*176*).

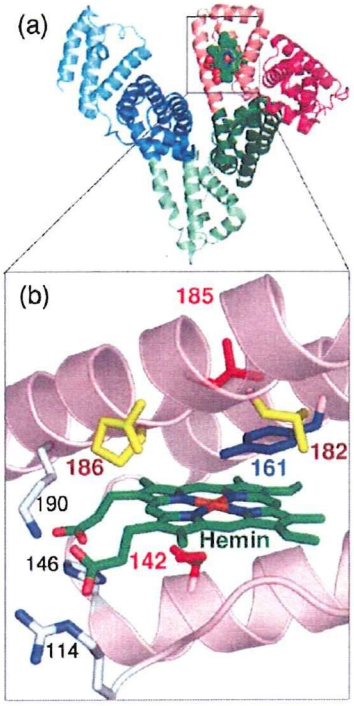
In the UV−vis absorption spectrum of rHSA(HL)-hemin, the ligand-to-metal charge transfer band at 625 nm was weakened because of the Y161L mutation. The MCD spectrum of rHSA(HL)-hemin showed a similar S-shaped pattern in the Soret band region resembling that of ferric Mb (*177, 178*). These results suggest that rHSA(HL)-hemin is in a predominantly ferric high-spin complex having a water molecule as the sixth ligand. The rHSA-hemin was easily reduced to the ferrous complex by adding a small molar excess of aqueous sodium dithionite under an Ar atmosphere (Figure 18). A single broad absorption band (λ_{max}: 559 nm) in the visible absorption spectrum and the MCD spectrum of rHSA(HL)-heme indicated the formation of a five-N-coordinate high-spin complex (*176, 177, 179*). The heme therefore appears to be accommodated in the mutated heme pocket with an axial coordination involving His-142. Upon exposure of rHSA(HL)-heme solution to O_2, the UV−vis absorption changed immediately to that of the O_2 adduct complex (Figure 18). It formed a carbonyl complex under a CO atmosphere. The single mutant rHSA(I142H)-heme, which retains Y161, was unable to bind O_2. The polar phenolate residue at the top of the porphyrin plane is likely to accelerate the proton-driven oxidation of the Fe^{2+} center. The replacement of Tyr-161 in rHSA(I142H)-heme by Leu enhanced stabilization of the O_2 adduct complex.

To evaluate the kinetics of O_2 and CO bindings to rHSA-hemes, laser flash photolysis experiments were carried out (Tables 7 and 8). It is noteworthy that the absorbance decay accompanying the CO recombination to rHSA(HL)-heme was composed of double-exponential profiles, which is normally not observed in Mb (the faster phase is defined as species I; the slower phase is defined as species II). The ratio of the amplitude of the species I and the species II was approximately 3:2. On the other hand, the rebinding of O_2 to rHSA(HL)-heme followed a simple monophasic decay. Numerous investigations of synthetic model hemes have helped to reveal the relation between the structure around the hemes and their O_2 and CO binding abilities (*4, 147, 148*). A bending strain in the proximal base coordination to the central Fe^{2+} atom, the "proximal-side steric (proximal pull) effect", is known to be capable of both increasing the dissociation rate for CO and decreasing the association rate. Simultaneously, it increases the O_2 dissociation rate without greatly altering the O_2 association kinetics. Consequently, one possible explanation for the existence of the two phases is that two different geometries of the axial His (His-142) coordination to the central ferrous ion of the heme might exist, each one accounting for a component of the biphasic kinetics of CO rebinding.

3.4. Modulation of O_2 Binding Property of rHSA-(mutant)−Heme. To control the O_2 binding affinity of rHSA-heme, we designed and produced diverse rHSA(mutant)−hemes in which bulky hydrophobic or hydrophilic amino acids were introduced around the O_2 binding site (Tyr-161, Leu-182, Leu-185, and Arg-186) (Figure 17). More recently, the beneficial effect of low-dose CO on the microcirculation by a hemoglobin-based artificial oxygen carrier has been discussed (*132, 182*). Control of the CO binding affinity of rHSA−heme is also tempting.

A. Substitution of Tyr-161 with Leu or Phe. The first, Tyr-161, was substituted to noncoordinating and hydrophobic amino acids (Leu or Phe). The O_2 and CO binding properties of rHSA(HL)−heme and rHSA(I142H/Y161F)−heme [rHSA(HF)−heme] showed that the presence of a Phe rather than a Leu at position 161 results in 6-fold and 4-fold increases in the O_2 binding affinity for species I and II, respectively (Table 7). This enhancement is mainly attributable to an increase in the O_2 association rate constant. The same trend was observed for CO binding [3-fold increase in k_{on}(CO)] (Table 8). The substitution of Leu-161 (102 $Å^3$) by Phe-161 (137 $Å^3$) (*183*) replaces an isopropyl group with a rigid benzyl group within the heme pocket. In rHSA(HL), the small side chain of Leu-161 might enable free rotation of the side chain of neighboring Leu-185, thereby reducing the volume on the distal side of the porphyrin plane (Figure 19a,b). On the other hand, the bulkier aromatic side chain of Phe-161 might prevent rotation of the isopropyl group of Leu-185 and thereby provide greater room of the distal pocket; this effect might provide easier access to the heme Fe^{2+} atom and account for the increased association rates for O_2 and CO.

B. Substitution of Leu-185 with Polar Amino Acid. Leu-185 was substituted with a more hydrophilic amino acid (Asn, Gln, or His), which was expected to interact with the coordinated O_2 by hydrogen bond and to stabilize the O_2 adduct complex similarly to Hb and Mb. In rHSA(mutant)−hemes in which Gln or His was introduced into Leu-185, they formed ferrous six-coordinated low-spin complexes under an Ar atmosphere. That



rHSA–heme	Position				
	142	161	182	185	186
Wild type	Ile	Tyr	Leu	Leu	Arg
I142H	***His***	Tyr	Leu	Leu	Arg
I142H/Y161L (HL)	***His***	***Leu***	Leu	Leu	Arg
I142H/Y161F (HF)	***His***	***Phe***	Leu	Leu	Arg
HL/L185N	***His***	***Leu***	Leu	***Asn***	Arg
HF/L185N	***His***	***Phe***	Leu	***Asn***	Arg
HL/L185Q	***His***	***Leu***	Leu	***Gln***	Arg
HF/L185Q	***His***	***Phe***	Leu	***Gln***	Arg
HF/L185H	***His***	***Phe***	Leu	***His***	Arg
HL/R186L	***His***	***Leu***	Leu	Leu	***Leu***
HL/R186F	***His***	***Leu***	Leu	Leu	***Phe***

Figure 17. (a) Crystal structure of HSA-hemin complex (1O9X) from ref 174. Hemin is shown in a space-filling representation. (b) Heme pocket structure in subdomain IB and positions of amino acids where site-specific mutations were introduced. Abbreviations of rHSA(mutant)s are shown in the table.

