Safety and Efficacy of Hemoglobin-Vesicles and Albumin-Hemes

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Summary. Keio University and Waseda University have worked together on artificial O_2 carrier research for 20 years in close cooperation. Two candidate materials have been selected from the viewpoints of safety, efficacy, and cost performance. One is Hemoglobin-vesicles (HbV) and the other is albumin-heme (rHSA-heme). This chapter summarizes our video presentation that introduced the recent results of our research into HbV and rHSA-heme.

Key words. Blood substitutes, Oxygen carriers, Hemoglobin-vesicles, Albumin-heme, Oxygen therapeutics

Introduction: Keio-Waseda Joint Research Project

For human beings to survive, it is necessary to continuously deliver oxygen that is needed for the respiration of all tissue cells. Red blood cell, a so-called moving internal-organ, reversibly binds and releases O_2 under physiological conditions. From this point of view, red blood cell substitutes, or O_2-Infusions, are very important. In order to promote this research, we have emphasized that the establishment of basic science for macromolecular complexes and molecular assemblies is essential. We have systematically studied the Metal Complexes (synthetic heme derivatives) embedded into a hydrophobic cluster, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. Therefore, the reaction activity and its rate constant are observed as cooperative phenomena with the

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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 Complexes and Synthesis of Functional Materials" [1,2].

Reproducing the O_2-binding ability of red blood cells (RBC), that is, the development of a synthetic O_2 carrier that does not need hemoglobin (Hb), was the starting point of our 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 must be prevented. We were able to detect the formation of the O_2-adduct complex, but for only several nano seconds, by utilizing the molecular atmosphere and controlling the electron density in the iron center. Based on this finding, we succeeded in 1983 with reversible and stable O_2 coordination and preparation of phospholipid vesicles embedded amphiphilic-heme, known as lipidheme/phospholipids vesicles (Fig. 1) [3–6]. This was the world's 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.

Soon after this discovery, Professor Kobayashi of Keio University asked Professor Tsuchida for a chance to evaluate the lipidheme solution with in vivo experiments. Since then the joint research and collaboration has continued since that time. We have synthesized over one hundred types of heme, and recently synthesized new lipidheme–bearing phospholipid groups, which complete self-organization in water to form stable vesicles. In 1985 Dr. Sekiguchi at Hokkaido Red Cross Blood Center proposed that Professor Tsuchida consider the utilization of outdated red blood cells and Hbs because, while the totally synthetic system is definitely promising it appeared that it

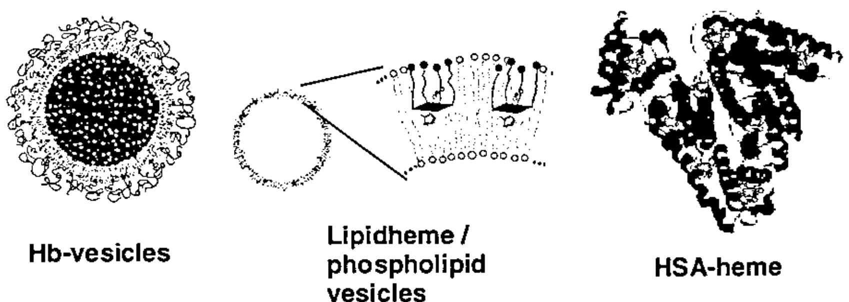


Fig. 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme

would take considerable time to arrive at a social consensus for its use. We started to produce Hb-vesicles (HbV) using purified Hbs and molecular assembly technologies. In the late 1990's, a mass-production system for recombinant human serum albumin (rHSA) was established and we then prepared albumin-heme hybrids (rHSA-heme) using its non-specific binding ability, which is now considered to be a promising synthetic material.

Based on our effective integration of 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, we have made strong progress in our research on the O_2-Infusion Project. During this period, we have received substantial funding support from the Japanese government. In the near future, mass production and clinical tests of O_2-Infusion will be started by a certain pharmaceutical industry.

Background and the Significance of HbV

Historically, the first attempt of Hb-based O_2 carrier in this area was to simply use stroma-free Hb. However, several problems became apparent, including dissociation into 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 intramolecular crosslinking, polymerization and polymer-conjugation. However, in some cases the significantly different structure in comparison with red blood cells resulted in side effects such as vasoconstriction [9].

Another idea is to encapsulate Hb with a lipid bilayer membrane to produce HbV that solves all the problems of molecular Hb [10]. Red blood cells have a biconcave structure with a diameter of about 8000 nm. Red blood cells 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. Purified Hb does not contain blood-type antigen and pathogen, thus serves as a safe raw material for HbV.

