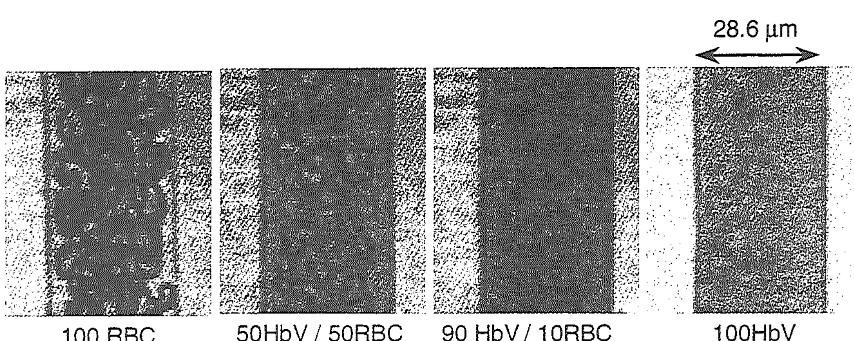

FIG. 9. Flow patterns of the mixture of HbV and RBC suspended in HSA in a narrow tube. HbV particles were homogeneously dispersed in a suspension medium. They tended to distribute in the marginal zone of the flow. The thickness of the RBC-free layer increased with the increasing amount of HbV. The RBC-free phase becomes darker and more semi-transparent, indicating the presence of HbV. Diameter of the tube = 28 μm; [Hb] = 10 g/dl; centerline flow velocity = 1 mm/s

similar with that from RBC alone. On the other hand, the addition of 50 vol% acellular Hb solution to RBC significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of the O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 8 nm) that affects their diffusion for the facilitated O_2 transport. It has been suggested that the faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation [110]. However, this concept is controversial regarding the recent finding that an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [111–113]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of nitric oxide as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

Effects on Hematological Functions

The biocompatibility of HbV is important to clinical use. Transient thrombocytopenia was one of the most significant hematological effects observed after infusion of liposome-encapsulated Hbs in rodents [114]. Exchange transfusion with unmodified HbV (containing DPPG as a lipid component) in anesthetized rats also resulted in a slightly decreased platelet count, although the change was insignificant [104]. These effects were also observed for administration of negatively charged liposomes [115,116]. The transient reduction in platelet counts caused by liposomes was also associated with

sequestration of platelets in the lung and liver. Platelet activation is necessary to prevent bleeding in vivo; however, nonphysiological activation leads to initiation and modulation of inflammatory responses because platelets contain an array of potent proinflammatory substance. RANTES (Regulated upon activation, normal T-cell expressed and presumably secreted), one of the C-C chemokines, is a useful marker for platelet activation as it is stored in α-granules of platelets and was shown to be released after stimulation. Accordingly, the biocompatibility of HbV was examined by estimating their effects on agonist-induced platelet aggregation response and RANTES release from platelets in vitro [117]. This study on biocompatibility was performed in collaboration with Dr. H. Ikeda at the Hokkaido Red Cross Blood Center (Sapporo), and his colleagues.

The effect of low concentration of HbV (Hb: 5.8 mg/dl) on platelet function was assessed by examining an agonist-induced aggregation response, and that of relatively high concentrations of HbV (Hb: 0.29, 1 and 2 g/dl) by measuring the release of RANTES from platelets, which is regarded as a marker of platelet activation. The pre-incubation of platelets with HbV at 5.8 mg/dl of Hb did not affect platelet aggregation induced by collagen, thrombin, and ristocetin. The pretreatment of platelet-rich plasma (PRP) with HbV at concentration up to 2 g/dl of Hb had no aberrant effects on the collagen-induced RANTES release. Furthermore, the collagen-induced release of RANTES from PRP was not affected by longer incubation with HbV at 2 g/dl of Hb. The basal levels of RANTES from PRP were unchanged in the presence of HbV. These results suggest that HbV, at the concentrations studied, have no aberrant effects on platelet functions in the presence of plasma.

The effect of HbV on the coagulation time (PT, APTT) was tested with human plasma. HbV was mixed with human plasma at the ratios of 20%, 40% and 60% v/v. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured automatically. The results were compared with saline and phosphate buffered saline. The PT value increased from 10 s to 15 s with increasing the mixing ratio; however, there was no significant difference between the groups. The APTT value increased from about 40 s to about 50 s with an increase of the mixing ratio; however, there were no significant differences between the groups. The delayed coagulation is due to the dilution of the blood components, and there is no significant effect on the blood coagulation system.

Polymorphonuclear neutrophils (PMNs) are essential cells in the host defense against a variety of infectious agents. Circulating PMNs require activation to migrate to inflammatory sites and then effectively kill pathogens. Previously in the field of drug delivery systems, sterically stabilized liposomes with PEG have been reported to reduce the chemotactic activity of human PMNs in response to zymosan and the bacterially derived peptide, *N*-formyl-

methionyl-leucyl-phenylalanine (fMLP) [118]. Therefore, the effects of the PEG-modified HbV on human PMNs in vitro were studied, focusing on the functional responses to fMLP as an agonist [119]. The pretreatment of PMNs with HbV up to a concentration of 56 mg/dl Hb did not affect the fMLP-triggered chemotactic activity. In parallel to these results, the fMLP-induced upregulation of CD11b (Mac-1) levels on HbV-pretreated PMNs was comparable to that of untreated cells. Furthermore, the pretreatment of PMNs with HbV even at 580 mg/dl Hb did not affect the gelatinase B [Matrix methalloproteinase-9 (MMP-9)] release, suggesting that the fMLP-induced release of secondary and tertiary granules was normal. In addition, the fMLP-triggered superoxide production of PMNs was unchanged by the pretreatment of HbV at 580 mg/dl Hb. Thus, these results suggest that HbV, at the concentrations studied, have no aberrant effects on the fMLP-triggered functions of human PMNs.

Hypertension and Vasoconstriction in Relation with NO and CO

As clinical trials of the chemically modified Hbs are extended to include larger numbers of individuals, it becomes apparent that the principal side effect consistently reported in the administration of acellular Hb solutions is hypertension presumably because of vasoconstriction. Hypertension, a well-defined reaction of the acellular intramolecularly cross-linked Hb (XLHb), was proposed to be beneficial in the treatment of hypotension concomitant to hemorrhagic shock [120]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [113,121]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [122,123]. This theory was validated indirectly using exteriorized rabbit aortic rings in organ baths, where constriction was observed following the addition of acellular Hb solutions as well as an NO synthase inhibitor [124,125]. Different modifications of the Hb molecule cause hypertension that is qualitatively and quantitatively different, and red blood cells (RBCs) and cellular HbV (liposome-encapsulated Hb) do not cause either vasoconstriction or hypertension [99,100,105]. Most evidence for the pressor response is obtained from measurements of systemic pressure, and direct evidence about the mechanism involved is scarce. In previous studies in conscious hamsters fitted with a dorsal skinfold, we found that small arteries of 130–160 μm diameter, termed resistance vessels, exhibit the greatest reactivity in hemorrhagic shock [126], playing a significant role in the regulation of blood flow. Constriction of these resistance vessels in this model was also directly correlated to the pressure response following administration of NO synthase inhibitor [127].

In collaboration with Prof. Intaglietta, we analyzed the relationship between the constriction of resistance vessel and hypertension after administration of acellular Hb and the extent to which the effect is dependent on the size of acellular Hb molecules modified by polymerization, polymer conjugation, and cellular liposome encapsulation [128]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheters inserted into the jugular vein, mean arterial pressure and heart rate were monitored through the jugular arterial catheter, and microvascular responses were monitored by an intravital microscopy. The Hb products included intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethyl-starch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb), and HbV. Their molecular diameters were 7, 22, 68, and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 ± 13 mmHg higher 3 h after infusion. There was a simultaneous decrease in the diameter of the resistance vessels (79 ± 8% of basal value) which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of O_2 carriers of greater molecular size resulted in lesser vasoconstriction and hypertension with HbV showing the smallest changes. Infusion of HSA was used as a control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of Hb-based O_2 carriers. Since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different Hb molecules.

The liver is a major organ that detoxifies excess amounts of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXα and CO. Under normal conditions, the liver contains at least two OH isozymes for physiologic degradation of the heme: HO-1 and HO-2. One of the important roles of the HO reaction is to generate CO that serves as an endogenous regulator that is necessary for maintaining microvascular blood flow [129]. Since Hb strongly binds with CO (about 200 times stronger than O_2), it is necessary to confirm the effects of HbV in hepatic microcirculation in comparison with stroma free Hb solution. Dr. Suematsu et al. studied the perfusion of a rat liver with an acellular Hb solution and HbV, and found out that the Hb solution increased vascular resistance by 30% [130]. The smaller acellular Hb molecules (7 nm) extravasate across the fenestrated endothelium with a pore size of about 100 nm, and reach to the space of Disse. Heme is excessively metabolized by HO-2 to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb in the space of Disse rapidly binds CO, resulting in vasoconstriction and

an increase in vascular resistance. On the other hand, HbV (250nm) is large enough to maintain in the sinusoid, and the vascular resistance is maintained.

These results indicate the importance of the size of the oxygen carriers, and that the size of HbV is appropriate for the maintenance of microvascular blood flow.

Biodistribution and Metabolism of HbV, and Influence on Organ Function

In the physiological condition, free Hb released from RBC is rapidly bound to haptoglobin, and removed from the circulation by hepatocytes. However, when the Hb concentration exceeds the haptoglobin binding capacity, unbound Hb is filtered through the kidney where it is actively absorbed. When the reabsorption capacity of the kidney is exceeded, hemoglobinuria and eventually renal failure occur. The encapsulation of Hb completely suppresses renal excretion, although HbV particles as well as phospholipid vesicles (liposomes) in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [131].

To examine the precise circulation persistence and biodistribution of HbV, we used radiolabelling technique, ^{99m}Tc-labelled HbV, in collaboration with Prof. Phillips at the University of Texas. The HbV co-encapsulated homocysteine (5mM) was successfully labeled with ^{99m}Tc by using the hexamethylpropylene amine oxime. The circulation half-life of ^{99m}Tc-HbV was determined to be 35h. In the gamma camera image, the radioactivity in the blood pool of the heart was gradually decreased and those of the liver and spleen were increased with time. The biodistribution data showed the major organs to eliminate the ^{99m}Tc-HbV from the blood circulation were the liver, bone marrow, and spleen, independent of the injection dose. [132].

The influence of HbV on RES, mainly liver and spleen, was studied with carbon clearance measurements and histopathological examination [133]. The HbV suspension was intravenously infused in male Wistar rats (200g) at dose rates of 10 and 20ml/kg, and the phagocytic activity was measured by monitoring the rate of carbon clearance at 8hs, and at 1, 3, 7 and 14 days after infusion. The phagocytic activity transiently decreased one day after infusion by about 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the original level at 7 days, and then returned to the original level at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with hematoxylin/eosin, and anti-human Hb antibody staining showed that HbV was metabolized within 7 days. Hemosiderin was slightly confirmed with Berlin blue staining at 3 and 7 days in the liver and spleen, although they disappeared at 14 days, indicating that the heme metabolism, excretion, or recycling of the iron ion proceeded smoothly and siderosis was minimal. Electron microscopic examination of the spleen and liver tissues clearly demonstrated the vesicular structure of HbV with a diameter of about 1/40 of RBCs in capillaries, and in phagosomes as entrapped in the spleen macrophages and Kupffer cells one day after infusion. The vesicular structure could not be observed at 7 days. Even though infusion of HbV modified the phagocytic activity for two weeks, it does not seem to cause any irreversible damage to the phagocytic organs from the histological point of view.