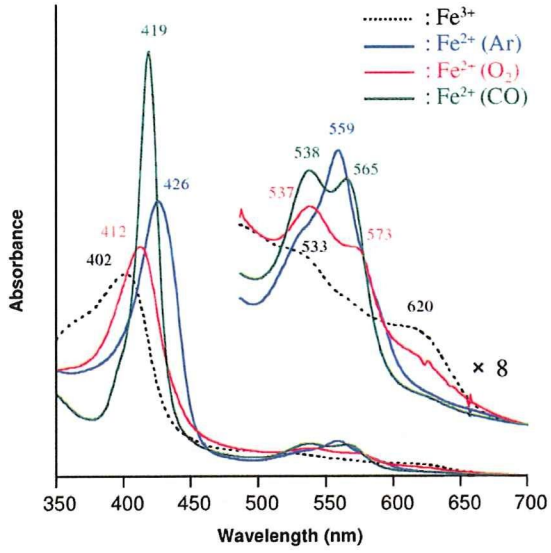


Figure 18. UV−vis absorption spectral changes of rHSA(HL)-heme in potassium phosphate buffered solution (pH 7.0).

result suggests that the introduced amino acid coordinates to the heme iron as a sixth ligand under an Ar atmosphere. Upon exposure of the solutions to O_2, they were oxidized. Bis-histidyl hemochromes are known to be oxidized by O_2 rapidly via an outer sphere mechanism (*184*−*186*). On the other hand, rHSA(HL/L185N)−heme and rHSA(HF/L185N)−heme in which Asn was introduced at Leu-185 formed ferrous five-coordinated high-spin complexes under an Ar atmosphere. They formed O_2 adduct complexes under O_2 atmosphere. The introduced Asn is estimated to be too far to coordinate to the heme.

Marked differences were apparent in a comparison of the O_2 and CO binding parameters for rHSA(HL)−heme and rHSA(HL/L185N)−heme. First, the presence of Asn rather than Leu at

Table 7. O_2 Binding Parameters of rHSA(Mutant)-Heme Complexes in Phosphate Buffered Solution (pH 7.0) at 22 °C

hemoproteins	$k_{on}(O_2)$ ($\mu M^{-1}s^{-1}$)	$k_{off}(O_2)$ ($m\ s^{-1}$)		$P_{50}(O_2)$ (Torr)	
		I	II	I	II
rHSA(HL)-heme	7.5	0.22	1.70	18	134
rHSA(HF)-heme	20	0.10	0.99	3	31
rHSA(HL/L185N)-heme	14	0.02	0.29	1	14
rHSA(HF/L185N)-heme	26	0.10	1.03	2	24
rHSA(HL/R186L)-heme	25	0.41	8.59	10	209
rHSA(HL/R186F)-heme	21	0.29	7.01	9	203
Mb[a]	14	0.012		0.51	
RBC[b]				8	

[a] Sperm whale myoglobin in 0.1 M potassium phosphate buffer (pH 7.0, 20 °C); ref 180. [b] Human red cell suspension in isotonic buffer (pH 7.4, 20 °C); ref 181.

Table 8. CO Binding Parameters of rHSA(Mutant)-Heme Complexes in Phosphate Buffered Solution (pH 7.0) at 22 °C

hemoproteins	$k_{on}(CO)$ ($\mu M^{-1}\ s^{-1}$)		$k_{off}(CO)$ (s^{-1})		$P_{50}(CO)$ (Torr)	
	I	II	I	II	I	II
rHSA(HL)-heme	2.0	0.27	0.013	0.079	0.0053	0.240
rHSA(HF)-heme	6.8	0.72	0.009	0.061	0.0011	0.068
rHSA(HL/L185N)-heme	6.8	1.60	0.008	0.039	0.0010	0.020
rHSA(HF/L185N)-heme	7.7	1.09	0.008	0.043	0.0008	0.032
rHSA(HL/R186L)-heme	5.0	0.57	0.011	0.165	0.0018	0.234
rHSA(HL/R186F)-heme	7.9	1.12	0.010	0.148	0.0010	0.107
Mb[a]	0.51		0.019		0.03	

[a] Sperm whale myoglobin in 0.1 M potassium phosphate buffer (pH 7.0, 20 °C); ref 180.

position 185 caused 2-fold and 3−6-fold increases, respectively, in the $k_{on}(O_2)$ and $k_{on}(CO)$ values. The Asn might partly rotate upward, which provides somewhat greater space of the distal pocket. Second, Asn-185 induced 18-fold and 10-fold increases in the O_2 binding affinity for species I and II, because the $k_{off}(O_2)$

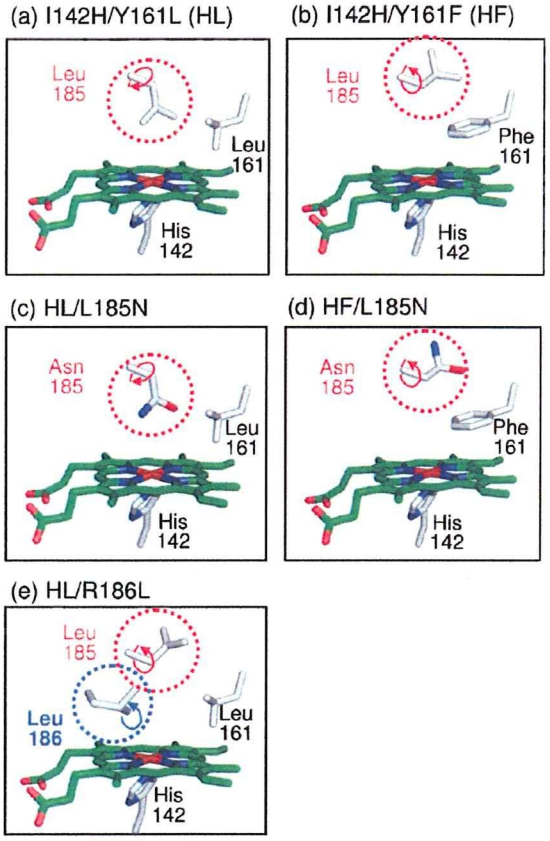


Figure 19. The proposed configuration of Leu-185 in (a) rHSA(HL)−heme and (b) rHSA(HF)−heme, Asn-185 in (c) rHSA(HL/L185N)−heme and (d) rHSA(HF/L185N)−heme, and Leu-186 in (e) rHSA(HL/R186L)-heme.

values were 1/6−1/11 of their former values. This corresponds to a free energy difference of −1.8 kcal mol^{-1} at 22 °C. The magnitude of the effect seems to be reasonable considering that, in HbO_2 and MbO_2, the distal His-64 stabilizes the coordinated O_2 by −0.6 to −1.4 kcal mol^{-1} because of the hydrogen bond (*187*). In contrast, The O_2 and CO binding parameters for rHSA(HF)−heme and rHSA(HF/L185N)−heme showed no significant differences. The bulky benzyl side chain of Phe-161 can prevent rotation of the polar amide group of Asn-185 and thereby decrease the effect of polarity and size on O_2 and CO binding parameters (Figure 19c,d) (*188*).

C. Substitution of Arg-186 with Leu or Phe. For administration into the human circulatory system, it would be better if the affinity were similar to the human RBC [$P_{50}(O_2)$: 8 Torr, 25 °C]. It is expected that providing a certain degree of hydrophobicity into the distal side of the heme by insertion of a nonpolar residue would reduce the O_2 binding affinity of the rHSA−heme complex. The most suitable position for that introduction might be at Arg-186, which is the entrance of the heme pocket and which is rather close to the central Fe(II) ion. Therefore, rHSA(HL/R186L)−hemin and rHSA(HL/R186F)−hemin were prepared. The O_2 dissociation rate constants of rHSA(HL/R186L)−heme and rHSA(HL/R186F)−heme were 3−4-fold higher than that of rHSA(HF)−heme, which reduced the O_2 binding affinities [larger $P_{50}(O_2)$]. This reduction might be attributable to the increased hydrophobicity in the distal pocket. The O_2 binding affinities of rHSA(HL/R186L)−heme [$P_{50}(O_2)$: 10 Torr] and rHSA(HL/R186F)−heme [$P_{50}(O_2)$: 9 Torr] have become equivalent to those of human RBC. The important structural factor in these mutants is Y161L, which enables the rotation of the isopropyl group of Leu-185 above the O_2 coordination site. Unexpectedly, the $k_{on}(O_2)$ and $k_{on}(CO)$ values of rHSA(HL/R186L)−heme and rHSA(HL/R186F)−heme were 3-fold and 3−4-fold higher than those of rHSA(HL)−heme and in the same range as that of rHSA(HF)−heme. In fact, Leu-161 is small, but the hydrophobic Leu-186 or Phe-186 might be integrated into the heme pocket from the entrance and might push up the neighboring Leu-185 residue (Figure 19e) (*188*).