HbV, with a diameter of 250 nm, do not have deformability but are small enough to penetrate capillaries or constrict vessels that RBC cannot penetrate. The surface of the vesicles is modified with polyethylene glycol (PEG) to ensure homogeneous dispersion when circulated in the blood and a shelf life of two years. The idea of Hb encapsulation with a polymer membrane mimicking the structure of RBC originated from Dr. Chang at McGill University [7]. After that, the encapsulation of Hb within a phospholipid vesicle was studied by Dr. Djordjevici at the University of Illinois in the 1970s [11]. However, it was not so easy to make HbV with a regulated diameter and ade-

quate O_2 transport capacity. We made a breakthrough in routinely producing HbV by using fundamental knowledge of macromolecular and supramolecular sciences. Some of the related technologies have already been published in academic journals [12–19]. Several liters of HbV are routinely prepared in a completely sterile condition. Hb is purified from outdated red blood cells, and concentrated to 40 g/dl. Virus removal is performed using a combination of pasteurization at 60°C 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 PEG chains. The suspension of Hb-vesicles is dated at the final stage.

The particle diameter of HbV is regulated to about 250 nm, therefore, the bottle of HbV is turbid, and is a suspension. One vesicle contains about 30,000 Hb molecules, and it does not show oncotic pressure. There is no chemical modification of Hb. Table 1 summarizes the physicochemical characteristics of HbV. 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 two years in a liquid state at room temperature [17]. There is little change in turbidity, diameter, and P_{50}. MetHb content decreases due to the presence of reductant inside the HbV, which reduces the

Table 1. Physicochemical characteristics of HbV suspended in 5% albumin (HSA)

Parameters	HbV/HSA	Human blood (RBC)	Analytical method
diameter (nm)	220–280	8000	Light scattering method
P_{50} (Torr)	27–34[1]	26–28	Hemox Analyzer
[Hb] (g/dl)	10 ± 0.5	12–17	CyanometHb method
[Lipid] (g/dl)	5.3–5.9	1.8–2.5[2]	Molibuden-blue method
[Hb]/[Lipid] (g/g)	1.6–2.0	6.7[3]	—
[PEG-lipid] (mol%)	0.3	—	^{1}H-NMR
metHb (%)	<3	<0.5	CyanometHb method
viscosity (cP)[4]	3.7	3–4	Capillary rheometer
osmolarity (mOsm)	300	ca. 300	(suspended in saline)
oncotic press. (Torr)	20	20–25	Wescor colloid osmometer
pH at 37°C	7.4	7.2–7.4	pH meter
Endotoxin (EU/mL)	<0.1	—	LAL assay
Pyrogen	Free	—	rabbit pyrogen test

[1] Adjustable, [2] Total cell membrane components, [3] Weight ratio of Hb to total cell membrane components, [4] At $230\,s^{-1}$.

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 with isovolemic hemodilution and resuscitation from hemorrhagic shock. Some of the results have already been published in academic journals in the fields of emergency medicine and physiology [20–28]. In this chapter two important facts 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.

To confirm the O_2 transporting ability of HbV, extreme hemodilution was performed with HbV suspended in human serum albumin (HSA) [21,23] (Fig. 2). The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex and skeletal muscle, 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

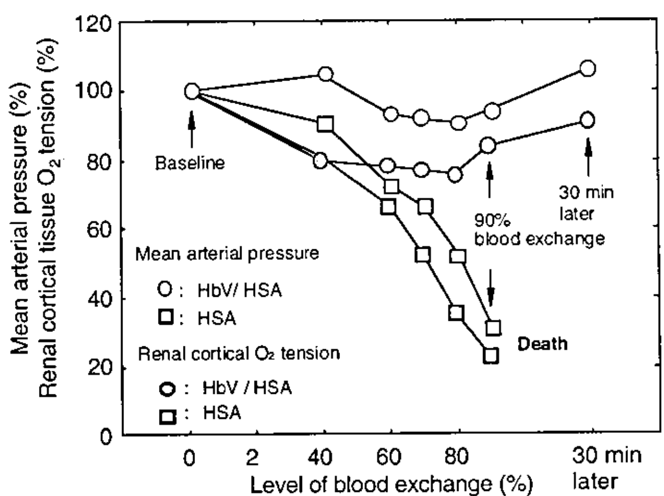


Fig. 2. 90% exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored

arterial pressure and renal cortical O_2 tension, and finally all the rats died of anemia. On the other hand, 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, we used the intravital microscopy equipped with all the units to measure blood flow rates, vascular diameter, O_2 tension, and so on. This system was developed by Professor Intaglietta at the University of California, San Diego. We used the hamster dorsal-skin fold preparation that allows observation of blood vessels from small arteries to capillaries. We evaluated the HbV suspension as a resuscitative fluid for hemorrhagic-shocked hamsters [26]. About 50% of the blood was withdrawn, and the blood pressure was maintained at around 40 mmHg for 1 h. The hamsters either received HbV suspended in HSA (HbV/HSA), HSA alone, or shed blood (Fig. 3). Immediately after infusion, all the groups showed increases in mean arterial pressure, however, only the albumin infusion resulted in incomplete recovery. 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 partial. Simultaneously,