We analyzed the influence of HbV on the organ functions by laboratory tests of plasma on a total of 29 analytes [134]. The HbV suspension was intravenously infused into male Wistar rats (20ml/kg). The blood was withdrawn at 8hs, and 1, 2, 3, and 7 days after infusion, and the plasma was ultracentrifuged to remove HbV in order to avoid its interference effect on the analytes. Enzyme concentrations, AST, ALT, ALP, and LAP showed significant, but minor changes, and did not show a sign of a deteriorative damage to the liver as one of the main organs for the HbV entrapment and the succeeding metabolism. The amylase and lipase activities showed reversible changes; however, there were no morphological changes in the pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Cholesterols, phospholipids, and β-lipoprotein transiently increased showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from the membrane components of HbV that are liberated from macrophages entrapping HbV. In conjunction with the previous report of the prompt metabolism of HbV in the reticuloendothelial system by histopathological examination, it can be concluded that HbV infusion transiently modified the values of the analytes without any irreversible damage to the corresponding organs at the bolus infusion rate of 20ml/kg.

In the series of safety evaluations, the repeated infusion of HbV in Wistar rats was performed at the dose rate of 10ml/kg/day for 14 days [135]. All the rats tolerated the infusion and body weight increased continuously. The hematological test, serum blood biochemistry, and histopathological examination did not raise any serious concern about the safety of HbV. One day after the final infusion spleen and liver weights increased significantly. Histopathological observation indicated significant HbV accumulation in liver and spleen; however, there was no sign of organ damage. Serum clinical laboratory tests indicated significant increases in lipid components derived provably from HbV particles. After a 2 week interval, spleen and liver weight returned to the original levels; however, a significant amount of hemosiderin was confirmed without serum iron increase. All the concentrations of the lipid

components returned to the original levels. Judging from these results, there was no sign of significant toxicity of HbV at the level of dosage employed.

Summary

The efficacy of HbV as oxygen carriers and their safety have been demonstrated. The advantages of cellular HbV can be summarized as follows:

1. The encapsulated Hb is extremely purified and free from virus, endotoxin, and blood type antigen.
2. There is no chemical modification of Hb. Dissociation of Hb tetramers to dimers is restrained and there is no release of Hb from HbV, preventing renal dysfunction.
3. The oxygen affinity is adequately adjusted and the metHb formation is restrained because both the allosteric effectors and metHb reduction systems can be coencapsulated in the vesicles.
4. HbV can be stored for over 2 years at room temperature, owing to both surface modification with PEG chains and deoxygenation.
5. The surface modification of HbV with PEG chains increases high dispersion stability and is effective to prevent aggregation in blood circulation.
6. The colloid osmotic pressure of the HbV suspension is close to zero. But it is adjustable with the addition of adequate colloids such as HSA, which is important to maintain blood volume. The solution viscosity can be adjusted equivalent to that of blood. This would be important for the shear stress on the vascular wall to regulate vascular tone.
7. HbV suspended in a plasma expander such as HSA and rHSA showed sufficient oxygen transporting capacity comparable with RBC for resuscitation from hemorrhagic shock and extreme hemodilution. It is also applicable for oxygenation of ischemic tissues.
8. The physiological activity of Hb such as binding with NO and CO, production of active oxygen species, heme release, and hemeoxygenase activation, can be minimized by encapsulation. Thus there is less vasoconstriction, hypertension, and oxygen injury.

According to the above achievements, significant efforts have been made to produce HbV with a facility of GMP standard, and to start preclinical and finally clinical trials. The combination of recombinant Hb-vesicles suspended in recombinant albumin would be the most ideal "artificial red blood cells" in the future.

Acknowledgments. This work has been supported by The Ministry of Education, Culture, Sports, Science and Technology, and The Ministry of Health, Labor and Welfare, Japan. The authors gratefully acknowledge, Dr. H.

Horinouchi, Dr. M. Watanabe, Dr. Y. Izumi (Keio Univ.), Dr. H. Ikeda (Hokkaido Red Cross Blood Center), Prof. M. Suematsu (Keio Univ.), Dr. M. Takaori (Higashitakarazuka Satoh Hospital), Prof. S. Hoka (Kitasato Univ.), Prof. R. Ogawa (Nippon Med. College), Prof. M. Intaglietta (UCSD), Prof. W.T. Phillips (Univ. Texas San Antonio), and Prof. N. Maeda (Ehime Univ.), and their active colleagues for the meaningful discussions and contributions to the research.

References

1. Tsuchida E, Takeoka S (1995) Stabilized hemoglobin vesicles. In: Tsuchida E (ed) Artificial red cells. John Wiley & Sons, Chichester, pp 35–64
2. Chang TMS (1991) Blood substitutes based on modified hemoglobin prepared by encapsulation or crosslinking: An overview. Biomater Artif Cells Immobilization Biotechnol 20:159–182
3. Toyoda T (1965) Artificial blood. Kagaku 35:7–13
4. Kitajima M, Sekiguchi W, Kondo A (1971) A modification of red blood cells by isocyanates. Bull Chem Soc Jpn 44:139–143
5. Bangham AD, Horne RW (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
6. Djordjevich L, Miller IF (1977) Lipid encapsulated hemoglobin as a synthetic erythrocyte. Fed Proc 36:567
7. Hunt CA, Burnette RR, MacGregor RD, et al (1985) Synthesis and evaluation of a protypal artificial red cell. Science 230:1165–1168
8. Kato A, Arakawa M, Kondo T (1984) Liposome-type artificial red blood cells stabilized with carboxymethylchitin. Nippon Kagaku Kaishi 6:987–991
9. Gaber BP, Farmer MC (1984) Encapsulation of hemoglobin in phospholipid vesicles: preparation and properties of a red cell surrogate. Prog Clin Biol Res 165:179–190
10. Hayward JA, Levine DM, Neufeld L, et al (1985) Polymerized liposomes as stable oxygen-carriers. FEBS Lett 187:261–266
11. Suzuki K, Miyauchi Y, Okamoto T, et al (1988) The characteristics and ability of NRC. Jpn J Artif Organs 17:708–711
12. Rudolph AS (1988) Freeze-dried preservation of liposome encapsulated hemoglobin: A potential blood substitute. Cryobiology 25:277–284
13. Jopski B, Pirkl V, Jaroni HW, et al (1989) Preparation of hemoglobin-containing liposomes using octyl glucoside and octyltetraoxyethylene. Biochim Biophys Acta 978:79–84
14. Mobed M, Chang TMS (1991) Preparation and surface characterization of carboxymethylchitin-incorporated submicron bilayer-lipid membrane artificial cells (liposomes) encapsulating hemoglobin. Biomater Artif Cells Immobil Biotechnol 19:731–744
15. Zheng S, Zheng Y, Beissinger R (1994) Efficacy, physical properties and pharmacokinetics of sterically-stabilized liposome-encapsulated hemoglobin. Artif Cells Blood Substitutes Immobil Biotechnol 22:487–501
16. Liu L, Yonetani T (1994) Preparation and characterization of liposome-encapsulated haemoglobin by a freeze-thaw method. J Microencapsulation 11:409–421
17. Tsuchida E (1998) Blood substitutes: present and future perspectives. Elsevier, Chichester

18. Sakai H, Takeoka S, Yokohama H, et al (1993) Purification of concentrated Hb using organic solvent and heat treatment. Protein Expression Purif 4:563–569
19. Fukutomi I, Sakai H, Takeoka S, Nishide H, Tsuchida E, Sakai K (2002) Carbonylation of oxyhemoglobin solution using a membrane oxygenator. J Artif Organs 5:102–107
20. Sakai H, Masada Y, Takeoka S, et al (2002) Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. J Biochem 131:611–617
21. Abe H, Ikebuchi K, Hirayama J, et al (2001) Virus inactivation in hemoglobin solution by heat treatment. Artif Cells Blood Substit Immobil Biotechnol 29:381–388
22. Huang Y, Takeoka S, Sakai H, et al (2002) Complete deoxygenation from a hemoglobin solution by an electro-chemical method and heat treatment for virus inactivation. Biotechnol Prog 18:101–107
23. Naito Y, Fukutomi I, Masada Y, et al (2002) Virus removal from hemoglobin solution using Planova membrane. J Artif Organs 5:141–145
24. Chung JE, Hamada K, Sakai H, et al (1995) Ligand exchange reaction of carbonylhemoglobin to oxyhemoglobin in a hemoglobin liquid membrane. Nippon Kagaku Kaishi 1995: 123–127
25. Takahashi A (1995) Characterization of neo red cells (NRCs), their function and safety in vivo tests. Artif Cells Blood Substitutes Immobil Biotechnol 23:347–354
26. Ogata Y, Goto H, Kimura T, et al (1997) Development of neo red cells (NRC) with the enzymatic reduction system of methemoglobin. Artif Cells Blood Substitutes Immobil Biotechnol 25:417–427
27. Teramura Y, Kanazawa H, Sakai H, et al (2003) The prolonged oxygen—carrying ability of Hb vesicles by coencapsulation of catalase in vivo. Bioconjugate Chem 14:1171–1176
28. Sakai H, Onuma H, Umeyama M, et al (2000) Photoreduction of methemoglobin by irradiation in near-ultraviolet region. Biochemistry 39:14595–14602
29. Takeoka S, Sakai H, Kose T, et al (1997) Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. Bioconjugate Chem 8:539–544 (1997)
30. Takeoka S, Ohgushi T, Sakai H, et al (1997) Construction of artificial metHb reduction system in Hb-vesicles. Artif Cells Blood Substitues Immobil Biotechnol 25:31–41
31. Sakai H, Takeoka S, Seino Y, et al (1994) Suppression of methemoglobin formation by glutathione in a concentrated hemoglobin solution and in a Hb-vesicles. Bull Chem Soc Jpn 67:1120–1125
32. Takeoka S, Sakai H, Nishide H, et al (1993) Preparation conditions of human hemoglobin-vesicles covered with lipid membranes. Jpn J Artif Organs 22:566–569
33. Takeoka S, Terase K, Sakai H, et al (1994) Interaction between phosphoslipid assemblies and hemoglobin (Hb). J Macromol Sci Pure Appl Chem A31:97–108
34. Takeoka S, Sakai H, Terease K, et al (1994) Characteristics of Hb-vesicles and encapsulation procedure. Artif Cells Blood Substitutes Immobilization Biotechnol 22:861–866
35. Takeoka S, Ohgushi T, Terase K, et al (1996) Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. Langmuir 12:1755–1759
36. Sakai H, Hamada K, Takeoka S, et al (1996) Physical properties of hemoglobin vesicles as red cell substitutes. Biotechnol Prog 12:119–125
37. Sou K, Naito Y, Endo T, et al (2003) Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. Biotechnol Prog 19:1547–1552

38. Shirasawa T, Izumizaki M, Suzuki Y, et al (2003) Oxygen affinity of hemoglobin regulates O_2 consumption, metabolism, and physical activity. J Biol Chem 278:5035–5043
39. Sakai H, Yuasa M, Onuma H, et al (2000) Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjug Chem 11:56–64
40. Benesch R, Behesch RE (1967) The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochem Biophys Res Commun 26:162–167
41. Wang L, Morizawa K, Tokuyama S, et al (1992) Modulation of oxygen-carrying capacity of artificial red cells (ARC). Polymer Adv Technol 4:8–11
42. Matsumura S, Yamaji K, Ohki H, et al (1992) Large scale production and characterization of lyophylized pyridoxalated hemoglobin polyoxyethylene (PHP). Biomater Artif Cells Immobil Biotechnol 20:435–438
43. Kerwin BA, Heller MC, Levin SH, et al (1998) Effects of Tween 80 and scrose on acute short-term stability and long-term storage at −20 °C of a recombinant hemoglobin. J Pharm Sci 87:1062–1068
44. Kerwin BA, Akers MJ, Apostol I, et al (1999) Acute and long-term stability studies of deoxy hemoglobin and characterization of ascorbate-induced modifications. J Pharm Sci 88:79–88
45. Levy A, Zhang L, Rifkind JM (1988) Hemoglobin: a source of superoxide radical under hypoxic conditions. Oxy-radicals Mol Pathol Proc Upjohn-UCLA Symp 11–25
46. Balagopalakrishna C, Manoharan PT, Abugo OO, et al (1996) Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. Biochemistry 35:6393–6398
47. Tsuchida E, Hasegawa E, Kimura N, et al (1992) Polymerization of unsaturated phospholipids as large unilamellar liposomes at low temperature. Macromolecules 25:207–212
48. Hosoi F, Omichi H, Akama K, et al (1997) Radiation-induced polymerization of phospholipid mixtures for the synthesis of artificial red blood cells. Nucl Instr Methods Phys Res B 131:329–334
49. Satoh T, Kobayashi K, Sekiguchi S, et al (1992) Characteristics of artificial red cells: hemoglobin encapsulated in poly-lipid vesicles. ASAIO J 38:M580–M584
50. Wang L, Takeoka S, Tsuchida E, et al (1992) Preparation of dehydrated powder of hemoglobin vesicles. Polymer Adv Technol 3:17–21
51. Sakai H, Takeoka S, Yokohama H, et al (1992) Encapsulation of Hb into unsaturated lipid vesicles and γ-ray polymerization. Polymer Adv Technol 3:389–394
52. Rudolph AS, Cliff RO (1990) Dry storage of liposome-encapsulated hemoglobin: a blood substitute. Cryobiology 27:585–590
53. Sakai H, Takisada M, Chung JE, et al (1995) Modification of hemoglobin-vesicles with oligosaccharide chains. Artif Organs Today 4:309–316
54. Woodle MC, Lasic DD (1992) Sterically stabilized liposomes. Biochim Biophys Acta 1113:171–199
55. Klibanov AL, Maruyama K, Torchilin VP, et al (1990) Amphipathic polyethylene glycols effectively prolongs the circulation time of liposomes. FEBS Lett 268:235–237
56. Yoshioka H (1991) Surface modification of haemoglobin-containing liposomes with poly(ethylene glycol) prevents liposome aggregation in blood plasma. Biomaterials 12:861–864
57. Sakai H, Tsai AG, Kerger H, et al (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