We have engineered mutant rHSA−heme complexes that can bind O_2. Principal modifications to the heme pocket that are necessary to confer reversible O_2 binding are (i) replacement of Tyr-161 by hydrophobic amino acid (Leu or Phe), and (ii) introduction of His as a proximal base at position Ile-142. Furthermore, (iii) modification of the distal amino acid has a considerable effect on the modulation of O_2 and CO binding affinities.

4. CONCLUSIONS

The structures of our artificial O_2 carriers differ greatly from those of sophisticated RBCs. However, clear advantages of simplified artificial O_2 carriers are readily apparent: the absence of blood-type antigens and infectious viruses, stability for long-term storage at room temperature for any emergency, all of which overwhelm the functionality of RBCs. The shorter half-life of artificial O_2 carriers in the bloodstream (ca. 3 days) limits their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties such as $P_{50}(O_2)$ and viscosity supports their possible development of tailor-made O_2 carriers to suit various clinical indications. The achievements of ongoing research described above give us confidence in advancing the further development with the expectation of its eventual realization.

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LITERATURE CITED

(1) Perutz, M. F. (1970) Stereochemistry of cooperative effects in haemoglobin. *Nature 228*, 726–739.
(2) Jones, R. D., Summerville, A., and Basolo, F. (1979) Synthetic oxygen carriers related to biological systems. *Chem. Rev. 79*, 139–179.
(3) Collman, J. P. (1977) Synthetic model for the oxygen-binding hemoproteins. *Acc. Chem. Res. 10*, 265–272.
(4) Momenteau, M., and Reed, C. (1994) Synthetic heme dioxygen complexes. *Chem. Rev. 94*, 659–698.
(5) Collman, J. P., Gagne, R. R., Halbert, T. R., Marchon, J.-C., and Reed, C. A. (1973) Reversible oxygen adduct formation in ferrous complexes derived from "picket fence" porphyrins. A model for oxymyoglobin. *J. Am. Chem. Soc. 73*, 7868–7870.

(6) Collman, J. P., Gagne, R. R., Reed, C. A., Halbert, T. R., Lang, G., and Robinson, W. T. (1975) "Picket fence porphyrins." Synthetic models for oxygen binding hemoproteins. *J. Am. Chem. Soc. 97*, 1427–1439.
(7) Matsushita, Y., Hasegawa, E., Eshima, K., and Tsuchida, E. (1983) Synthesis of amphiphilic porphinatoiron complexes having phosphocholine groups. *Chem. Lett. 1983*, 1387–1389.
(8) Tsuchida, E. (1985) Liposome-embedded iron-porphyrins as an artificial oxygen carrier. *Ann. N.Y. Acad. Sci. 446*, 429–442.
(9) Tsuchida, E., and Nishide, H. (1986) Hemoglobin model - artificial oxygen carrier composed of porphinatoiron complexes. *Top. Curr. Chem. 1132*, 63–99.
(10) Komatsu, T., Moritake, M., Nakagawa, A., and Tsuchida, E. (2002) Self-organized lipid-porphyrin bilayer membranes in vesicular form: nanostructure, photophysical properties and dioxygen coordination. *Chem. Eur. J. 8*, 5469–5480.
(11) Komatsu, T., Ando, K., Kawai, N., Nishide, H., and Tsuchida, E. (1995) O_2-transport albumin - a new hybrid hemoprotein incorporating tetraphenylporphinatoiron(II). *Chem. Lett.* 813–814.
(12) Savitsky, J. P., Doczi, J., Black, J., and Arnold, J. D. (1978) A clinical safety trial of stroma-free hemoglobin. *Clin. Pharm. Ther. 23*, 73–80.
(13) Greenwald, R. B., Pendri, A., Martinez, A., Gilbert, C., and Bradley, P. (1996) PEG thiazolidine-2-thione, a novel reagent for facile protein modification: conjugation of bovine hemoglobin. *Bioconjugate Chem. 7*, 638–641.
(14) Kluger, R., and Li, X. (1997) Efficient chemical introduction of a disulfide cross-link and conjugation site into human hemoglobin at β-lysine-82 utilizing a bifunctional aminoacyl phosphate. *Bioconjugate Chem. 8*, 921–926.
(15) Sakai, H., Yuasa, M., Onuma, H., Takeoka, S., and Tsuchida, E. (2000) Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjugate Chem. 11*, 56–64.
(16) Hai, T. T., Pereira, D. E., Nelson, D. J., Catarello, J., and Srnak, A. (2000) Surface modification of diaspirin cross-linked hemoglobin (DCLHb) with chondroitin-4-sulfate derivatives. Part 1. *Bioconjugate Chem. 11*, 705–713.
(17) Manjula, B. N., Tsai, A., Upadhya, R., Perumalsamy, K., Smith, P. K., Malavalli, A., Vandegriff, K., Winslow, R. M., Intaglietta, M., Prabhakaran, M., Friedman, J. M., and Acharya, A. S. (2003) Site-specific PEGylation of hemoglobin at Cys-93(β): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate Chem. 14*, 464–472.
(18) Murray, J. A., Ledlow, A., Launspach, J., Evans, D., Loveday, M., and Conklin, J. L. (1995) The effects of recombinant human hemoglobin on esophageal motor function in humans. *Gastroenterology 109*1241–1248.
(19) Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, S. L., Arnone, A., and Walder, J. A. (1986) Isolation and characterization of a new hemoglobin derivative cross-linked between the a chains (lysine 99$\alpha_1 \gg$ lysine 99α_2). *J. Biol. Chem. 261*, 9929–9937.
(20) Iwashita, Y. (1991) Pyridoxalated hemoglobin-polyoxyethylene conjugate (PHP) as an O_2 carrier. *Artif. Organs Today 1*, 89–114.
(21) Riess, J. G. (2001) Oxygen carriers ("blood substitutes") - raison d'etre, chemistry, and some physiology. *Chem. Rev. 101*, 2797–2920.
(22) de Figueiredo, L. F., Mathru, M., Solanki, D., Macdonald, V. W., Hess, J., and Kramer, G. C. (1997) Pulmonary hypertension and systemic vasoconstriction might offset the benefits of acellular hemoglobin blood substitutes. *J. Trauma 42*, 847–854.
(23) Tsai, A. G., Kerger, H., and Intaglietta, M. (1995) Microcirculatory consequences of blood substitution with $\alpha\alpha$-hemoglobin. In *Blood Substitutes: Physiological Basis of Efficacy* (Winslow, R. M., Vandegriff, K., Intaglietta, M., Eds.) pp155–174, Birkhauser, Boston.
(24) Goda, N., Suzuki, K., Naito, M., Takeoka, S., Tsuchida, E., Ishimura, Y., Tamatani, T., and Suematsu, M. (1998) Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J. Clin. Invest. 101*, 604–612.
(25) Sakai, H., Hara, H., Yuasa, M., Tsai, A. G., Takeoka, S., Tsuchida, E., and Intaglietta, M. (2000) Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol. 279*, H908–H915.
(26) Burhop, K., Gordon, D., and Estep, T. (2004) Review of hemoglobin-induced myocardial lesions. *Artif. Cells Blood Substit. Immobil. Biotechnol. 32*, 353–374.
(27) Neragi-Miandoab, S., and Vlahakes, G. J. (2006) Elevated troponin I level with hemoglobin based oxygen carrying solutions (HBOCs) as a priming solution despite improved left ventricular function. *Interact. Cardiovasc. Thorac. Surg. 5*, 135–138.
(28) Natanson, C., Kern, S. J., Lurie, P., Banks, S. M., and Wolfe, S. M. (2008) Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA 299*, 2304–2312.
(29) Balla, J., Jacob, H. S., Balla, G., Nath, K., Eaton, J. W., and Vercellotti, G. M. (1993) Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc. Natl. Acad. Sci. U.S.A. 90*, 9285–9289.
(30) Sakai, H., Sato, A., Masuda, K., Takeoka, S., and Tsuchida, E. (2008) Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J. Biol. Chem. 283*, 1508–1517.
(31) Sakai, H., Sato, A., Sobolewski, P., Takeoka, S., Frangos, J. A., Kobayashi, K., Intaglietta, M., and Tsuchida, E. (2008) NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers. *Biochim. Biophys. Acta 1784*, 1441–1447.
(32) Martini, J., Cabrales, P., Tsai, A. G., and Intaglietta, M. (2006) Mechanotransduction and the homeostatic significance of maintaining blood viscosity in hypotension, hypertension and haemorrhage. *J. Intern. Med. 259*, 364–372.
(33) Sakai, H., Suzuki, Y., Kinoshita, M., Takeoka, S., Maeda, N., and Tsuchida, E. (2003) O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs. *Am. J. Physiol. Heart Circ. Physiol. 285*, H2543–H2555.
(34) Vandegriff, K. D., and Olson, J. S. (1984) The kinetics of O_2 release by human red blood cells in the presence of external sodium dithionite. *J. Biol. Chem. 259*, 12609–12618.
(35) Chang, T. M. S. (2005) Therapeutic applications of polymeric artificial cells. *Nat. Rev. Drug Discov. 4*, 221–235.
(36) Toyoda, T. (1965) Artificial blood. *Kagaku 35*, 7–13 (in Japanese).
(37) Kimoto, S., Hori, M., Toyoda, T., and Sekiguchi, W. (1968) Artificial red cells. *Gekachiryou (Surgical Therapy) 19*, 324–332 (in Japanese).
(38) Bangham, A. D., and Horne, R. W. (1964) Negative staining of phospholipids and their structure modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol. 8*, 660–668.
(39) Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol. 13*, 238–252.
(40) Djordjevich, L., and Miller, I. F. (1977) Lipid encapsulated hemoglobin as a synthetic erythrocyte. *Fed. Proc. 36*, 567–571.
(41) Farmer, M. C., Rudolph, A. S., Vandegriff, K. D., Hayre, M. D., Bayne, S. A., and Johnson, S. A. (1988) Liposome-encapsulated hemoglobin: oxygen binding properties and respiratory function. *Biomater. Artif. Cells Artif. Organs 16*, 289–299.
(42) Rudolph, A. S., Klipper, R. W., Goins, B., and Phillips, W. T. (1991) In vivo biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. *Proc. Natl. Acad. Sci. U.S.A. 88*, 10976–10980.