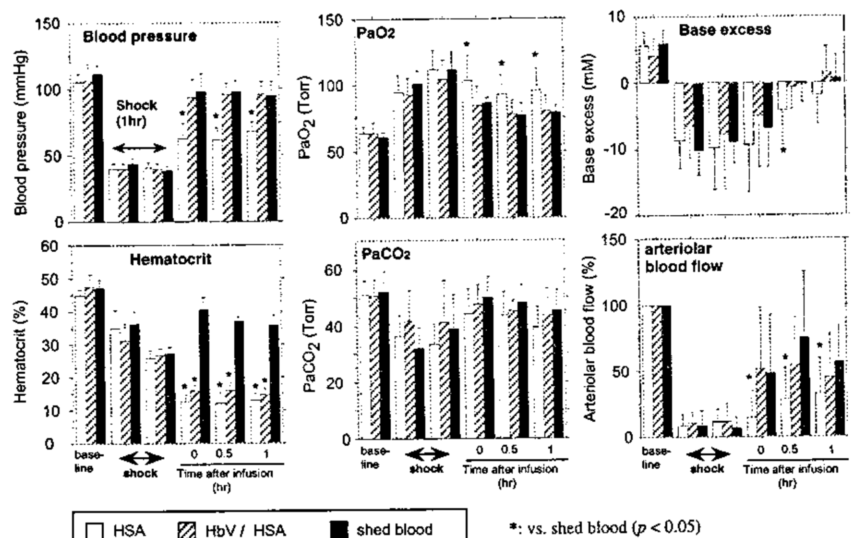


FIG. 3. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD

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 significantly lower value one hour 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.

In Vivo Safety of HbV

We further examined the safety profile of HbV such as cardiovascular responses, pharmacokinetics, influence on reticulo endothelial system (RES), influence on clinical measurements and daily repeated infusion [29–35].

We observed the responses to the infusion of intra-molecularly cross-linked Hb (XLHb) and HbV into 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. 4). On the other hand, HbV at 250 nm, showed minimal change. 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. Intra-moleculary cross-linked Hb traps nitric oxide (NO) as an endothelium-derived relaxation factor, and induces vasoconstriction, and hypertension. On the other hand, the large HbV stay in the lumen and do 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

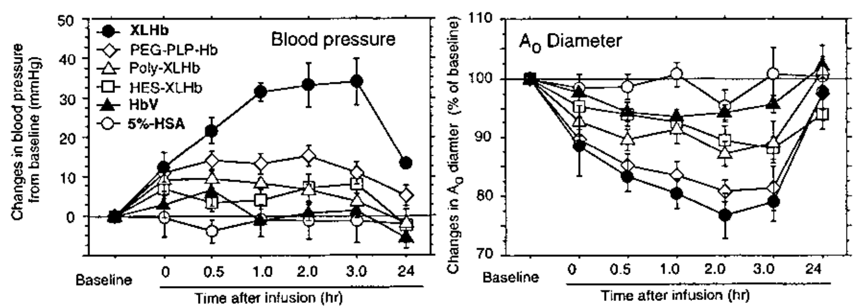


FIG. 4. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2 carriers. Mean ± SD

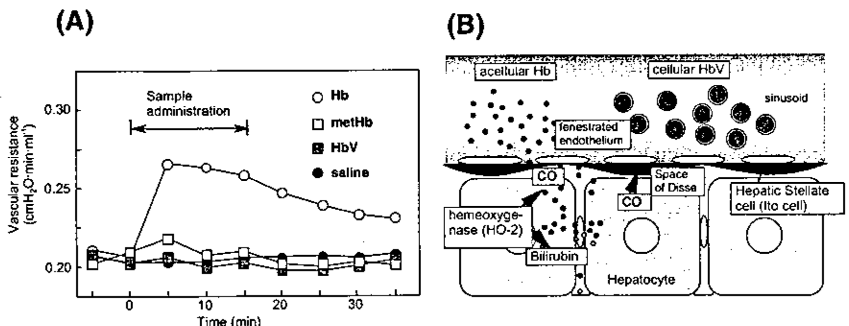


Fig. 5. A Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. B Schematic representation of hepatic microcirculation: The small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolised 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. On the other hand, the larger HbV retains in the sinusoid and there is no extravasation and vasoconstriction 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. 5). 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 molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. On the other hand, 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. On the other hand, HbV (250 nm in diameter) is large enough to remain in the sinusoid, and the vascular resistance is maintained.