58. Sakai H, Takeoka S, Park SI, et al (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
59. Phillips WT, Klipper RW, Awasthi VD, et al (1999) Poly(ethylene glycol)-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. J Pharm Exp Ther 288:665–670
60. Singh M, Ferdous AJ, Jackson TL (1999) Stealth monensis liposomes as a potentiator of adriamycin in cancer treatment. J Controlled Release 59:43–53
61. Meyer O, Kirpotin D, Hong K, et al (1998) Cationic liposomes coated with polyethyle glycol as carriers for oligonucleotide. J Biol Chem 273:15621–15627
62. Sakai H, Tomiyama K, Sou K, et al (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
63. Fletcher JR, Ramwell PW (1980) The effects of prostacyclin on endotoxin shock and endotoxin-induced platelet aggregation in dogs. Circ Shock 7:299–308
64. Shibayama Y, Asaka S, Nakata K (1991) Endotoxin hepatotoxicity augmented by alcohol. Exp Mol Pathol 55:196–202
65. U.S. Department of Health and Human Services Public Health Service, Food and Drug Administration (1987) Guideline on validation of the limulus amebocyte lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices
66. Levin J, Bang FB (1964) The role of endotoxin in the extracellulalr coagulation of limulus blood. Bull Johns Hopkins Hospital 115:265–274
67. Fujiwara H, Ishida S, Shimazaki Y, et al (1990) Measurement of endotoxin in blood products using an endotoxin specific Limulus test reagent and its relation to pyrogenic activities in rabbit. Yakugaku Zasshi 110:332–340 (in Japanese)
68. Carr C Jr, Morrison DC (1984) Lipopolysaccharide interaction with rabbit erythrocyte membranes. Infect Immun 43:600–606
69. Kaca W, Roth RI, Levin J (1994) Hemoglobin, a newly recognized lipopolysaccharide (LPS)-binding protein that enhances LPS biological activity. J Biol Chem 269:25078–25084
70. Roth RI, Levin J, Chapman KW, et al (1993) Production of modified crosslinked cell-free hemoglobin for human use: the role of quantitative determination of endotoxin contamination. Transfusion 33:919–924
71. Yin ET, Galanos C, Kinsky S, et al (1972) Picogram-sensitive assay for endotoxin: Gelation of Limulus polyphemus blood cell lysate induced by purified lipopolysaccharides and lipid A from gram-negative bacteria. Biochim Biophys Acta 261:284–289
72. Richardson EC, Banerji B, Seid RC Jr, et al (1983) Interactions of lipid A and liposome-associated lipid A with Limulus polyphemus amoebocytes. Infect Immun 39:1385–1391
73. Sakai H, Hisamoto S, Fukutomi I, et al (2004) Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. J Pharm Sci 93:310–321
74. Jurgens G, Muller M, Koch MHJ, et al (2001) Interaction of hemoglobin with enterobacterial lipopolysaccharide and lipid A. Eur J Biochem 268:4233–4242
75. Cliff RO, Kwasiborski V, Rudolph AS (1995) A comparative study of the accurate measurement of endotoxin in liposome encapsulated hemoglobin. Artif Cells Blood Substitutes Immobil Biotechnol 23:331–336. Links
76. Harmon P, Cabral-Lilly D, Reed RA, et al (1997) The release and detection of endotoxin from liposomes. Anal Biochem 250:139–146

77. Piluso LG, Martinez MY (1999) Resolving liposomal inhibition of quantitative LAL methods. PDA J Pharm Sci Technol 53:260–263
78. Minobe S, Nawata M, Watanabe T, et al (1991) Specific assay for endotoxin using immobilized histidine and Limulus amebocyte lysate. Anal Biochem 198:292–297
79. Alayash AI (1999) Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? Nat Biotechnol 17:545–549
80. Yamamoto Y, Brodsky MH, Baker JC, et al (1987) Detection and characterization of lipid hydroperoxides at picomole levels by high-performance liquid chromatography. Anal Biochem 160:7–13
81. Grisham MB, Gaginell TS, Von Ritter C, et al (1990) Effects of neutrophil-derived oxidants on intestinal permeability, electrolyte transport, and epithelial cell viability. Inflammation 14:531–542
82. Nagababu E, Rifkind JM (2000) Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. Biochemistry 39:12503–12511
83. Gunther MR, Sampath V, Caughey WS (1999) Potential roles of myoglobin autoxidation in myocardial ischemia-reperfusion injury. Free Radic Biol Med 26:1388–1395
84. Svistunenko DA, Patel RP, Voloshchenko SV, et al (1997) The globin-based free radical of ferryl hemoglobin is detected in normal human blood. J Biol Chem 272:7114–7121
85. Clark MR (1988) Senescence of red blood cells: progress and problems. Physiol Rev 68:503–554
86. Mcleod LL, Alayash AI (1999) Detection of a ferrylhemoglobin intermediate in an endothelial cell model after hypoxia-reoxygenation. Am J Physiol Heart Circ Physiol 277:H92–H99
87. Goldman DW, Breyer RJ III, Yeh D, et al (1998) Acellular hemoglobin-mediated oxidative stress toward endothelium: a role for ferryl iron. Am J Physiol Heart Circ Physiol 275:H1046–H1053
88. D'Agnillo F, Alayash AI (2000) Interactions of hemoglobin with hydrogen peroxide alters thiol levels and course of endothelial cell death. Am J Physiol Heart Circ Physiol 279:H1880–H1889
89. Takeoka S, Teramura Y, Atoji T, et al (2002) Effect of Hb-encapsulation with vesicles on H_2O_2 reaction and lipid peroxidation. Bioconjugate Chem 13:1302–1308
90. Glick MR, Ryder KW (1993) Double trouble: hemolysis and stabilized hemoglobins (so you think you're seeing red now?). Clin Chem 39:1761–1763
91. Ma Z, Monk TG, Goodnough LT, et al (1997) Effect of hemoglobin- and perflubron-based oxygen carriers on common clinical laboratory tests. Clin Chem 43:1732–1737
92. Chance JJ, Norris EJ, Kroll MH (2000) Mechanism of interference of a polymerized hemoglobin blood substitutes in an alkaline phosphatase method. Clin Chem 46:1331–1337
93. Kazmierczak SC, Catrou PG, Best AE, et al (1999) Multiple regression analysis of interference effects from a hemoglobin-based oxygen carrier solution. Clin Chem Lab Med 37:453–464
94. Kazmierczak SC, Catrou PG, Boudreau D (1998) Simplified interpretative format for assessing test interference: studies with hemoglobin-based oxygen carrier solutions. Clin Chem 44:2347–2352
95. Sakai H, Tomiyama K, Masada Y, et al (2003) Pretreatment of serum containing Hb-vesicles (oxygen carriers) to avoid their interference in laboratory tests. Clin Chem Lab Med 41:222–231
96. Nolte D, Pickelmann S, Lang M, et al (2000) Compatibility of different colloid plasma expanders with Peflubron emulsion. Anesthesiology 93:1261–1270

97. Yoshizu A, Yamahata T, Izumi Y, et al (1997) The oxygen transporting capability of hemoglobin vesicle, an artificial oxygen carrier, evaluated in a rat hemorrhagic shock model. Artif Blood 5:18–22
98. Yoshizu A, Izumi Y, Park SI, et al (2004) Hemorrhagic shock resuscitation with an artificial oxygen carrier Hemoglobin Vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma necrosis factor alpha (TNF α). ASAIO J (submitted)
99. Sakai H, Takeoka S, Wettstein R, et al (2002) Systemic and Microvascular responses to the hemorrhagic shock and resuscitation with Hb-vesicles. Am J Physiol Heart Circ Physiol 283:H1191–H1199
100. Sakai H, Horinouchi H, Masada Y, et al (2004) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. Crit Care Med 32:539–545
101. Izumi Y, Sakai H, Hamada K, et al (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
102. Sakai H, Izumi Y, Yamahata T, et al (1995) Evaluation of oxygen transport of hemoglobin vesicles by exchange transfusion into rats. Artif Blood 3:81–86
103. Kobayashi K, Izumi Y, Yoshizu A, et al (1997) The oxygen carrying capability of hemoglobin vesicles evaluated in rat exchange transfusion models. Artif Cells Blood Substitues Immobil Biotechnol 25:357–366
104. Izumi Y, Sakai H, Takeoka S, et al (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
105. Sakai H, Tsai AG, Rohlfs RJ, et al (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. Erni D, Wettstein R, Schramm S, et al (2003) Normovolemic hemodilution with hemoglobin-vesicle solution attenuates hypoxia in ischemic hamster flap tissue. Am J Physiol Heart Circ Physiol 284:H1702–H1709
107. Contaldo C, Schramm S, Wettstein R, et al (2003) Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. Am J Physiol Heart Circ Physiol 285:H1140–H1147
108. Tsai AG, Intaglietta M (2001) High viscosity plasma expanders: Volume restitution fluid for lowering the perfusion trigger. Biorheology 38:229–237
109. Sakai H, Suzuki Y, Kinoshita M, et al (2003) O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permaeble tube: comparison with RBCs and acellular Hbs. Am J Physiol Heart Circ Physiol 285:H2543–H2551
110. Page TC, Light WR, McKay CB, et al (1998) Oxygen transport by erythrocyte/ hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance. Microvasc Res 55:54–66
111. Baines AD, Adamson G, Wojciechowski P, et al (1998) Effect of modifying O_2 diffusivity and delivery on glomerular and tubular function in hypoxic perfused kidney. Am J Physiol Renal Physiol 274:F744–F752
112. Rohlfs RJ, Bruner E, Chiu A, et al (1998) Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem 273:12128–12134
113. Tsai AG, Kerger H, Intaglietta M (1995) Microcirculatory consequences of blood substitution with αα-hemoglobin. In: Winslow RM, Vandegriff K, Intaglietta M (eds) Blood substitutes: physiological basis of efficacy. Birkhauser, Boston, pp 155–174
114. Rabinovici R, Rudolph AS, Yue TL, et al (1990) Biological responses to liposome-encapsulated hemoglobin (LEH) are improved by a PAF antagonist. Circ Shock 31:431–445
115. Loughrey HC, Bally MB, Reinish LW, et al (1990) The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. Thromb Haemost 64:172–176
116. Doerschuk CM, Gie RP, Bally MB, et al (1989) Platelet distribution in rabbits following infusion of liposomes. Thromb Haemost 61:392–396
117. Wakamoto S, Fujihara M, Abe H, et al (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
118. Hatipoglu U, Gao X, Verral S, et al (1998) Sterically stabilized phospholipids attenuate human neutrophils chemotaxis in vitro. Life Sci 63:693–699
119. Ito T, Fujihara M, Abe H, et al (2001) Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on N-formyl-methionyl-leucyl-phenylalanine-induced responses of polymorphonuclear neutrophils in vitro. Artif Cells Blood Substit Immobil Biotechnol 29:427–437
120. Abassi Z, Kotob S, Pieruzzi F, et al (1997) Effect of polymerization on the hypertensive action of diaspirin cross-linked hemoglobin in rats. J Lab Clin Med 129:603–610
121. Gardiner SM, Compton AM, Bennett T, et al (1990) Control of regional blood flow by endothelium-derived nitric oxide. Hypertension 15:486–492
122. Doherty, DH, Doyle MP, et al (1988) Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. Nat Biotechnol 16:672–676
123. Moncada, S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–131
124. Rioux, F, Drapeau G, Marceau F (1995) Recombinant human hemoglobin (rHb1.1) selectively inhibits vasorelaxation elicted by nitric oxide donors in rabbit isolated aortic rings. J Cardiovasc Pharmacol 25:587–594
125. Nakai, K, Ohta T, Sakuma I, et al (1996) Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. J Cardiovasc Pharmacol 28:115–123
126. Sakai, H, Hara H, Tsai AG, et al (1999) Changes in resistance vessels during hemorrhagic shock and resuscitation in conscious hamster model. Am J Physiol Heart Circ Physiol 276:H563–H571
127. Sakai H, Hara H, Tsai AG, et al (2000) Constriction of resistance arteries determines L-NAME-induced hypertension in a conscious hamster model. Microvasc Res 60:21–27
128. Sakai H, Hara H, Yuasa M, et al (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
129. Makino N, Suematsu M, Sugiura Y, et al (2001) Altered expression of heme oxygenase-1 in the livers of patients with portal hypertensive diseases.Hepatology 33:32–42
130. Goda N, Suzuki K, Naito M, et al (1998) Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. J Clin Invest 101:604–612
131. Rudolph AS, Klipper RW, Goins B, et al (1991) In vivo biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. Proc Natl Acad Sci USA 88:10976–10980