(43) Phillips, W. T., Klipper, R. W., Awasthi, V. D., Rudolph, A. S., Cliff, R., Kwasiborski, V., and Goins, B. A. (1999) Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. *J. Pharmacol. Exp. Ther. 288*, 665–670.

(44) Usuba, A., Osuka, F., Kimura, T., Sato, R., Ogata, Y., Gotoh, H., Kimura, T., and Fukui, H. (1998) Effect of liposome-encapsulated hemoglobin, neo red cells, on hemorrhagic shock. *Surg. Today 28*, 1027–1035.

(45) Yoshioka, H. (1991) Surface modification of haemoglobin-containing liposomes with polyethylene glycol prevents liposome aggregation in blood plasma. *Biomaterials 12*, 861–864.

(46) Sakai, H., Hamada, K., Takeoka, S., Nishide, H., and Tsuchida, E. (1996) Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol. Prog. 12*, 119–125.

(47) Takeoka, S., Ohgushi, T., Terase, K., Ohmori, T., and Tsuchida, E. (1996) Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir 12*, 1755–1759.

(48) Sou, K., Naito, Y., Endo, T., Takeoka, S., and Tsuchida, E. (2003) Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol. Prog. 19*, 1547–1552.

(49) Sakai, H., Takeoka, S., Yokohama, H., Seino, Y., Nishide, H., and Tsuchida, E. (1993) Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif. 4*, 563–569.

(50) Abe, H., Ikebuchi, K., Hirayama, J., Fujihara, M., Takeoka, S., Sakai, H., Tsuchida, E., and Ikeda, H. (2001) Virus inactivation in hemoglobin solution by heat treatment. *Artif. Cells Blood Substit. Immobil. Biotechnol. 29*, 381–388.

(51) Chung, J., Hamada, K., Sakai, H., Takeoka, S., and Tsuchida, E. (1995) Ligand-exchange reaction of carbonylhemoglobin to oxyhemoglobin in a hemoglobin liquid membrane. *Nippon Kagaku Kaishi 2*, 123–127 (in Japanese).

(52) Takeoka, S., Sakai, H., Kose, T., Mano, Y., Seino, Y., Nishide, H., and Tsuchida, E. (1997) Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. *Bioconjugate Chem. 8*, 539–544.

(53) Teramura, Y., Kanazawa, H., Sakai, H., Takeoka, S., and Tsuchida, E. (2003) Prolonged oxygen-carrying ability of hemoglobin vesicles by coencapsulation of catalase *in vivo*. *Bioconjugate Chem. 14*, 1171–1176.

(54) Sakai, H., Masada, Y., Onuma, H., Takeoka, S., and Tsuchida, E. (2004) Reduction of methemoglobin via electron transfer from photoreduced flavin: restoration of O_2-binding of concentrated hemoglobin solution coencapsulated in phospholipid vesicles. *Bioconjugate Chem. 15*, 1037–1045.

(55) Atoji, T., Aihara, M., Sakai, H., Tsuchida, E., and Takeoka, S. (2006) Hemoglobin vesicles containing methemoglobin and L-tyrosine to suppress methemoglobin formation *in vitro* and *in vivo*. *Bioconjugate Chem. 17*, 1241–1245.

(56) Sakai, H., Takeoka, S., Seino, Y., and Tsuchida, E. (1994) Suppression of methemoglobin formation by glutathione in a concentrated hemoglobin solution and in a Hb-vesicle. *Bull. Chem. Soc. Jpn. 67*, 1120–1125.

(57) Rameez, S., Alosta, H., and Palmer, A. F. (2008) Biocompatible and biodegradable polymersome encapsulated hemoglobin: a potential oxygen carrier. *Bioconjugate Chem. 19*, 1025–1032.

(58) Zhao, J., Liu, C. S., Yuan, Y., Tao, X. Y., Shan, X. Q., Sheng, Y., and Wu, F. (2007) Preparation of hemoglobin-loaded nano-sized particles with porous structure as oxygen carriers. *Biomaterials 28*, 1414–1422.

(59) Rudolph, A. S. (1988) The freeze-dried preservation of liposome encapsulated hemoglobin: a potential blood substitute. *Cryobiology 25*, 277–284.

(60) Rabinovici, R., Rudolph, A. S., Vernick, J., and Feuerstein, G. (1994) Lyophilized liposome encapsulated hemoglobin: evaluation of hemodynamic, biochemical, and hematologic responses. *Crit. Care Med. 22*, 480–485.

(61) Ringsdorf, H., Schlarb, B., and Venzmer, J. (1988) Molecular architecture and function of polymeric oriented systems - models for the study of organization, surface recognition, and dynamics of biomembranes. *Angew. Chem., Int. Ed. 27*, 113–158.

(62) Kato, A., Arakawa, M., and Kondo, T. (1984) Preparation and stability of liposome-type artificial red blood cells stabilized with carboxymethylchitin. *J. Microencapsul. 1*, 105–112.