So, what is the optimal molecular dimension of Hb-based O_2 carriers? The upper limitation is below the capillary diameter to prevent capillary plugging, and for sterilization by membrane filters (Fig. 6). On the other hand, 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. We have clarified the influence of HbV on the RES, because the fate of HbV is RES trapping.

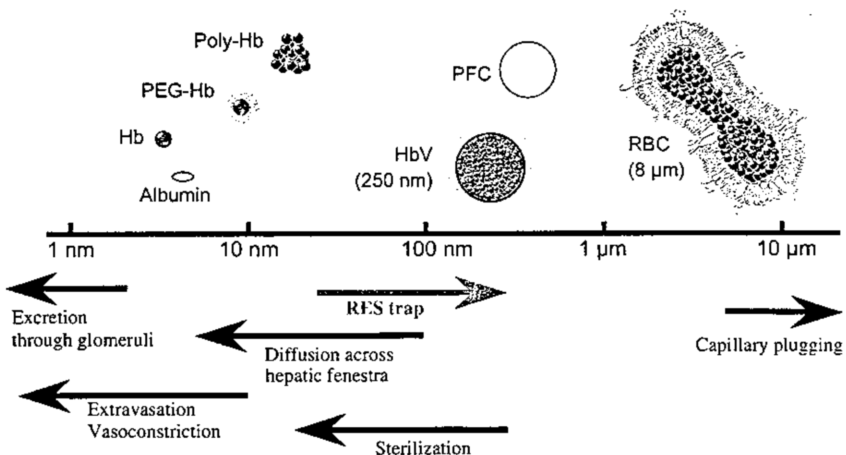
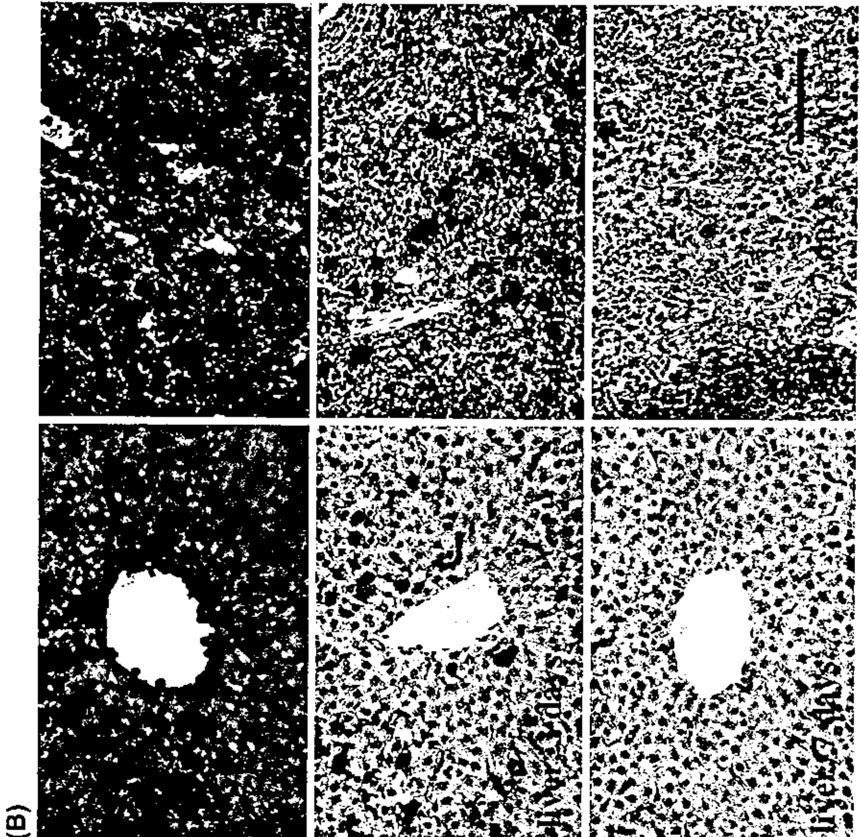


Fig. 6. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process

Circulation persistence was measured by monitoring the concentration of radioisotope-labeled HbV in collaboration with Dr. W.T. 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 24 h. The circulation time in the case of the human body can be estimated to be twice or three times longer; or about 2 or 3 days at the same dose rate. Gamma camera images of radioisotope-labeled HbV showed the time course of biodistribution. Just after infusion, HbV remains in the blood stream so that the heart and liver that contain a lot of blood showed strong intensity. However, after it is finished playing its role in O_2 transport, a total of 35% of HbV are finally distributed mainly in the liver, spleen and bone marrow.