132. Sou K, Klipper R, Goins B, et al (2003) Pharmacokinetics of the hemoglobin-vesicles (HbV) in rats. Artif Blood 11:117 (Abstract)
133. Sakai H, Horinouchi H, Tomiyama K, et al (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. Am J Pathol 159:1079–1088
134. Sakai H, Horinouchi H, Masada Y, et al (2004) Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. Biomaterials 25:4317–4325
135. Sakai H, Masada Y, Horinouchi H, et al (2003) Daily repeated infusion of Hb-vesicels (HbV) into Wistar rats for two weeks: A preliminary safety study. Artif Blood 11:72 (Abstract)

Oxygen-Carrying Plasma Hemoprotein Including Synthetic Heme

TERUYUKI KOMATSU and EISHUN TSUCHIDA

Summary. Recombinant human serum albumin (rHSA) incorporating tetraphenylporphyrinatoiron(II) derivative with four pivaloylamino substituents (FepivP), albumin-heme, is an entirely synthetic hemoprotein that can reversibly bind and release O_2 under physiological conditions. We have recently found that replacing the substituent groups of FepivP with more hydrophobic 1-methylcyclohexanoylamino groups, affording FecycP, substantially stabilizes the formed O_2–adduct complex. The O_2- and CO-binding abilities and blood compatibility of this new rHSA-heme hybrid (rHSA-FecycP) have been investigated by spectroscopy. The maximum number of FecycP binding to one albumin was determined to be eight. Because the isoelectric point and circular dichroism (CD) spectral pattern were identical to those of rHSA itself, the two-dimensional structure of the host albumin could be unchanged after the incorporation of FecycP. Laser-flash photolysis experiments gave the association and dissociation rate constants for O_2 and CO (k_{on}, k_{off}). The rebinding kinetics of these gaseous ligands consists of multiple exponentials. We conjectured that the O_2- and CO-binding reactions are affected by the molecular environment around each of the active heme sites. rHSA-FecycP showed almost the same O_2-binding affinity($P_{1/2}^{O_2}$ 34 torr at 37°C) and thermodynamic parameters (ΔH, ΔS) for the oxygenation as rHSA-FepivP. In contrast, the half-life of the O_2-adduct complex (9h, 37°C) became significantly longer than that of rHSA-FepivP (by a factor of 4.5), which is close to that of myoglobin. The obtained red solution was stable and demonstrated a long shelf life (>2 years) at room temperature. The equivalent mixture of rHSA-FecycP and whole blood exhibited no coagulation or precipitation, indicating its high blood compatibility.

Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan

Key words. Human serum albumin, Albumin-heme, Synthetic hemoprotein, Oxygen-binding ability, Red blood cell substitute

Introduction

Human serum albumin (HSA) used for clinical treatment in Japan amounted to 1.9 million l (in terms of a blood source) in 2002 [1]. Most was administered to hemorrhagic shocked patients as a resuscitation fluid. If HSA can transport oxygen (O_2) like red blood cells, it could be of extreme medical importance not only as a blood replacement but also as an O_2 therapeutic agent.

In our circulatory system, free hemin, an iron(III) complex of protoporphyrin IX dissociated from methemoglobin, is potentially toxic because it may (1) intercalate phospholipid membranes, (2) be a major source of iron for bacterial pathogens, and (3) catalyze the formation of free radicals. Hemopexin has high affinity for binding protein with hemin, having the highest binding constant of any known protein ($K > 10^{12} M^{-1}$), but it releases it into liver cells via specific surface receptors [2]. Crystal structure analysis of the hemopexin-hemin complex revealed that the hemin is tightly bound by double histidine coordinations to the central ferric ion and multiple hydrogen bondings with the amino acid residues [3]. Nevertheless, the concentration of hemopexin in the plasma is rather low (<17 µM). HSA may also provide reserve binding capacity of hemin in various conditions (e.g., trauma, inflammation, hemolysis). In fact, HSA binds hemin with a relatively high affinity ($K = 10^8 M^{-1}$) [4]. We have determined the single crystal structure of the HSA-hemin-myristate complex with a resolution of 3.2 Å [5]. Hemin is accommodated into the narrow D-shaped pocket in subdomain IB; and proximal coordination with Tyr-161 and three hydrogen bondings with basic amino acids contribute to maintaining the assembly. Addition of a sodium dithionate into this solution under an N_2 atmosphere reduced the central ferric ion to the ferrous state, although exposure to O_2 gas immediately oxidized the iron(II) center (T. Komatsu, N. Ohmichi, E. Tsuchida, unpublished data, 2004).

We have found that tetraphenylporphyrinatoiron(II) derivative with four pivaloylamino substituents (FepivP) (Fig. 1) was also incorporated into HSA, and the obtained albumin-heme (HSA-FepivP) can reversibly bind and release O_2 under physiological conditions in the same manner as hemoglobin (Hb) and myoglobin (Mb) [6–12]. Because recombinant HSA (rHSA) was manufactured on a large scale by expression in *Pichia pastoris* [13], rHSA-heme hybrid has become entirely synthetic and absolutely free of infectious pathogens. Our animal experiments have also demonstrated that rHSA-heme works as an "oxygen-carrying plasma hemoprotein" in the bloodstream [14; T. Komatsu et al., unpublished data, 2004].

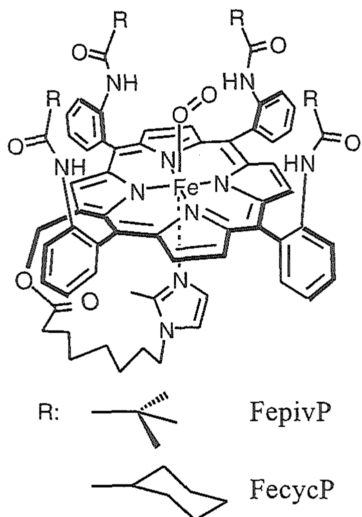

Fig. 1. Structures of the new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) and pivaloylamino substituents (FepivP), and the simulated structure of oxygenated FecycP. The extensible systematic forcefield (ESFF) simulation was performed using an Insight II system (Molecular Simulations, San Diego, CA, USA). The structure was generated by alternative minimization and annealing dynamic calculations from 1000 K to 100 K. The dielectric constant was fixed at 2.38 D, corresponding to the toluene solution. The *dotted surface* represents the van der Waals radius

Half of the Hb-based O_2 carrier in advanced clinical trials still exhibited vasoconstriction, which increased blood pressure and decreased cardiac output [15–19]. The precise mechanism of this hypertension is controversial, but many investigators suspect that the Hb molecules penetrate the vascular endothelium and bind the endothelial-derived relaxing factor (EDRF), namely nitric oxide [20–27]. Others believe that excessive delivery of O_2 to arteriolar vascular walls induces autoregulatory vasoconstriction [28–33]. Interestingly, rHSA-heme does not induce such a vasopressor effect [34]. The electrostatic repulsion between the albumin surface and glomerular basement membrane around the endothelial cell retards rapid leakage of the rHSA-heme molecule and quick scavenging of NO. Albumin-heme is now recognized to be one of the most promising materials as a new class of red blood cell substitute.

To improve the O_2-binding ability of rHSA-FepivP, we have synthesized new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) (Fig. 1) [35]. rHSA-FecycP forms a significantly stable O_2-adduct complex with

a long half-life compared to that of FepivP (by a factor of 4.5). We herein report the O_2- and CO-binding abilities of this entirely synthetic albumin-based O_2 carrier.

Incorporation of Heme into rHSA

Based on quantitative analysis of the absorption intensity for the Soret band of aqueous rHSA-FecycP, the maximum number of FecycP binding to an rHSA was determined to be eight using a molar extinction coefficient [35]. FecycP is accommodated into certain domains of rHSA with binding constants of 10^6–$10^4\,M^{-1}$.

The isoelectric points (*p*I) of the obtained rHSA-FecycP hybrid (FecycP/rHSA = 1–8 mol/mol) were 4.8, exactly the same as those of rHSA. Fatty acid binding, for example, induced a reduction in the *p*I value due to partial neutralization of the surface charge. The FecycP molecule without any ionic side chain interacts nonspecifically with a hydrophobic subdomain of rHSA, so its surface charge distributions are unaltered. Consequently, the essential biological roles as serum albumin [i.e., control of colloid osmotic pressure (COP) and plasma expansion] are essentially sustained after the incorporation of FecycP.

The secondary and tertiary structures of rHSA and the deformation upon FecycP binding were measured by circular dichroism (CD) spectroscopy. The spectral pattern showed typical double-minimum negative peaks in the ultraviolet (UV) region independent of the number of FecycP molecular bound (Fig. 2). The estimated α-helix content was approximately 67%, suggesting that the FecycP association did not cause any high-ordered structural change in the host albumin. Moreover, rHSA-FecycP showed no induced CD in the Soret region (400–500 nm). The hemin binding to the serum albumin is accompanied by a rise in the extrinsic negative Cotton effect in the Soret region because it binds to albumin through axial coordination, allowing a large degree of immobilization [36,37]. We concluded that hydrophobic interaction is the major molecular force of FecycP binding, and its incorporation does not induce any changes in the highly ordered structure or in the surface net charges of rHSA.