(63) Mobed, M., Nishiya, T., and Chang, T. M. (1992) Preparation of carboxymethylchitin-incorporated submicron bilayer-lipid membrane artificial cells (liposomes) encapsulating hemoglobin. *Biomater. Artif. Cells Immobil. Biotechnol. 20*, 365–368.

(64) Li, S., Nickels, J., and Palmer, A. F. (2005) Liposome-encapsulated actin-hemoglobin (LEAcHb) artificial blood substitutes. *Biomaterials 26*, 3759–3769.

(65) Tsuchida, E., Hasegawa, E., Kimura, N., Hatashita, M., and Makino, C. (1992) Polymerization of unsaturated phospholipids as large unilamellar liposomes at low temperature. *Macromolecules 25*, 2007–212.

(66) Sakai, H., Takeoka, S., Yokohama, H., Nishide, H., and Tsuchida, E. (1992) Encapsulation of Hb into unsaturated lipid vesicles and gamma-ray polymerization. *Polym. Adv. Technol. 3*, 389–394.

(67) Akama, K., Awai, K., Yano, Y., Tokuyama, S., and Nakano, Y. (2000) In vitro and in vivo stability of polymerized mixed liposomes composed of 2,4-octadecadienoyl groups of phospholipids. *Polym. Adv. Technol 11*, 280–287.

(68) Sakai, H., Takeoka, S., Park, S. I., Kose, T., Nishide, H., Izumi, Y., Yoshizu, A., Kobayashi, K., and Tsuchida, E. (1997) Surface-modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. *Bioconjugate Chem. 8*, 23–30.

(69) Sakai, H., Tsai, A. G., Kerger, H., Park, S. I., Takeoka, S., Nishide, H., Tsuchida, E., and Intaglietta, M. (1998) Subcutaneous microvascular responses to hemodilution with red cell substitutes consisting of polyethylene glycol-modified vesicles encapsulating hemoglobin. *J. Biomed. Mater. Res. 40*, 66–78.

(70) Sakai, H., Okamoto, M.,; Ikeda E., Horinouchi H., Kobayashi K., and Tsuchida, E. (2008) Histopathological changes of rat brain after direct injection of hemoglobin vesicles (oxygen carriers) and neurological impact in an intracerebral hemorrhage model. *J. Biomed. Mater. Res., Part A* (in press).

(71) Sakai, H., Tomiyama, K., Sou, K., Takeoka, S., and Tsuchida, E. (2000) Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin vesicles as oxygen carriers in a liquid state. *Bioconjugate Chem. 11*, 425–432.

(72) Sou, K., Endo, T., Takeoka, S., and Tsuchida, E. (2000) Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjugate Chem. 11*, 372–379.

(73) Sato, T., Sakai, H., Sou, K., Buchner, R., and Tsuchida, E. (2007) Poly(ethylene glycol)-conjugated phospholipids in aqueous micellar solutions: hydration, static structure, and interparticle interactions. *J. Phys. Chem. B 111*, 1393–1401.

(74) Szebeni, J. (2005) Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. *Toxicology 216*, 106–121.

(75) Van de Velde, M., Wouters, P. F., Rolf, N., Van Aken, H., and Vandermeersch, E. (1998) Comparative hemodynamic effects of three different parenterally administered lipid emulsions in conscious dogs. *Crit. Care Med. 26*, 132–137.

(76) Vercellotti, G. M., Hammerschmidt, D. E., Craddock, P. R., and Jacob, H. S. (1982) Activation of plasma complement by perfluorocarbon artificial blood: probable mechanism of adverse pulmonary reactions in treated patients and rationale for corticosteroids prophylaxis. *Blood 59*, 1299–1304.

(77) Phillips, W. T., Klipper, R., Fresne, D., Rudolph, A. S., Javors, M., and Goins, B. (1997) Platelet reactivity with liposome-encapsulated hemoglobin in the rat. *Exp. Hematol. 25*, 1347–1356.

(78) Szebeni, J., Fontana, J. L., Wassef, N. M., Mongan, P. D., Morse, D. S., Dobbins, D. E., Stahl, G. L., Bunger, R., and Alving, C. R. (1999) Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. *Circulation 99*, 2302–2309.

(79) Izumi, Y., Sakai, H., Kose, T., Hamada, K., Takeoka, S., Yoshizu, A., Horinouchi, H., Kato, R., Nishide, H., Tsuchida, E., and Kobayashi, K. (1997) Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J. 43*, 289–297.

(80) Loughrey, H. C., Bally, M. B., Reinish, L. W., and Cullis, P. R. (1990) The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. *Thromb. Haemost. 64*, 172–176.

(81) Chonn, A., Cullis, P. R., and Devine, D. V. (1991) The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol. 146*, 4234–4241.

(82) Sou, K., and Tsuchida, E. (2008) Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: effect of the structure of acidic groups. *Biochim. Biophys. Acta - Biomembr. 1778*, 1035–1041.

(83) Abe, H., Azuma, H., Yamaguchi, M., Fujihara, M., Ikeda, H., Sakai, H., Takeoka, S., and Tsuchida, E. (2007) Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. *Artif. Cells Blood Substit. Biotechnol. 35*, 157–172.

(84) Abe, H., Fujihara, M., Azuma, H., Ikeda, H., Ikebuchi, K., Takeoka, S., Tsuchida, E., and Harashima, H. (2006) Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: effects on coagulation, kallikrein-kinin, and complement systems. *Artif. Cells Blood Substit. Immobil. Biotechnol. 34*, 1–10.

(85) Wakamoto, S., Fujihara, M., Abe, H., Yamaguchi, M., Azuma, H., Ikeda, H., Takeoka, S., and Tsuchida, E. (2005) Effects of hemoglobin vesicles on resting and agonist-stimulated human platelets *in vitro*. *Artif. Cells Blood Substit. Immobil. Biotechnol. 33*, 101–111.

(86) Wakamoto, S., Fujihara, M., Abe, H., Sakai, H., Takeoka, S., Tsuchida, E., Ikeda, H., and Ikebuchi, K. (2001) Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on agonist-induced platelet aggregation and RANTES release *in vitro*. *Artif. Cells Blood Substit. Immobil. Biotechnol. 29*, 191–201.

(87) Takeoka, S., Mori, K., Ohkawa, H., Sou, K., and Tsuchida, E. (2000) Synthesis and assembly of poly(ethylene glycol)-lipids with mono-, di-, and tetraacyl chains and a poly(ethylene glycol) chain of various molecular weights. *J. Am. Chem. Soc. 122*, 7927–7935.

(88) Okamura, Y., Maekawa, I., Teramura, Y., Maruyama, H., Handa, M., Ikeda, Y., and Takeoka, S. (2005) Hemostatic effects of phospholipid vesicles carrying fibrinogen gamma chain dodecapeptide *in vitro* and *in vivo*. *Bioconjugate Chem. 16*, 1589–1596.

(89) Ohkawa, H., Teramura, Y., Takeoka, S., and Tsuchida, E. (2000) Synthesis of multiacyl poly(ethylene glycol) for the conjugation of cytochrome c to phospholipid vesicle. *Bioconjugate Chem. 11*, 815–821.

(90) Sou, K., Goins, B., Takeoka, S., Tsuchida, E., and Phillips, W. T. (2007) Selective uptake of surface-modified phospholipid vesicles by bone marrow macrophages *in vivo*. *Biomaterials 28*, 2655–2666.

(91) Teramura, Y., and Iwata, H. (2008) Islets surface modification prevents blood-mediated inflammatory responses. *Bioconjugate Chem. 19*, 1389–1395.

(92) Obata, Y., Suzuki, D., and Takeoka, S. (2008) Evaluation of cationic assemblies constructed with amino acid based lipids for plasmid DNA delivery. *Bioconjugate Chem. 19*, 1055–1063.