The time course of liver uptake was monitored with a confocal fluorescence microscope. Hb-vesicle was stained with a lipid fluorophore. The liver of an anesthetized hamster was exposed and a fluorescence-labeled-HbV was infused intravenously. Due to the motion of respiration, the picture oscillates. However, a static frame can be obtained. The individual particles of HbV cannot be recognized. When the vesicles are accumulated in phagosomes of Kupffer cells, they can be recognized with a strong fluorescence. How is HbV metabolized in macrophages? The transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes [34] (Fig. 7). Red blood cells and HbV contain a lot of ferric iron with a high electron density, so that they show



strong contrast in TEM. However, after 7 days, the HbV structure cannot be observed. We confirmed no abnormalities in the tissues and no irreversible damage to the organs or complete metabolism within a week. A Polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV. This antibody does not recognize rat Hb. The red-colored parts indicate the presence of Hb in HbV, and almost disappear after 7 days in both the spleen and liver. This shows that HbV can be metabolized quite promptly.

One issue of the Hb-based O_2 carriers is that they have a significant influence on clinical laboratory tests. They remain in the plasma phase in hematocrit capillaries after centrifugation of blood samples, and interfere with the colorimetric and turbidimetric measurements. However, HbV can be simply removed from blood plasma either by ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran to enhance precipitation. We can obtain a very clear supernatant for accurate analyses [35]. This is one advantage of HbV in comparison with acellular Hb solutions. Accordingly we examined the influence on organ functions by serum clinical laboratory tests after the bolus infusion of HbV at a dose rate of 20 ml/kg. Albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH), which reflect the liver function, moves their values within normal range. Concentrations of bilirubin and ferric ion are maintained at a low level. The concentration of lipids transiently changed. In particular, the cholesterols increased significantly. And phospholipids slightly increased, however, they returned to the original level after 7 days. These results indicate that the membrane components of HbV, once they reappear from RES, are metabolized on the physiological pathway.

We recently tested a daily repeated infusion of HbV in Wistar rats as a safety study. The dose rate was a 10-ml/kg/day infusion for 14 days. All rats well tolerated and survived. Body weight showed a monotonous but slightly depressed increase in comparison with the saline group. However, after 2 weeks there was no significant difference with the saline control group. All the rats seemed very healthy and active. There was no piloerection. As for the hematological parameters, the numbers of white blood cells and platelets did not exhibit a significant difference from the HbV group and the saline control group. Hematocrit showed a slight reduction for the HbV group, probably due to the accumulation of the large amount of HbV in the blood. Histopathological examination one day after the final infusion of HbV showed signifi-

◄ Fig. 7. **A** Transmission electron microscopy of rat spleen one day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. **B** Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. They disappeared within 7 days. Cited from: Sakai et al (2001) Am J Pathol 159:1079–1088

cant accumulation of HbV in spleen macrophages, and liver Kupffer cells. Berlin Blue staining revealed the presence of hemosiderin indicating that the metabolism of Hb was initiated. There were no other morphological abnormalities, and the serum clinical chemistry indicated transient but reversible increases in lipid components. AST and ALT were within the normal range. From these results we are confident with the safety of HbV.

Design and Physicochemical Properties of rHSA-Heme

We have been conducting research on totally synthetic O_2 carriers, or so-called albumin-heme that does not require Hb. Human serum albumin is the most abundant plasma protein in our blood stream, but its crystal structure has not been elucidated for long time. In 1998, Dr. Stephen Curry of the Imperial College of London was the first elucidate the crystal structure of the human serum albumin complexed with seven molecules of myristic acids [36]. He found that the dynamic conformational changes of albumin take place by the binding of fatty acid.

In Japan, recombinant human serum albumin is now manufactured on a large scale by expression in the yeast Pichia pastoris, and it will appear on the market soon [37]. A large-scale plant, which can produce one million vials per year, has been already established. From the viewpoint of clinical application, O_2-carrying albumin is quite exciting and may be of extreme medical importance. With this background, we have found that synthetic heme derivative is efficiently incorporated into recombinant human serum albumin (rHSA), creating a red-colored rHSA-heme hybrid. This rHSA-heme can reversibly bind and release O_2 molecules under physiological conditions in the same manner as Hb. In other words, our rHSA-heme hybrid is a synthetic O_2-carrying hemoprotein, and we believe that its saline solution will become a new class of red blood cell substitute. We have already published these chemistry findings and technologies in international journals [38–49].