O_2-Binding Property of rHSA-Heme

The UV-visible absorption spectrum of the aqueous rHSA hybrid that included carbonyl FecycP showed the formation of the typical CO-coordinated low-spin tetraphenylporphyrinatoiron(II) derivative (λ_{max}: 429, 545 nm). Light irradiation of this solution under an O_2 atmosphere led to

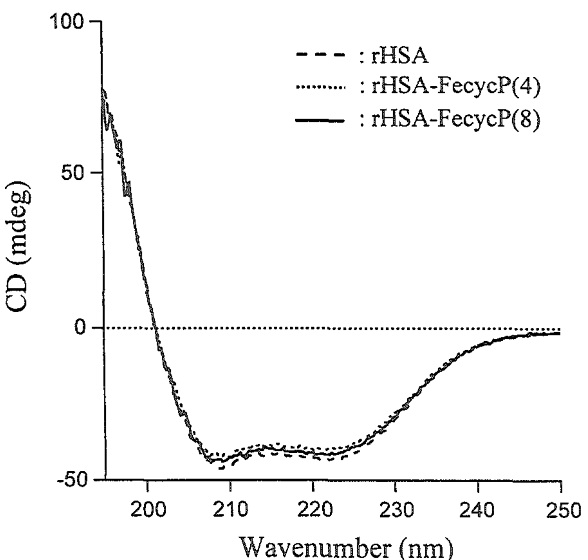

Fig. 2. Circular dichroism (*CD*) spectra of recombinant human serum albumin (*rHSA*) and rHSA-FecycP in water at 25°C

CO dissociation, giving the O_2-adduct complex (λ_{max}: 428, 555 nm). Upon exposure of the oxygenated rHSA-FecycP to N_2, the UV-visible absorption pattern changed to that of the five-*N*-coordinated high-spin iron(II) complex with an intramolecularly coordinated proximal imidazole (λ_{max}: 445, 543, 567 nm). This oxygenation was reversibly dependent on the O_2 partial pressure and sufficiently stable under physiological conditions (37°C, pH 7.4) (Fig. 3). The rate of irreversible oxidation is satisfactorily slow (vide infra).

The O_2 coordination to FecycP in human serum albumin is expressed by Eq. 1.

$$\mathrm{FeP} + \mathrm{O_2} \underset{k_{off}^{O_2}}{\overset{k_{on}^{O_2}}{\rightleftarrows}} \mathrm{FeP\text{-}O_2} \quad (1)$$

$$\left[P_{1/2}^{\ O_2} = \left(K^{O_2}\right)^{-1} = k_{on}^{\ O_2} / k_{off}^{\ O_2}\right]$$

The O_2 association and O_2-dissociation rate constants ($k_{on}^{O_2}$, $k_{off}^{O_2}$) were explored by laser flash photolysis (Table 1) [9,35,38–40]. The detailed kinetic evaluation of rHSA-FecycP gave the following results.

1. The absorption decays accompanying O_2 recombination were composed of three phases of first-order kinetics; the curves were fit by a triple-exponential equation [9]. The minor (<10%) and fastest component was

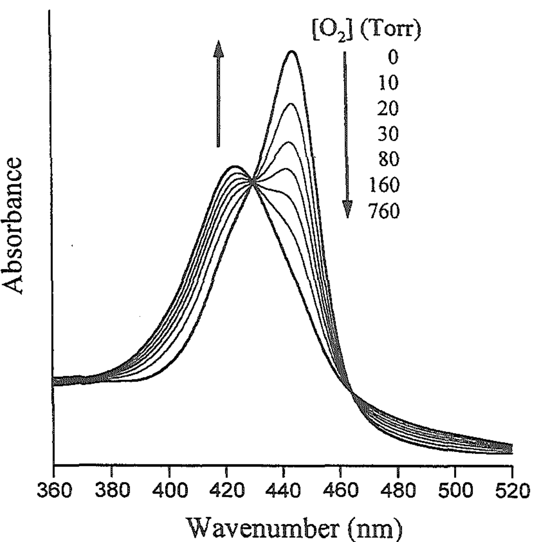

FIG. 3. Ultraviolet-visible. Absorption spectral changes of rHSA-FecycP(4) dependent on the O_2 partial pressure in phosphate-buffered solution (pH 7.3) at 37°C. The number in parenthesis is molar ratio of FecycP and rHSA

TABLE 1. O_2 association and dissociation rate constants for rHSA-FecycP in phosphate-buffered solution (pH 7.3) at 25°C

Substance	k_{on}[$M^{-1}s^{-1}$]		k_{off}[s^{-1}]	
	Fast	Slow	Fast	Slow
rHSA-FecycP(8)	4.6×10^7	7.3×10^6	9.8×10^2	1.6×10^2
rHSA-FepivP(8)[a]	3.4×10^7	9.5×10^6	7.5×10^2	2.0×10^2
Hb (T-state)α[b]	2.9×10^6		1.8×10^2	

rHSA, recombinant human serum albumin; FecycP, tetraphenylporphyrinatoiron(II) derivative with 1-methyl cyclohexanoylamino groups; FepivP, tetraphenyl porphyrinatoiron (II) with pivaloyamino substitute; Hb, hemoglobin.
[a] Ref. [9].
[b] pH 7, 20°C; Ref. [40].
The numbers in parenthesis is molar ratio of porphyrin and rHSA.

independent of the O_2 concentrations. It should be correlated with a base elimination [41].
2. Based on careful inspection of the two slower phases, the association rate constants for the fast and slow rebinding [k_{on}(fast) and k_{on}(slow)] of O_2 were calculated. The k_{on}(fast) values are four- to fivefold higher than the k_{on}(slow) volues.
3. The concentration ratios of the fast and slow reactions were 2:1 to 3:1.

Based on these findings, we can conclude that the O_2 association with FecycP in the hydrophobic domains of rHSA is influenced by the molecular

TABLE 2. O_2-binding equilibrium parameters and half-lifetime of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

Substance	$P_{1/2}$(torr)[a]	ΔH[kJ mol^{-1}]	ΔS[J $K^{-1}mol^{-1}$]	$\tau_{1/2}$[h][a]
rHSA-FecycP(4)	34	−59	−108	9
rHSA-FecycP(8)	35	−59	−107	9
rHSA-FepivP(4)[b]	36	−60	−114	2
rHSA-FepivP(8)[b]	33	−60	−112	2
Red cells[c]	27			
Hbα	40[d]	−57 to −65[e]	−116 to −133[e]	35[f]
Mb[d]	40[d]	−57 to −65[e]	−116 to −133[e]	12[g]

[a] At 37°C.
[b] Ref. [8].
[c] pH 7.4; ref. [42].
[d] T-state, pH 7, 20°C; ref. [40].
[e] pH 7.4; ref. [43].
[f] At 37°C, pH 7.2; ref. [44].
[g] At 35°C, pH 7.0; ref. [45].
The number in parenthesis is molar ratio of porphyrin and rHSA.

microenvironment around each O_2 coordination site (e.g., steric hindrance of the amino acid residue and difference in polarity).

The O_2-binding affinity for such oxygenation could be directly determined. Adequate isosbestic behavior was maintained during the course of a spectrophotometric titration of O_2 (Fig. 3). According to the kinetic experiments, the $P_{1/2}$ values were divided into two components using our previously reported equation [9]. The calculated $P_{1/2}$ for the fast and slow phases were identical in each case (Table 2). The thermodynamic parameters (ΔH, ΔS) of oxygenation were also measured by the van't Hoff plots of the K^{O_2} values (Fig. 4) [8]. The $P_{1/2}{}^{O_2}$, ΔH, and ΔS values for oxygenation of rHSA-FecycP resembled those of Hb and Mb [8,40,42–45]. Moreover, we could not find significant differences in these parameters for rHSA-FepivP and rHSA-FecycP. This result indicates that the substituent structure on the porphyrin plane does not cause any substantial change in the O_2 equilibria and kinetics of rHSA-heme.

Stability of O_2-Adduct Complex of Albumin-Heme

Accompanying the autooxidation of the central iron(II), the absorption band (λ_{max} 555 nm) slowly disappeared at 37°C, leading to formation of the inactive ferric porphyrin. The effect of the heme structure on the half-life of the O_2-adduct complex against the ferric state ($\tau_{1/2}$) was marked. The rHSA-FecycP had a $\tau_{1/2}$ of 9 h, which is 4.5-fold longer than that of rHSA-FepivP and close to that of the Mb (12 h at 37°C) [46].

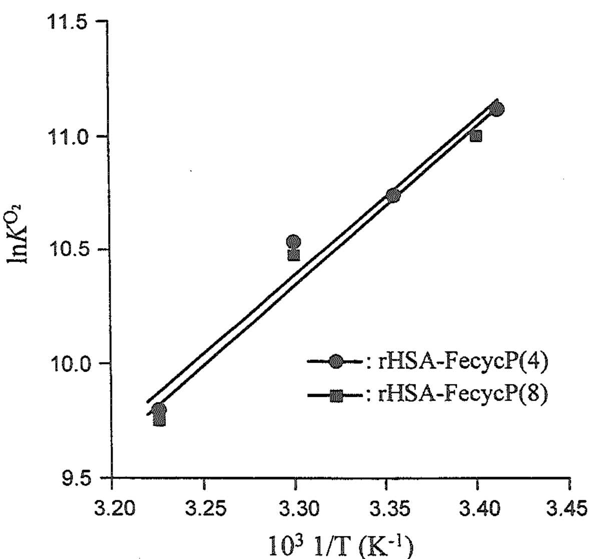

Fig. 4. Van't Hoff plots of O_2-binding affinity of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

CO-Binding Property of rHSA-Heme

Upon addition of CO gas through the deoxy or oxy state of the rHSA-FecycP solution, the spectrum immediately exhibited formation of the carbonyl complex. The CO-binding affinity ($P_{1/2}^{CO}$) of rHSA-FecycP became 2.5-fold higher than that of rHSA-FepivP (Table 3) [9,47,48]. Kinetically, this is due to the low CO dissociation rate constant, k_{off}^{CO}. More recently, CO/O_2 discrimination of Hb and Mb has not been based mainly on distal steric constraints in the heme pocket; the emphasis has shifted to polar interactions in the binding pocket [49,50]. That is, a polar environment could favor the highly polarized coordinated Fe-O_2 unit over the apolar Fe-CO moiety. In FecycP, the hydrophobic cavity around the central ferrous ion probably contributes to the rise in CO-binding affinity. This interpretation is in good agreement with assumptions by other investigators.

Blood Compatibility

The red rHSA-FecycP solution showed a long shelf life (>2 years) at temperatures of 4°–37°C without any aggregation or precipitation. The solution properties also satisfied physiological requirements. The specific gravity was 1.013 (FecycP/rHSA = 1–8 mol/mol). The viscosity of 1.2 cP (at a high shear rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cP) and exhibited Newtonian-type shear rate dependence similar to that of rHSA itself (Fig. 5). Furthermore, the viscosity of the mixed dispersion with freshly drawn blood (1:1, v/v) showed 2.0 cP (at 230 s^{-1}), indicating that rHSA-FecycP had good compatibility with blood. Optical microscopic observations also revealed that the homogeneous morphology of the red blood cells was not affected by mixing with whole blood (not shown).

Table 3. CO-binding parameters of rHSA-FecycP in phosphate-buffered solution (pH 7.3) at 25°C

Substance	$P_{1/2}^{CO}$(torr)	k_{on}($M^{-1}s^{-1}$)	
		Fast	Slow
rHSA-FecycP(8)	0.04	5.9×10^6	8.9×10^5
rHSA-FepivP(8)	0.10	4.9×10^6	6.7×10^5
Hb (T-state)α[a]	0.30	2.2×10^5	

[a] Aqueous, pH 7.0–7.4, 20°C; refs. [47, 48].
The number in parenthesis is molar ratio of porphyrin and rHSA.

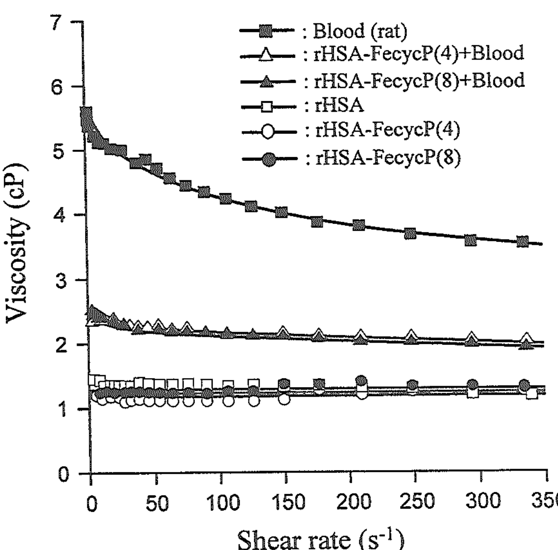

Fig. 5. Viscosity of rHSA-FecycP solution with whole blood at 37°C

Conclusions

Human serum albumin incorporating synthetic heme formed an O_2-adduct complex under physiological conditions. In particular, oxygenated rHSA-FecycP showed high stability compared to the previous rHSA-FepivP, and its half-life reached a value similar to that of the native Mb. It has been also found

that another rHSA-heme complex incorporating an FecycP analogue with a histidyl base at the porphyrin periphery had an extremely long half-life of the oxygenated complex (25 h) under the same conditions (in this case the O_2-binding affinity is quite high) [35]. rHSA–FecycP with a $P_{1/2}$ value (34 torr at 37°C) similar to that of red blood cells is now the most promising material to be used as an artificial O_2 carrier. Exchange transfusion with rHSA-FecycP into anesthetized beagles to evaluate its clinical safety and efficacy is now under investigation.