(93) Sou, K., Inenaga, S., Takeoka, S., and Tsuchida, E. (2008) Loading of curcumin into macrophages using lipid-based nanoparticles. *Int. J. Pharm. 352*, 287–293.

(94) Sou, K., Klipper, R., Goins, B., Tsuchida, E., and Phillips, W. T. (2005) Circulation kinetics and organ distribution of Hb vesicles developed as a red blood cell substitute. *J. Pharmacol. Exp. Ther. 312*, 702–709.

(95) Bradley, A. J., Devine, D. V., Ansell, S. M., Janzen, J., and Brooks, D. E. (1998) Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch. Biochem. Biophys. 357*, 185–194.

(96) Hu, Q., and Liu, D. (1996) Co-existence of serum-dependent and serum-independent mechanisms for liposome clearance and involvement of non-Kupffer cells in liposome uptake by mouse liver. *Biochim. Biophys. Acta 1284*, 153–161.

(97) Shibuya-Fujiwara, N., Hirayama, F., Ogata, Y., Ikeda, H., and Ikebuchi, K. (2001) Phagocytosis *in vitro* of polyethylene glycol-modified liposome-encapsulated hemoglobin by human peripheral blood monocytes plus macrophages through scavenger receptors. *Life Sci. 70*, 291–300.

(98) Sakai, H., Horinouchi, H., Tomiyama, K., Ikeda, E., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am. J. Pathol. 159*, 1079–1088.

(99) Sakai, H., Horinouchi, H., Masada, Y., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2004) Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials 25*, 4317–4325.

(100) Sakai, H., Masada, Y., Horinouchi, H., Ikeda, E., Sou, K., Takeoka, S., Suematsu, M., Takaori, M., Kobayashi, K., and Tsuchida, E. (2004) Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J. Pharmacol. Exp. Ther. 311*, 874–884.

(101) Sakai, H., Horinouchi, H., Yamamoto, M., Ikeda, E., Takeoka, S., Takaori, M., Tsuchida, E., and Kobayashi, K. (2006) Acute 40% exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. *Transfusion 46*, 339–347.

(102) Sakai, H., Seishi, Y., Obata, Y., Takeoka, S., Horinouichi, H., Tsuchida, E., and Kobayashi, K. (2009) Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: Profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days, *Shock 31*, 192−200.

(103) Yamaoka, T., Tabata, Y., and Ikada, Y. (1994) Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *J. Pharm. Sci. 83*, 601–606.

(104) Ohki, N., Kimura, T., and Ogata, Y. (1998) The reduction of methemoglobin in Neo Red Cell. *Artif. Cells Blood Substit. Immobil. Biotechnol. 26*, 477–485.

(105) Sakai, H., Tsai, A. G., Rohlfs, R. J., Hara, H., Takeoka, S., Tsuchida, E., and Intaglietta, M. (1999) Microvascular responses to hemodilution with Hb vesicles as red cell substitutes: Influences of O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol. 276*, H553−H562.

(106) Wang, L., Morizawa, K., Tokuyama, S., Satoh, T., and Tsuchida, E. (1992) Modulation of oxygen-carrying capacity of artificial red cells (ARC). *Polym. Adv. Technol. 4*, 8–11.

(107) Cabrales, P., Sakai, H., Tsai, A. G., Takeoka, S., Tsuchida, E., and Intaglietta, M. (2005) Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am. J. Physiol. Heart Circ. Physiol. 288*, H1885−H1892.

(108) Izumi, Y., Sakai, H., Hamada, K., Takeoka, S., Yamahata, T., Kato, R., Nishide, H., Tsuchida, E., and Kobayashi, K. (1996) Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical oxygen tension. *Crit. Care Med. 24*, 1869–1873.

(109) Sakai, H., Takeoka, S., Wettstein, R., Tsai, A. G., Intaglietta, M., and Tsuchida, E. (2002) Systemic and Microvascular responses to the hemorrhagic shock and resuscitation with Hb vesicles. *Am. J. Physiol. Heart Circ. Physiol. 283*, H1191–H1199.

(110) Sakai, H., Masada, Y., Horinouchi, H., Yamamoto, M., Ikeda, E., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2004) Hemoglobin vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit. Care Med. 32*, 539–545.

(111) Yoshizu, A., Izumi, Y., Park, S. I., Sakai, H., Takeoka, S., Horinouchi, H., Ikeda, E., Tsuchida, E., and Kobayashi, K. (2004) Hemorrhagic shock resuscitation with an artificial oxygen carrier hemoglobin vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma necrosis factor alpha (TNFα). *ASAIO J. 50*, 458–463.

(112) Terajima, K., Tsueshita, T., Sakamoto, A., and Ogawa, R. (2006) Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. *Shock 25*, 184–189.

(113) Yamazaki, M., Aeba, R., Yozu, R., and Kobayashi, K. (2006) Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. *Circulation 114* (1 Suppl), I220–I225.

(114) Tsai, A. G., Vandegriff, K. D., Intaglietta, M., and Winslow, R. M. (2003) Targeted O_2 delivery by low-P_{50} hemoglobin: a new basis for O_2 therapeutics. *Am. J. Physiol. Heart Circ. Physiol. 285*, H1411–H1419.

(115) Plock, J. A., Tromp, A. E., Contaldo, C., Spanholtz, T., Sinovcic, D., Sakai, H., Tsuchida, E., Leunig, M., Banic, A., and Erni, D. (2007) Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue. *Crit. Care Med. 35*, 899–905.

(116) Nolte, D., Pickelmann, S., Lang, M., Keipert, P., and Messmer, K. (2000) Compatibility of different colloid plasma expanders with perflubron emulsion: an intravital microscopic study in the hamster. *Anesthesiology 93*, 1261–1270.

(117) Jouan-Hureaux, V., Audonnet-Blaise, S., Lacatusu, D., Krafft, M. P., Dewachter, P., Cauchois, G., Stoltz, J. F., Longrois, D., and Menu, P. (2006) Effects of a new perfluorocarbon emulsion on human plasma and whole-blood viscosity in the presence of albumin, hydroxyethyl starch, or modified fluid gelatin: an in vitro rheologic approach. *Transfusion 46*, 1892–1898.

(118) Vandegriff, K. D., McCarthy, M., Rohlfs, R. J., and Winslow, R. M. (1997) Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated. *Biophys. Chem. 69*, 23–30.

(119) Kobayashi, K. (2006) Summary of recombinant human serum albumin development. *Biologicals 34*, 55–59.

(120) Meyuhas, D., Nir, S., and Lichtenberg, D. (1996) Aggregation of phospholipid vesicles by water-soluble polymers. *Biophys. J. 71*, 2602–2612.

(121) Neu, B., and Meiselman, H. J. (2002) Depletion-mediated red blood cell aggregation in polymer solutions. *Biophys. J. 83*, 2482–2490.

(122) Sakai, H., Sato, A., Takeoka, S., and Tsuchida, E. (2007) Rheological property of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma substitute aqueous solutions. *Langmuir 23*, 8121–8128.

(123) Tsai, A. G., Friesenecker, B., McCarthy, M., Sakai, H., and Intaglietta, M. (1998) Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. *Am. J. Physiol. Heart Circ. Physiol. 275*, H2170–H2180.

(124) Intaglietta, M., Cabrales, P., and Tsai, A. G. (2006) Microvascular perspective of oxygen-carrying and -noncarrying blood substitutes. *Annu. Rev. Biomed. Eng. 8*, 289–321.

(125) Rebel, A., Ulatowski, J. A., Kwansa, H., Bucci, E., and Koehler, R. C. (2003) Cerebrovascular response to decreased hematocrit: effect of cell-free hemoglobin, plasma viscosity, and CO_2. *Am. J. Physiol. Heart Circ. Physiol. 285*, H1600–H1608.