Figure 8 summarizes the structure of the albumin-heme molecule. The Maximal binding numbers of heme to one albumin are eight, and the magnitude of the binding constants ranged from 10^6 to 10^4 (M^{-1}). The isoelectric point of rHSA-heme was found to be 4.8, independent of the binding numbers of heme. This value is exactly the same as that of albumin itself. Furthermore, the viscosity and density did not change after the incorporation of heme molecules, and the obtained solution showed a long shelf life of almost two years at room temperature. The O_2-binding sites of rHSA-heme are iron-porphyrin, therefore the color of the solution changed in a similar way to Hb. Upon addition of O_2 gas through this solution, the visible absorption pattern immediately changed to that of the O_2-adduct complex. Moreover, after bubbling carbon monoxide gas, albumin-heme formed a very stable carbonyl complex.

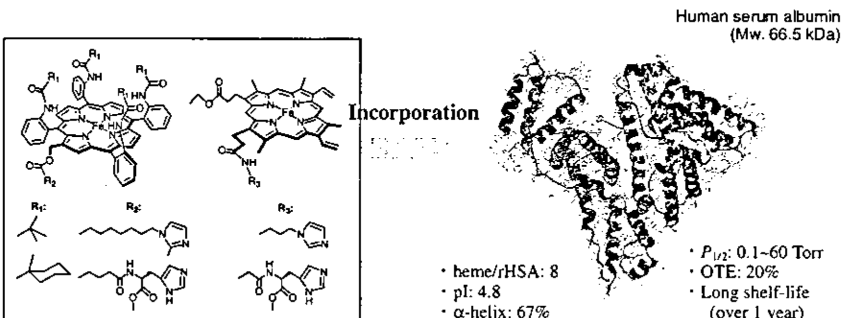


Fig. 8. Structure of the albumin-heme molecule

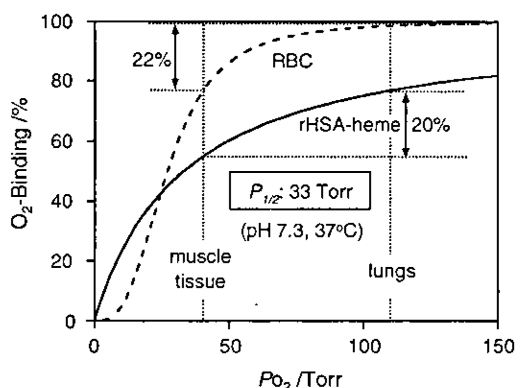


Fig. 9. O_2-binding equilibrium curve of albumin-heme

Figure 9 shows the O_2-binding equilibrium curve of rHSA-heme. The O_2-binding affinity, of rHSA-heme is always constant, independent of the number of heme, and the O_2-binding profile does not show cooperativity. However, the O_2-transporting efficiency of albumin-heme between the lungs where PaO_2 is 110 Torr and muscle tissue where PtO_2 is 40 Torr increases to 20%, which is similar to 22% efficiency of red blood cells. The O_2-binding property of albumin-heme can be controlled by changing the chemical structure of heme derivatives incorporated. More recently, we have found that a protoheme derivative is also incorporated into albumin and can bind and release O_2 as well [50].

In Vivo Safety and Efficacy of rHSA-Heme

Based on these findings, we can say that rHSA-heme can become an entirely synthetic O_2-carrier, and satisfy the initial clinical requirements for a red blood cell substitute. However, we have another problem to solve before we can use this material as an O_2-carrier in the circulatory system. This problem is NO scavenging. Of course, it can bind NO, and it may be anticipated that the injection of rHSA-heme also induce hypertensive action. We have evaluated the efficacy and safety of this rHSA-heme solution with animal experiments.

As described before, small Hb molecules extravasate through the vascular endothelium and react with NO, thus inducing vasoconstriction and acute increases in systemic blood pressure. Contrary to the expectations, the observation of the intestinal microcirculation after the infusion of rHSA-heme into an anesthetized rat revealed that the diameters of the venules and arterioles were not deformed at all [51]. Indeed, only a small change in the mean arterial pressure was observed after the administration of the rHSA-heme solution (Fig. 10). In contrast, the infusion of Hb elicited an acute increase in blood pressure. Why does albumin-heme not induce vasoconstriction or hypertension? The answer probably lies in the negatively charged molecular surface of albumin. One of the unique characteristics of serum albumin is its low permeability through the capillary pore, which is less than 1/100 that for Hb due to the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells.