Acknowledgments. This work was partially supported by Health Science Research Grants from MHLW, Grant-in-Aid for Scientific Research (No. 16350093) from JSPS, and Grant-in-Aid for Exploratory Research (No. 16655049) from MEXT. The authors are grateful to NIPRO Corp. for their supporting the oxygen-infusion project. We also thank Mr. Seiji Ishihara (Waseda University) for his skilful physicochemical experiment.

References

1. Homepage of Ministry of Health, Labor, and Welfare, Japan (2003) (http://www.mhlw.go.jp/shingi/2003/06/s0627-12.html)
2. Tolosano E, Altruda F (2002) Hemopexin: structure, function, and regulation. DNA Cell Biol 21:297–306
3. Paoli M, Anderson BF, Baler HM, et al (1999) Crystal structure of hemopexin reveals a novel high-affinity heme site formed between two β-propeller domains. Nat Struct Biol 6:926–931
4. Adams PA, Berman MC (1980) Kinetics and mechanism of the interaction between human serum albumin and monomeric heamin. Biochem J 191:95–102
5. Zunszain PA, Ghuman J, Komatsu T, et al (2003) Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. BMC Struct Biol 3:6
6. Komatsu T, Hamamatsu K, Wu J, et al (1999) Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*-pivamido)phenylporphyrinatoiron(II) derivatives. Bioconjug Chem 10:82–86
7. Komatsu T, Hamamatsu K, Tsuchida E (1999) Cross-linked human serum albumin dimers incorporating sixteen (tetraphenylporphinato)iron(II) derivatives: synthesis, characterization, and O_2-binding property. Macromolecules 32:8388–8391
8. Tsuchida E, Komatsu T, Mastukawa, et al (1999) Human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinato-iron(II) derivative as a totally synthetic O_2-carrying hemoprotein. Bioconjug Chem 10:797–802
9. Komatsu T, Matsukawa Y, Tsuchida E (2000) Kinetics of CO and O_2 binding to human serum albumin-heme hybrid. Bioconjug Chem 11:772–776
10. Komatsu T, Matsukawa Y, Tsuchida E (2001) Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphyrinatoiron(II) derivatives. Bioconjug Chem 12:71–75
11. Komatsu T, Okada T, Moritake M, et al (2001) O_2-binding properties of double-sided porphyrinatoiron(II)s with polar substituents and their human serum albumin hybrids. Bull Chem Soc Jpn 74:1695–1702

12. Huang Y, Komatsu T, Nakagawa A, et al (2003) Compatibility in vitro of albumin-heme (O_2 carrier) with blood cell components. J Biomed Mater Res 66A:292–297
13. Sumi A, Ohtani W, Kobayashi K, et al (1993) Purification and physicochemical properties of recombinant human serum albumin. Biotechnol Blood Proteins 227:293–298
14. Tsuchida E, Komatsu T, Hamamatsu K, et al (2000) Exchange transfusion of 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. Bioconjug Chem 11:46–50
15. Chang TMS (1997) Recent and future developments in modified hemoglobin and microencapsulated hemoglobin as red blood cell substitutes. Artif Cells Blood Substit Immobil Biotechnol 25:1–24
16. Tsuchida E (1998) Perspectives of blood substitutes. In: Tsuchida E (ed) Blood substitutes: present and future perspectives. Elsevier Science, Lausanne, pp 1–14
17. Winslow RM (1998) The role of blood substitutes in emerging healthcare systems. In: Tsuchida E (ed) Blood substitutes: present and future perspectives. Elsevier Science, Lausanne, pp 15–32
18. Winslow RM (1999) New transfusion strategies: red cell substitutes. Annu Rev Med 50:337–353
19. Squires JE (2002) Artificial blood. Science 295:1002–1005
20. Keipert P, Chang T (1998) Pyridoxylated-polyhemoglobin solution: a low viscosity oxygen-delivery blood replacement fluid with normal oncotic pressure and long term storage feasibility. Biomater Artif Cells 16:185–196
21. Keipert PE, Gonzales A, Gomez CL, et al (1993) Acute changes in systemic blood pressure and urine output of conscious rats following exchange transfusion with diaspirin-crosslinked hemoglobin solution. Transfusion 33:701–708
22. Hess JR, MacDonald VW, Brinkley WW (1993) Synthetic and pulmonary hypertension after resuscitation with cell-free hemoglobin. J Appl Physiol 74:1769–1778
23. Schultz SC, Grady B, Cole F, et al (1993) A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med 122:301–308
24. Thompson A, McGarry AE, Valeri CR, et al (1994) Stroma-free hemoglobin increases blood pressure and GFR in the hypotensive rat: role of nitric oxide. J Appl Physiol 77:2348–2354
25. Sharma AC, Singh G, Gulati A (1995) Role of NO mechanism in cardiovascular effects of diaspirin cross-linked hemoglobin in anesthetized rats. Am J Physiol 269:H1379–H1399
26. Moisan S, Drapeau G, Burhop KE, et al (1998) Mechanism of the acute pressor effect and bradycardia elicited by disapirin crosslinked hemoglobin in anesthetized rats. Can J Physiol Pharmacol 76:434–442
27. Abassi Z, Kotob S, Pieruzzi F, et al (1997) Effects of polymerization on the hypertensive action of diaspirin cross-linked hemoglobin in rats. J Lab Clin Med 129:603–610
28. Guyton AC, Ross JM, Carrier O, et al (1964) Evidence for tissue oxygen demand as the major factor causing autoregulation. Circ Res 14:1–60
29. Johnson PC (1986) Autoregulation of blood flow. Circ Res 59:483–495
30. Vandegriff KD, Winslow RM (1995) A theoretical analysis of oxygen transport: a new strategy for the design of hemoglobin-based red cell substitutes. In: Winslow RM, Vandegriff KD, Intaglietta M (eds). Blood substitutes: physiological basis of efficiency. Birkhäuser, Boston, pp 143–154
31. Tsai AG, Kerger H, Intaglietta M (1995) Microcirculatory consequences of blood substitution with αα-hemoglobin. In: Winslow RM, Vandegriff KD, Intaglietta M (eds) Blood substitutes: physiological basis of efficiency. Birkhäuser, Boston, pp 155–174

32. Rohlfs RJ, Bruner E, Chiu A, et al (1998) Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem 273:12128–12134
33. Winslow RM (2000) αα-Crosslinked hemoglobin: was failure predicted by preclinical testing? Vox Sang 79:1–20
34. Tsuchida E, Komatsu T, Matsukawa Y, et al (2003) Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging. J Biomed Mater Res 64A:257–261
35. Komatsu T, Matsukawa Y, 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. Bioconjug Chem 13:397–402
36. Beaven H, Chen CH, D'Albis A, et al (1974) A spectroscopic studies of the haemin-human-serum-albumin system. Eur J Biochem 41:539–546
37. Casella L, Gullotti M, Ploi S, et al (1993) Haem-protein interactions: the binding of haem complexes to serum albumin. Gazz Chim Itali 123:149–154
38. Traylor TG, Tsuchiya S, Campbell D, et al (1985) Anthracene heme cyclophanes: steric effects in CO, O_2, and RNC binding. J Am Chem Soc 107:604–614
39. Collman JP, Brauman JI, Iverson BL, et al (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
40. Sawicki CA, Gibson QH (1977) Properties of the T state of human oxyhemoglobin stideid by laser flash photolysis. J Biol Chem 252:7538–7547
41. Geibel J, Cannon J, Campbell D, et al (1978) Model compounds for R-state and T-state hemoglobins. J Am Chem Soc 100:3575–3585
42. Severinghaus JW (1966) Blood gas calculator. J Appl Physiol 21:1108–1116
43. Imai K, Yonetani T (1975) Thermodynamical studies of oxygen equilibrium of hemoglobin. J Biol Chem 250:7093–7098
44. Sugawara Y, Shikama K (1980) Autoxidation of native oxymyoglobin. Eur J Biochem 110:241–246
45. Mansouri A, Winterhalter H (1973) Nonequivalence of chains in hemoglobin oxidation. Biochemistry 12:4946–4949
46. Sugawara Y, Shikama K (1980) Autoxidation of native oxymyoglobin. Eur J Biochem 110:241–246
47. Steinmeier RC, Parkhurst LJ (1975) Kinetic studies on the five principle components of normal adult human hemoglobin. Biochemistry 14:1564–1573
48. Sharma VS, Schmidt MR, Ranney HM (1976) Dissociation of CO from carboxyhemoglobin. J Biol Chem 251:4267–4272
49. Springer BA, Sligar SG, Olson JS, et al (1994) Mechanism of ligand recognition in myoglobin. Chem Rev 94:699–714
50. Matsuura M, Tani F, Naruta Y (2002) Formation and characterization of carbon monoxide adducts of iron "twin coronet" porphyrins: extremely low CO affinity and a strong negative polar effect on bound CO. J Am Chem Soc 124:1941–1950

POLYMERS FOR ADVANCED TECHNOLOGIES
Polym. Adv. Technol. 2005; **16**: 73–83
Published online 7 January 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/pat.559

PAT

Oxygen infusions (hemoglobin-vesicles and albumin-hemes) based on nano-molecular sciences†

Eishun Tsuchida[1]*, Hiromi Sakai[1], Teruyuki Komatsu[1], Shinji Takeoka[1], Yubin Huang[1], Keitaro Sou[1], Akito Nakagawa[1], Yuji Teramura[1] and Koichi Kobayashi[2]

[1]Advanced Research Institute for Science & Engineering, Waseda University, Tokyo 169-8555, Japan
[2]Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan

Since the discovery of a red-colored saline solution of a heme derivative that reversibly binds and releases oxygen (1983), significant efforts have been made to realize an oxygen infusion as a red cell substitute based on the sciences of both molecular assembling phenomena and macromolecular metal complexes. The authors have specified that hemoglobin (Hb)-vesicles (HbV) and recombinant human serum albumin-hemes (rHSA-heme) would be the best systems that meet the clinical requirements. (A) Hb is rigorously purified from outdated, donated red cells via pasteurization and ultrafiltration, to completely remove blood type antigen and pathogen. The HbV encapsulates thus purified concentrated Hb solution with a phospholipid bimolecular membrane (diameter, 250 nmø), and its solution properties can be adjusted comparable with blood. Surface modification of HbV with a water-soluble polymer ensures stable dispersion state and storage over a year at 20°C. *In vivo* tests have clarified the efficacy for extreme hemodilution and resuscitation from hemorrhagic shock, and safety in terms of biodistribution, metabolism in reticuloendothelial system (RES), clinical chemistry, blood coagulation, etc. The HbV does not induce vasoconstriction thus maintains blood flow and tissue oxygenation. (B) rHSA is now manufactured in Japan as a plasma-expander. The rHSA can incorporate eight heme derivatives (axial base substituted hemes) as oxygen binding sites, and the resulting rHSA-heme is a totally synthetic O_2-carrier. Hb binds endothelium-derived relaxation factor, NO, and induces vasoconstriction. The rHSA-heme binds NO as Hb does, however, it does not induce vasoconstriction due to its low pI (4.8) and the resulting low permeability across the vascular wall (1/100 of Hb). A 5%-albumin solution possesses a physiologic oncotic pressure. Therefore, to increase the O_2-transporting capacity, albumin dimer is effective. Albumin dimer can incorporate totally 16 hemes with a regulated oncotic pressure. The rHSA-heme is effective not only as a red cell substitute but also for oxygen therapeutics (e.g. oxygenation for tumor). Significant efforts have been made to produce HbV and rHSA-heme with a facility of Good Manufacturing Practice (GMP) standard, and to start preclinical and finally clinical trials.
Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: oxygen infusion; blood substitutes; surface modification; water-soluble polymers; biomaterials

INTRODUCTION

For human beings to survive, it is necessary to continuously deliver O_2 that is needed for the respiration of all tissue cells. Blood, a so-called moving internal-organ, reversibly binds and releases O_2 under physiological conditions. From this point of view, realization of red blood cell (RBC) substitutes, or O_2-infusions, would contribute significantly to human health and welfare. In this research field, the basic sciences for macromolecular complexes, molecular assemblies, and nano-molecular sciences play fundamental roles. The authors have systematically studied the metal complexes (synthetic heme derivatives) embedded into a hydrophobic cluster in aqueous medium, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. As a result, the reaction activity is observed as cooperative phenomena with the properties of the molecular atmosphere. In other words, the development of our O_2-infusion has been based on "the regulation of the electronic process on macromolecular metal complexes".[1,2]

To reproduce the O_2-binding ability of RBCs, that is, the development of a synthetic O_2-carrier that does not need hemoglobin (Hb), was the starting point of the idea for this study. In general, central ferric iron of a heme is immediately oxidized by O_2 in water, preventing the O_2 coordination process from being observed. Therefore, the electron transfer

*Correspondence to: E. Tsuchida, Professor, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan.
E-mail: eishun@waseda.jp
†Selected paper presented at the 7th International Symposium on Polymers for Advanced Technologies, 21–24 September 2003, Fort Lauderdale, Florida, USA.