(126) Dimino, M. L., and Palmer, A. F. (2007) High O_2 affinity hemoglobin-based oxygen carriers synthesized via polymerization of hemoglobin with ring-opened 2-chloroethyl-β-D-fructopyranoside and 1-o-octyl-β-D-glucopyranoside. *Biotechnol. Bioeng. 97*, 462–472.

(127) Plock, J. A., Contaldo, C., Sakai, H., Tsuchida, E., Leunig, M., Banic, A., Menger, M. D., and Erni, D. (2005) Is the Hb in Hb vesicles infused for isovolemic hemodilution necessary to improve oxygenation in critically ischemic hamster skin? *Am. J. Physiol. Heart Circ. Physiol. 289*, H2624–H2631.

(128) Contaldo, C., Plock, J., Sakai, H., Takeoka, S., Tsuchida, E., Leunig, M., Banic, A., and Erni, D. (2005) New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue. *Crit. Care Med. 33*, 806–812.

(129) Verdu, E. F., Bercik, P., Huang, X. X., Lu, J., Al-Mutawaly, N., Sakai, H., Tompkins, T. A., Croitoru, K., Tsuchida, E., Perdue, M., and Collins, S. M. (2008) The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection. *Am. J. Physiol. Gastrointest. Liver Physiol. 295*, G664–G670.

(130) Komatsu, H., Furuya, T., Sato, N., Ohta, K., Matsuura, A., Ohmura, T., Takagi, S., Matsuura, M., Yamashita, M., Itoda, M., Itoh, J., Horinouchi, H., and Kobayashi, K. (2007) Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. *Neurosci. Lett. 421*, 121–125.

(131) Yamamoto, M., Izumi, Y., Horinouchi, H., Teramura, Y., Sakai, H., Kohno, M., Watanabe, M., Kawamura, M., Adachi, T., Ikeda, E., Takeoka, S., Tsuchida, E., Kobayashi, K. (2009) Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation. *J. Surg. Res.* doi:10.1016/j.jss. 2007 12 770.

(132) Sakai, H., Horinouchi, H., Tsuchida, E., Kobayashi, K. (2008) Hemoglobin-vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model. *Shock.* doi:10 1097/SHK.0b013e318189017a.

(133) Peters, T. (1996) *All about Albumin*: Biochemistry, Genetics and Medical Applications, Academic Press, San Diego.

(134) He, X. M., and Carter, D. C. (1992) Atomic structure and chemistry of human serum albumin. *Nature 358*, 209–215.

(135) Carter, D. C., and Ho, J. X. (1994) Structure of serum albumin. *Adv. Protein Chem. 45*, 153–203.

(136) Curry, S., Madelkow, H., Brick, P., and Franks, N. P. (1998) Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Biol. 5*, 827–835.

(137) Bhattacharya, A. A., Grune, T., and Curry, S. (2000) Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J. Mol. Biol. 303*, 721–732.

(138) Ghuman, J., Zunszain, P. A., Petitpas, I., Bhattacharya, A. A., Otagiri, M., and Curry, S. (2007) Structural basis of the drug-binding specificity of humans serum albumin. *J. Mol. Biol. 353*, 38–52.

(139) Tsuchida, E., Komatsu, T., Matsukawa, Y., Hamamatsu, K., and Wu, J. (1999) Human serum albumin incorporating tetrakis(o-pivalamido)phenyl porphinatoiron(II) derivative as a totally synthetic O_2-carrying hemoprotein. *Bioconjugate Chem. 10*, 797–802.

(140) Komatsu, T., Hamamatsu, K., Wu, J., and Tsuchida, E. (1999) Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis-(o-pivalamido) phenylporphyrinatoiron(II) derivatives. *Bioconjugate Chem. 10*, 82–86.

(141) Tsuchida, E., Komatsu, T., Yanagimoto, T., and Sakai, H. (2003) Preservation stability and *in vivo* administration of albumin-heme hybrid solution as an entirely synthetic O_2-carrier. *Polym. Adv. Technol. 13*, 848–850.

(142) Huang, Y., Komatsu, T., Nakagawa, A., Tsuchida, E., and Kobayashi, S. (2003) Compatibility *in vitro* of albumin-heme

(O_2 carrier) with blood cell components. *J. Biomed. Mater. Res. 66A*, 292–297.

(143) Komatsu, T., Huang, Y., Wakamoto, S., Abe, H., Fujihara, M., Azuma, H., Ikeda, H., Yamamoto, H., Horinouchi, H., and Kobayashi, K. (2007) Influence of O_2-carrying plasma hemoprotein "albumin-heme" on complement system and platelet activation *in vitro* and physiological responses to exchange transfusion. *J. Biomed. Mater. Res. 81A*, 821–826.

(144) Tsuchida, E., Nakagawa, A., and Komatsu, T. (2003) Coordination structure of active site in synthetic hemoprotein (albumin-heme) with dioxygen and carbon monoxide. *Macromol. Symp. 195*, 275–280.

(145) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2000) Nitrosyl iron(II) complex of *meso*-tetrakis(α,α,α,α-o-pivalamidophenyl)porphyrin with a covalently linked 2-methylimidazolylalkyl group. *Chem. Lett.* 1060–1061.

(146) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2001) Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(II) derivatives. *Bioconjugate Chem. 12*, 71–75.

(147) Collman, J. P., Brauman, J. I., Iverson, B. L., Sessler, J. L., Morris, R. M., and Gibson, Q. H. (1983) O_2 and CO binding to iron(II) porphyrins: A comparison of the "picket fence" and "pocket" porphyrins. *J. Am. Chem. Soc. 105*, 3052–3064.

(148) Traylor, T. G., Tsuchiya, S., Campbell, D., Mitchell, M., Stynes, D., and Koga, N. (1985) Anthracene-heme cyclophanes. Sterice in CO, O_2 and RNC binding. *J. Am. Chem. Soc. 107*, 604–614.

(149) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2000) Kinetics of CO and O_2 binding to human serum albumin-heme hybrid. *Bioconjugate Chem. 11*, 772–776.

(150) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2002) Effect of heme structure on O_2-binding properties of human serum albumin-heme hybrids: intramolecular histidine coordination provides a stable O_2-adduct complex. *Bioconjugate Chem 13*, 397–402.

(151) Nakagawa, A., Komatsu, T., Iizuka, M., and Tsuchida, E. (2008) O_2 binding to human serum albumin incorporating iron porphyrin with a covalently linked methyl-L-histidine isomer. *Bioconjugate Chem. 19*, 581–584.

(152) Nakagawa, A., Komatsu, T., and Tsuchida, E. (2007) meso-Tetrakis(α,α,α,α-o-amidophenyl) porphinatoiron(II) bearing a proximal histidyl group at the β-pyrrolic position via an acyl bond: synthesis and O_2 coordination in aqueous media. *Chem. Lett. 36*, 640–641.

(153) Komatsu, T., Okada, T., Moritake, M., and Tsuchida, E. (2001) O_2-binding properties of double-sided porphinatoiron(II)s with polar substituents and their human serum albumin hybrids. *Bull. Chem. Soc. Jpn. 74*, 1695–1702.

(154) Nakagawa, A., Komatsu, T., Iizuka, M., and Tsuchida, E. (2006) Human serum albumin hybrid incorporating tailed porphyrinatoiron(II) in the α,α,α,β-conformer as an O_2-binding site. *Bioconjugate Chem. 17*, 146–151.

(155) Nakagawa, A., Komatsu, T., Ohmichi, N., and Tsuchida, E. (2003) Synthetic dioxygen-carrying hemoprotein. human serum albumin including iron(II) complex of protoporphyrin IX with an axially coordinated histidylglycyl-propionate. *Chem. Lett. 32*, 504–505.