We are now evaluating the O_2-transporting ability of this albumin-heme molecule in the circulatory system with further animal experiments [52]. First, we determined the physiological responses to exchange transfusion with rHSA-heme solution into rats after 70% hemodilution and 40% hemorrhage (Fig. 11). The declined mean arterial pressure and blood flow after a 70% exchange with albumin and further 40% bleeding of blood showed a significant recovery of up to 90% of the baseline values by the infusion of the rHSA-heme solution. On the other hand, all rats in the control group only injected with albumin died within 30 min. Furthermore, muscle tissue O_2-tension significantly increased. These responses indicate the in vivo O_2-delivery of the rHSA-heme solution.

More recently, we have synthesized human serum albumin dimer, which can incorporate sixteen hemes in its hydrophobic domain [53]. The human serum rHSA-heme dimer solution dissolves 1.2 times more O_2 compared to that of red blood cells and keeps its colloid osmotic pressure at the same level as the physiological value.

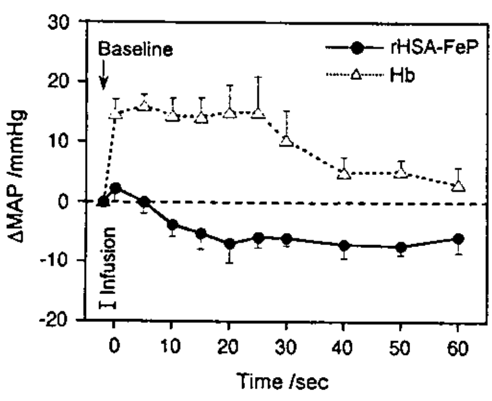


Fig. 10. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats (n = 5). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± S.E. Basal value is 90.1 ± 3.0 mmHg

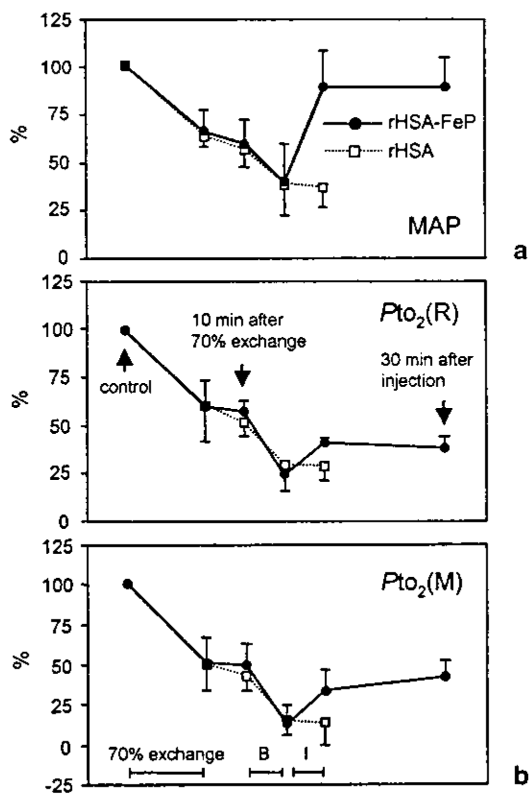


Fig. 11. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats (n = 5). All data are shown as changes from the basal values and expressed as mean ± S.E.

Potential Applications of Artificial O_2 Carriers

For almost 20 years our group at Keio University in collaboration with Dr. Tsuchida's group at Waseda University have been trying to produce artificial O_2 carriers. To date, we have produced several types of O_2 carriers and evaluated their efficacy and biocompatibility. In this chapter, we have shown what we have done to produce O_2 carriers. Below, we would like to show you the potential applications of artificial O_2 carriers, as well as a glimpse of the vast possibilities that lie ahead.

Tumor Oxygenation

Unlike vessels in normal tissues, the development of a vasculature in a tumor lacks normal course of angiogenesis and is hence, highly heterogeneous. Consequently, areas of hypoxia are quite common in tumors. In these hypoxic regions, it can be added that tumor cells acquire resistance to treatments such as chemotherapy and radiation. Our rHSA-heme was injected into the responsible artery that supplies circulation to an implanted tumor (Fig. 12) [54]. O_2 tension of the tumor rises immediately after intra-arterial infusion of albumin heme up to 2.4 times that of the baseline value. Our findings in animals indicate that tumor tissue O_2 levels can be elevated by the administration of artificial O_2 carriers due to the difference in O_2 transporting properties from red blood cells. Whether this increase in tissue O_2 can potentiate cancer treatment is currently under investigation.