Copyright © 2005 John Wiley & Sons, Ltd.

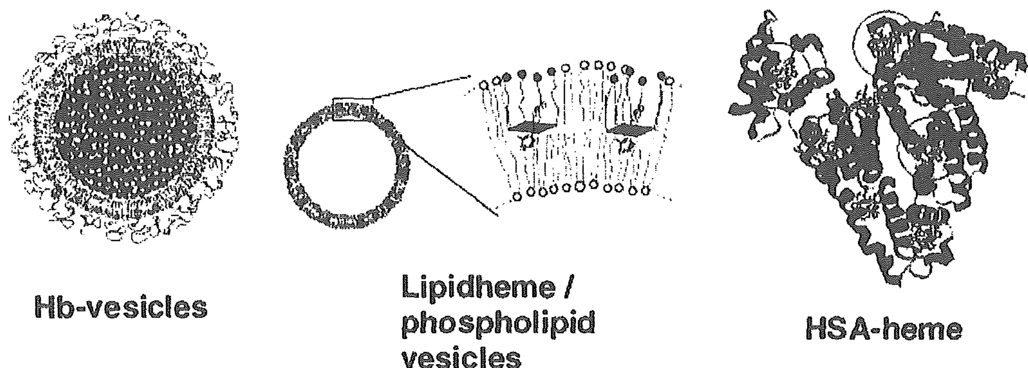

Figure 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme.

must be prevented. Fortunately, the formation of the O_2-adduct complex could be detected but for only several nano-seconds by utilizing the molecular atmosphere and controlling the electron density in the iron center. Based on this finding, the authors succeeded in reversible and stable O_2-coordination in 1983 and preparing phospholipid vesicles embedded amphiphilic-heme, known as lipidheme/phospholipids vesicles (Fig. 1).[3–5] This was the first example of reversible O_2-binding taking place under physiological conditions. For example, human blood can dissolve about 27 ml of O_2 per dl, however a 10 mM lipidheme-phospholipid vesicle solution can dissolve 29 ml of O_2 per dl. This material is suitable for "O_2-infusion". Thus over hundred types of heme derivatives have been synthesized, and recently new lipidheme bearing phospholipid groups have been synthesized, which completes self-organization in water to form stable vesicles.[6]

In 1985, Dr Sekiguchi at Hokkaido Red Cross Blood Center proposed Waseda group to consider the utilization of Hb in outdated RBCs. Thus the research of Hb-vesicles (HbV) based on molecular assembly technologies was started. In the latter 1990s, a mass-production system for recombinant human serum albumin (rHSA) was established and then albumin-heme hybrids (rHSA-heme) using its non-specific binding ability was prepared, which is now considered to be a promising synthetic material. Based on the effective integration of nano-molecular science and technologies for functional materials developed by Waseda University, and the outstanding evaluation system of safety and efficacy developed by Keio University using animal experiments, strong progress on the research of the O_2-infusion project has been made. In the near future, mass production and clinical tests of O_2-infusion will be started by the pharmaceutical industry.

DEVELOPMENT OF Hb-BASED O_2-CARRIERS AND THE CHARACTERISTICS OF HbV

Historically, the first attempt of Hb-based O_2-carrier in this area was to simply use stroma-free Hb (Fig. 2). However, several problems became apparent, including dissociation into

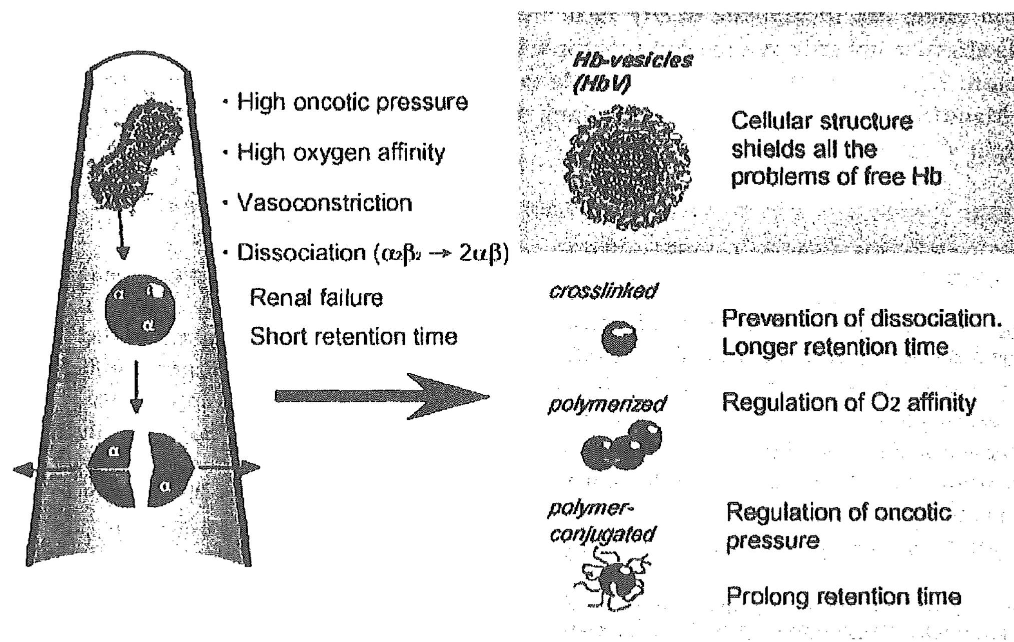

Figure 2. Approaches to solve the problems of utilization of Hb as an O_2-carrier, chemical modification or encapsulation of Hb.

Copyright © 2005 John Wiley & Sons, Ltd.

dimers that have a short circulation time, renal toxicity, high oncotic pressure and high O_2-affinity. Since the 1970s, various approaches were developed to overcome these problems.[7,8] This includes intra-molecular crosslinking, polymerization and polymer-conjugation. However, in some cases the significantly different structure in comparison with RBCs resulted in side effects such as vasoconstriction.[9]

Another idea is to encapsulate Hb with a lipid bilayer membrane to solve all the problems of molecular Hb.[10] RBCs have a biconcave structure with a diameter of about 8000 nm. RBCs can deform to a parachute-like configuration to pass through narrow capillaries. The possibility of infection and blood-type mismatching, and short shelf life are the main problems. The idea of Hb encapsulation with a polymer membrane mimicking the structure of RBC is originated from Dr Chang at McGill University.[7] After that, the encapsulation of Hb within a phospholipid vesicle was studied by Dr Djordjevich at the University of Illinois in the 1970s.[11] However, it was not so easy to make HbV with a regulated diameter and adequate O_2-transport capacity, the authors made a breakthrough in routinely producing HbV by using fundamental knowledge of macromolecular and supramolecular sciences.[12–19] Several liters of HbV are routinely prepared in a completely sterile condition. Hb is purified from outdated RBCs, and concentrated to 40 g/dl. Virus removal is performed using a combination of pasteurization at 60° and filtration with a virus removal filter. The Hb encapsulation with phospholipids bilayer membrane and size regulation was performed with an extrusion method. The vesicular surface is modified with polyethylene glycol (PEG) chains. The suspension of Hb-vesicles is deoxygenated at the final stage.

The particle diameter of HbV is regulated to about 250 nm, therefore, the bottle of HbV is turbid. One vesicle contains about 30,000 Hb molecules so that it does not show oncotic pressure. There is no chemical modification of Hb. O_2-affinity is controllable with an appropriate amount of allosteric effectors, pyridoxal 5-phosphate. Hb concentration is regulated to 10 g/dl, and the weight ratio of Hb to total lipid approaches 2.0 by using an ultra pure and concentrated Hb solution of 40 g/dl, which is covered with a thin lipid bilayer membrane. The surface is modified with 0.3 mol% of PEG-lipid. Viscosity, osmolarity, and oncotic pressure are regulated according to the physiological conditions.

HbV can be stored for over 2 years in a liquid state at room temperature.[17] There is little change in turbidity, diameter, and P_{50}. Methemoglobin (MetHb) content decreases due to the presence of reductant inside the HbV, which reduces the trace amount of metHb during storage. This excellent stability is obtained by deoxygenation and PEG-modification. Deoxygenation prevents metHb formation. The surface modification of HbV, with PEG chains prevents vesicular aggregation and leakage of Hb and other reagents inside the vesicles. Liquid state storage is convenient for emergency infusion compared to freeze-dried powder or the frozen state.

IN VIVO EFFICACY OF HbV

The efficacy of HbV has been confirmed mainly with isovolemic hemodilution and resuscitation from hemorrhagic shock.[20–28] In this review two important cases are described. One is isovolemic hemodilution with 90% blood exchange in a rat model. The other is resuscitation from hemorrhagic shock in a hamster model.

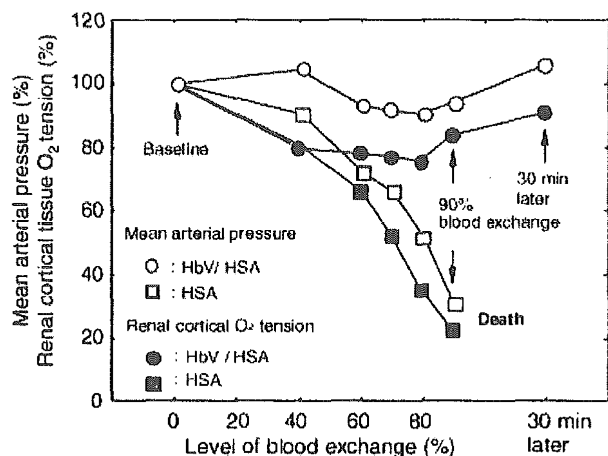

Figure 3. Ninety per cent exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored.

To confirm the O_2-transporting ability of HbV, extreme hemodilution was performed with HbV suspended in human serum albumin (HSA)[21,23] (Fig. 3). The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex, and the blood flow rate in the abdominal aorta was measured with the pulsed Doppler method. Hemodilution with albumin alone resulted in significant reductions in mean arterial pressure and renal cortical O_2 tension, and finally all the rats died of anemia. However, hemodilution with HbV, suspended in HSA sustained both blood pressure and renal cortical O_2 tension, and all the rats survived. These results clearly demonstrate that HbV has sufficient O_2 transporting capability.