(156) Nakagawa, A., Komatsu, T., Ohmichi, N., and Tsuchida, E. (2004) Synthesis of protoheme IX derivatives with a covalently linked proximal base and their human serum albumin hybrids as artificial hemoprotein. *Org. Biomol. Chem. 2*, 3108–3112.

(157) Wang, R.-M., Komatsu, T., Nakagawa, A., and Tsuchida, E. (2005) Human serum albumin bearing covalently attached iron(II) porphyrins as O_2-coordination sites. *Bioconjugate Chem. 16*, 23–26.

(158) Harris, J. M. (1992) *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications* (Harris, J. M., Ed.) Plenum Press, New York.

(159) Veronese, F. M., and Harris, J. M. (2002) Introduction and overview of peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54*, 453–456.

(160) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54*, 459–476.

(161) Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405–417.

(162) Nucci, M. L., Shorr, R., and Abuchowski, A. (1991) The therapeutic value of poly(ethylene glycol) modified proteins. *Adv. Drug Delivery Rev. 6*, 133–151.

(163) Kawahara, N. Y., and Ohno, H. (1997) Induced thermostability of poly(ethylene oxide)-modified hemoglobin in glycols. *Bioconjugate Chem. 8*, 643–648.

(164) Huang, Y., Komatsu, T., Wang, R.-M., Nakagawa, A., and Tsuchida, E. (2006) Poly(ethylene glycol)-conjugated human serum albumin including iron porphyrins: surface modification improves the O_2-transporting ability. *Bioconjugate Chem. 17*, 393–398.

(165) Cabrales P., Tsai, A. G., Intaglietta, M. (2004) Microvascular pressure and functional capillary density in extreme hemodilution with low- and high-viscosity dextran and a low-viscosity Hb-based O_2 carrier. *Am. J. Physiol. Heart Circ. Physiol. 287*, H363−H373.

(166) Martini, J., Cabrales, P., K, A., Acharya, S. A., Intaglietta, M., Tsai, A. G. (2008) Survival time in severe hemorrhagic shock after perioperative hemodilution is longer with PEG-conjugated human serum albumin than HES 130/0.4: a microvasucular perspective. *Crit. Care 12*, R54.

(167) Sugawara, Y., and Shikama, K. (1980) Autoxidation of native oxymyoglobin. *Eur. J. Biochem. 110*, 241–246.

(168) Tsuchida, E., Komatsu, T., Hamamatsu, K., Matsukawa, Y., Tajima, A., Yoshizu, A., Izumi, Y., and Kobayashi, K. (2000) Exchange transfusion with albumin-heme as an artificial O_2-infusion into anesthetized rats: physiological responses, O_2-delivery, and reduction of the oxidized hemin sites by red blood cells. *Bioconjugate Chem. 11*, 46–50.

(169) Huang, Y., Komatsu, T., Yamamoto, H., Horinouchi, H., Kobayashi, K., and Tsuchida, E. (2006) PEGylated albumin-heme as an oxygen-carrying plasma expander: exchange transfusion into acute anemia rat model. *Biomaterials 27*, 4477–4483.

(170) Tsuchida, E., Komatsu, T., Matsukawa, Y., Nakagawa, A., Sakai, H., Kobayashi, K., and Suematsu, M. (2003) Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging. *J. Biomed. Mater. Res. 64A*, 257–261.

(171) Nakagawa, A., Komatsu, T., Huang, H., Lu, G., and Tsuchida, E. (2007) O_2-binding albumin thin films: solid membranes of poly(ethylene glycol)-conjugated human serum albumin incorporating iron porphyrin. *Bioconjugate Chem. 18*, 1673–1677.

(172) Goa, K. L., and Benfield, P. (1994) Hyaluronic-acid - a review of its pharmacology and use as a surgical and in ophthalmology, and its therapeutic potential in joint disease and wound-healing. *Drugs 47*, 536–566.

(173) Adams, P. A., and Berman, M. C. (1980) Kinetics and mechanism of the interaction between human serum albumin and monomeric haemin. *Biochem. J. 191*, 95–102.

(174) Zunszain, P. A., Ghuman, J., Komatsu, T., Tsuchida, E., and Curry, S. (2003) Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. *BMC Struct. Biol. 3*, 6.

(175) Wardell, M., Wang, Z., Ho, J. X., Robert, J., Ruker, F., Ruble, J., and Carter, D. C. (2002) The atomic structure of human methemalbumin at 1.9 Å. *Biochem. Biophys. Res. Commun. 291*, 813–819.

(176) Komatsu, T., Ohmichi, N., Zunszain, P. A., Curry, S., and Tsuchida, E. (2004) Dioxygenation of human serum albumin having a prosthetic-heme group in a tailor-made heme pocket. *J. Am. Chem. Soc. 126*, 14304–14305.

(177) Komatsu, T., Ohmichi, N., Nakagawa, A., Zunszain, P. A., Curry, S., and Tsuchida, E. (2005) O_2 and CO binding properties of artificial hemoproteins formed by complexing iron protoporphyrin IX with human serum albumin mutants. *J. Am. Chem. Soc. 127*, 15933–15942.

(178) Vickery, L., Nozawa, T., and Sauer, K. (1976) Magnetic circular dichroism studies of myoglobin complexes. Correlation

with-heme spin state and axial ligation. *J. Am. Chem. Soc. 98*, 343–350.
(179) Antonini, E., and Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*, p 18, North-Holland Publishers, Amsterdam.
(180) Rohlfs, R., Mathews, A. J., Carver, T. E., Olson, J. S., Springer, B. A., Egeberg, K. D., and Sligar, S. G. (1990) The effects of amino acid substitution at position E7 (residue 64) on the kinetics of ligand binding to sperm whale myoglobin. *J. Biol. Chem. 265*, 3168–3176.
(181) Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970) Studies of the function of abnormal hemoglobins I. An improved method for automatic measurement of the oxygen equilibrium curve of hemoglobin. *Biochim. Biphys. Acta 200*, 189–197.
(182) Vandegriff, K. D., Young, M. A., Lohman, J., Bellelli, A., Samaja, M., Malavalli, A., and Winslow, R. M. (2008) CO-MP4, a polyethylene glycol-conjugated haemoglobin derivative and carbon monoxide carrier that reduces myocardial infarct size in rats. *Br. J. Pharmacol. 154*, 1649–1561.
(183) Creighton, T. E. (1992) *Proteins: Structures and Molecular Properties*, p 242, W. H. Freeman and Co., New York.
(184) Chu, M. M. L., Castro, C. E., and Hathaway, G. M. (1978) Oxidation of low-spin iron(II) porphyrins by molecular oxygen. An outer sphere mechanism. *Biochemistry 17*, 481–486.
(185) Tsuchida, E., Nishide, H., Sato, Y., and Kaneda, M. (1982) The preparation of protoheme mono-N-[5-(2-methyl-1-imidazolyl)pentyl]amide and its oxygenation. *Bull. Chem. Soc. Jpn. 55*, 1890–1895.
(186) Uno, T., Sakamoto, R., and Tomisugi, Y. (2003) Inversion of axial coordination in myoglobin to create a "proximal" ligand binding pocket. *Biochemistry 42*, 10191–10199.
(187) Springer, B. A., Sligar, S. G., Olson, J. S., and Philips, G. N. (1994) Mechanisms of a ligand recognition in myoglobin. *Chem. Rev. 94*, 699–714.
(188) Komatsu, T., Nakagawa, A., Zunszain, P. A., Curry, S., and Tsuchida, E. (2007) Genetic engineering of the heme pocket in human serum albumin: modulation of O_2 binding of iron protoporphyrin IX by variation of distal amino acids. *J. Am. Chem. Soc. 129*, 11286–11295.

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