To observe the microcirculatory response to the infusion of Hb products, intravital microscopy was used equipped with all the units to measure blood flow rates, vascular diameter, O_2 tension, and so on, in collaboration with Dr Intaglietta at the University of California, San Diego. The hamster dorsal-skin fold preparation allows observation of blood vessels from small arteries down to capillaries. The HbV suspension, as a resuscitative fluid for hemorrhagic-shocked hamsters was evaluated.[26] About 50% of the blood was withdrawn, and the blood pressure was maintained at around 40 mmHg for 1 hr, and the hamsters either received HbV suspended in HSA (HbV/HSA), HSA alone, or shed blood (Fig. 4). Immediately after infusion, all the groups showed increases in mean arterial pressure. However, only the albumin infusion resulted in incomplete recovery. However, the HbV/HSA group showed the same recovery with the shed autologous blood infusion. During the shock period, all the groups showed significant hyperventilation that was evident from the significant increase in arterial O_2 tension. Simultaneously, base excess and pH decreased significantly. Immediately after resuscitation, all the groups tended to recover. However, only the HSA group showed sustained hyperventilation. Base excess for the HSA group remained at a

Copyright © 2005 John Wiley & Sons, Ltd.

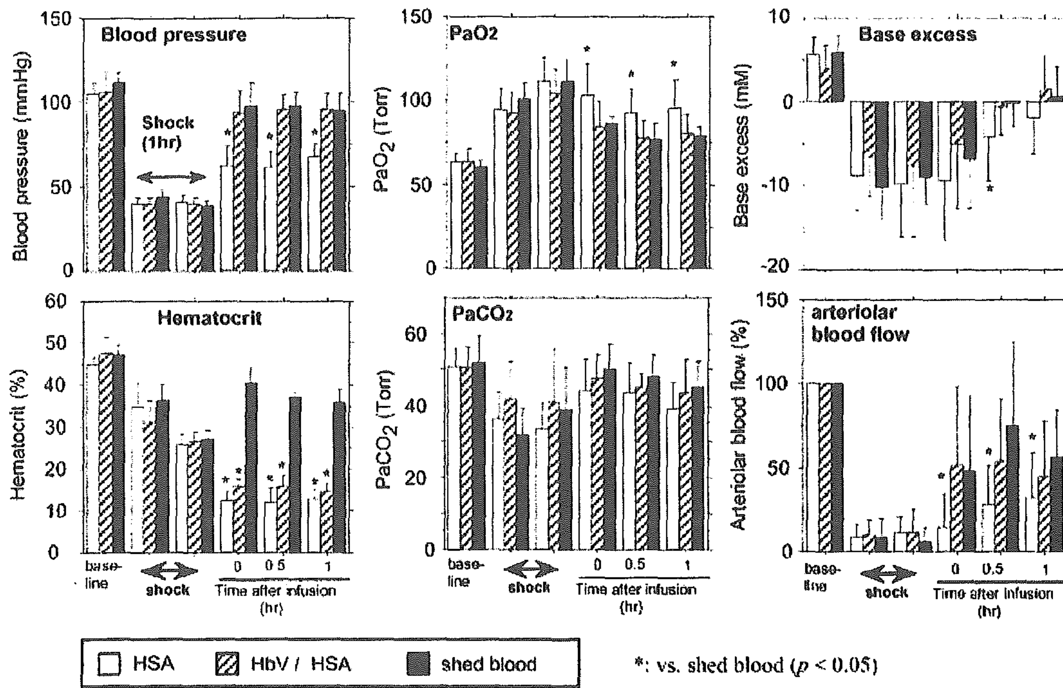

Figure 4. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD.

significantly lower value 1 hr after resuscitation. Blood flow decreased significantly in arterioles to 11% of basal value during shock. The HbV/HSA and shed autologous blood groups immediately showed significant increases in blood flow rate after resuscitation, while the albumin group showed the lowest recovery.

SAFETY EVALUATION OF HbV

The safety profile of HbV such as cardiovascular responses, pharmacokinetics, influence on RES, influence on clinical measurements and daily repeated infusions were further examined.[29–37]

The microvascular responses to the infusion of intramolecularly crosslinked Hb (XLHb) and HbV were studied using conscious hamsters. XLHb (7 nm in diameter) showed a significant increase in hypertension equal to 35 mmHg, and simultaneous vasoconstriction of the resistance artery equal to 75% of the baseline levels[30] (Fig. 5). However, HbV with diameter of 250 nm showed minimal changes. The small acellular XLHb is homogeneously dispersed in the plasma, and it diffuses through the endothelium layer of the vascular wall and reaches the smooth muscle. XLHb traps nitric oxide (NO) as an endothelium-derived relaxation factor, and induces vasoconstriction, and hypertension. However, the large HbV stay in the lumen and does not induce vasoconstriction. Several mechanisms are proposed for Hb-induced vasoconstriction. These include NO-binding, excess O_2 supply, reduced shear stress, or the presence of Hb recognition site on the endothelium. But it is clear that Hb-encapsulation shields against the side effects of acellular Hbs.

Professor Suematsu at Keio University has revealed the effects of Hb-based O_2 carriers in hepatic microcirculation[29,32] (Fig. 6). On the vascular wall of the sinusoid in hepatic microcirculation, there are many pores, called fenestration, with a diameter of about 100 nm. The small Hb

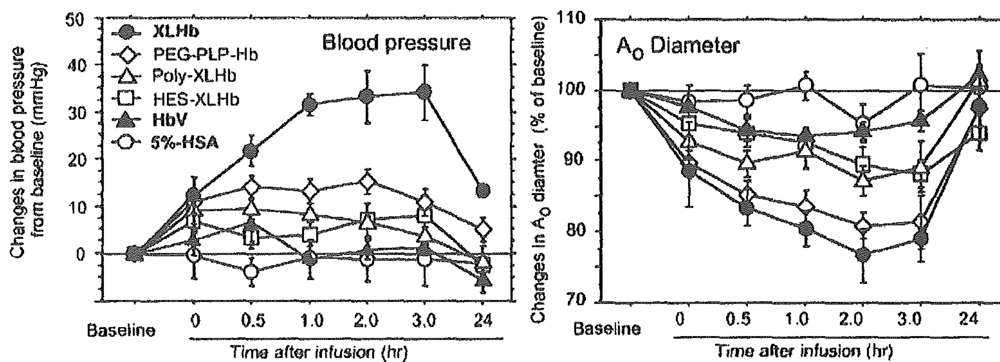

Figure 5. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2-carriers. Mean ± SD.

Copyright © 2005 John Wiley & Sons, Ltd.

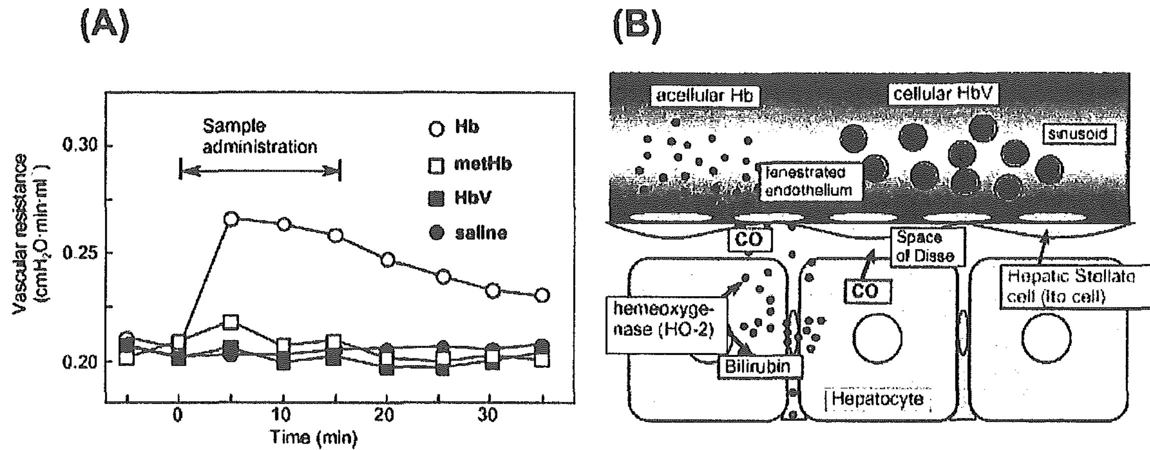

Figure 6. (A) Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. (B) Schematic representation of hepatic microcirculation: the small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolized by hemeoxygenase-2 (HO-2) and CO is released as a vasorelaxation factor. However, the excess amount of the extravasated Hb traps CO and induces vasoconstriction and the resulting higher vascular resistance. However, the larger HbV retains in the sinusoid and there is no extravasation and vasoconstriction.

molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. However, HbV particles, which are larger than the pores, do not extravasate. Heme of extravasated Hb is excessively metabolized by hemeoxygenase-2 in hepatocyte to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb rapidly binds CO, resulting in the vasoconstriction and an increase in vascular resistance. Furthermore, HbV (250 nm in diameter) is large enough to remain in the sinusoid, and the vascular resistance is maintained.

From these results, the optimal molecular dimension of Hb-based O_2 carriers can be proposed. The upper limitation is below the capillary diameter to prevent capillary plugging, and for sterilization by membrane filters (Fig. 7). However, smaller sizes exhibit a higher rate of vascular wall permeability with side effects such as hypertension and neurological disturbances. HbV exhibits a very low level of vascular wall permeability. Therefore, the HbV appears to be appropriate from the viewpoint of hemodynamics. However, the influence of HbV on the RES has to be clarified, because the fate of HbV is RES trapping.

Circulation persistence was measured by monitoring the concentration of radioisotope-labeled HbV in collaboration with Dr Phillips at the University of Texas at San Antonio. The circulation half-life is dose dependent, and when the dose rate was 14 ml/kg, the circulation half-life was 35 hr in rats. The circulation time in the case of the human body can be estimated to be twice as long; or about 3 days at the same dose rate. Gamma camera images of radioisotope-labeled HbV

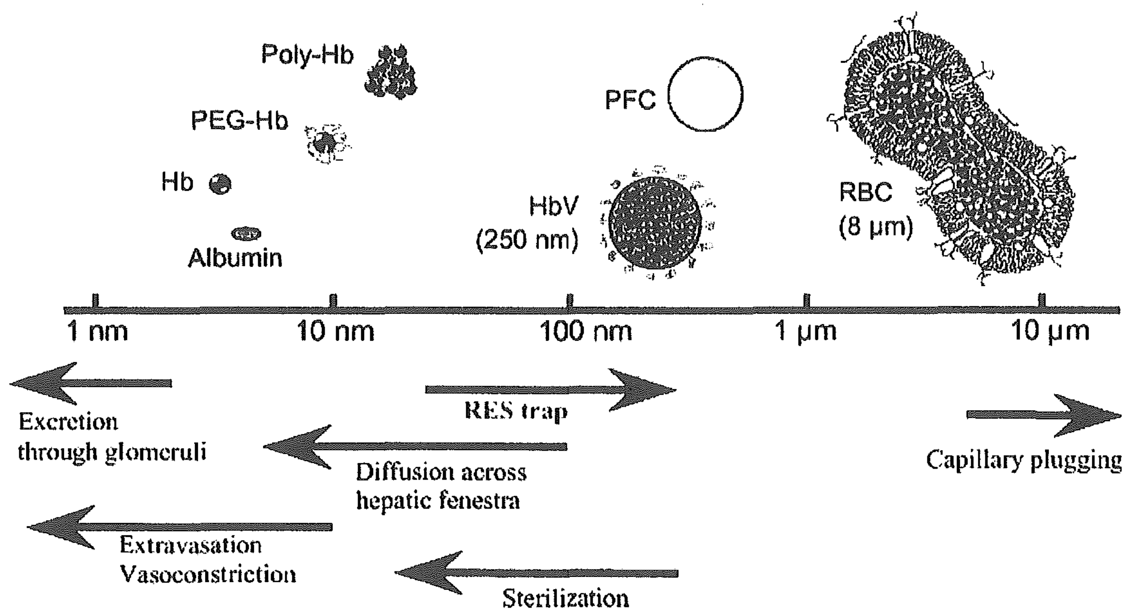

Figure 7. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process.

Copyright © 2005 John Wiley & Sons, Ltd.