Organ Preservation

One of the most important agenda in transplantation medicine is long-term organ preservation and circumvention of ischemia reperfusion injuries. We

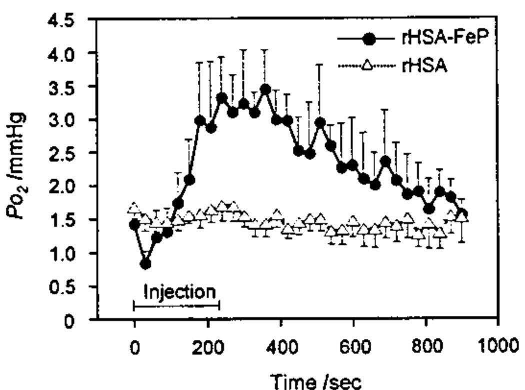


Fig. 12. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats ($n = 4$ each). All data are shown as changes from the basal values (Po_2) just before the infusion and expressed as mean ± S.E.

think that artificial O_2 carriers can be applied as a perfusate for donor tissue in order to overcome these problems. In particular, its O_2 carrying capacity has the potential to significantly extend the preservation period. This will make it easier to transport organs. Also, utilizing the extra time, we may in the future be able to perform additional organ tests for better compatibility, or even perform genetic modifications during this period. We believe that through these applications, the concept of organ preservation can be expanded to organ culture, and furthermore to include the preservation of cells derived from donor tissues.

Extracorporeal Circulation

Extracorporeal circulation is quite common in cardiac surgery. Improvements are being made in the priming solutions but red cells are often still required to fill the device circuit, particularly in compromised cases and in children [55]. We believe that the use of artificial O_2 carriers in the priming solution can decrease or completely eliminate the need for a transfusion in such cases, and hence reduce the incidence of infection or GVHD.

Tissue Ischemia

Tissue ischemia can ensue from impairment of peripheral perfusion due to a variety of diseases such as arteriosclerosis obliterans, diabetes, and Burger's disease. The key event in the progression of ischemic diseases is the inability of red cells to flow through the capillaries, beyond obstruction ulceration and gangrene formation become imminent. We believe that this critical phase can be avoided or delayed by the application of artificial O_2 carriers, which can be designed to flow through these damaged capillaries or collateral circulation [27,28]

Liquid Ventilation for Acute Lung Injury

For patients who present acute lung injury or acute respiratory distress syndrome (ARDS), gas exchange in the lung exhibits severe deterioration and sometimes even the newest mechanical ventilation method fails to establish adequate oxygenation of the blood. In this type of critical case, liquid ventilation using an artificial O_2 carrier can establish optimal oxygenation of the blood and may reproduce the integrity of lung parenchyma [56]. Briefly explained, oxygenated liquid ventilation fluid is administered into the lung through trachea and O_2 molecules are transferred through diseased alveolus by diffusion and oxygenate the blood. Currently, this method is thought to be effective for patients with congenital diaphragmatic herniation. Efficacy for adult acute lung injuries is now under investigation. Perfluorochemicals are the main fluid used for clinical use, however, aqueous artificial O_2 carriers may have the potential to be used for liquid ventilation.

Epilogue

The research field of the red cell substitutes is moving forward very rapidly. Also as you have seen, the paradigm in this field is expanding from red cell substitutes to "O_2 therapeutics". The quality control and the pre-clinical test will be completed on the carriers produced at the pilot plant, after which clinical trials will proceed. We look forward to the day that our research will play an effective role in treating patients.

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Hemoglobin-Vesicles (HbV) as Artificial Oxygen Carriers

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Summary. Considering the physiological significance of the cellular structure of a red blood cell (RBC), it may be reasonable to mimic its structure for designing a hemoglobin (Hb)-based oxygen carrier. In this chapter, we have summarized the characteristics and performances of Hb-vesicles (HbV) that have been developed on the basis of molecular assembly. Collaborative in vitro and in vivo studies have revealed sufficient safety and efficacy of HbV.

Key words. Blood substitutes, Hemoglobin-vesicles, Red blood cells, Oxygen transport, Liposome

Introduction: Importance of Cellular Structure

When we design an artificial oxygen carrier based on hemoglobin (Hb) molecules, we may have to reconsider why Hb is encapsulated in RBCs in our body. Barcroft et al. (1923) insisted that the reasons for Hb encapsulation in RBCs were: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of Hb from blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, NADPH, etc.) and other electrolytes [1]. Moreover, during the long history of development of Hb-based oxygen carriers, many side effects of molecular Hb have become apparent such as renal toxicity due to the dissociation of tetrameric Hb subunits to two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$), which may induce renal toxicity and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction,

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Efficacy of HbV as Oxygen Carriers in Vivo

The advantages of the HBOCs are the absence of blood-type antigens and infectious viruses, and stability for long-term storage that overwhelm the RBC transfusion. The shorter half lives of HBOCs in the blood stream (2–3 days) limit their use but they are applicable for a shorter period of use such as: (1) a resuscitative fluid for hemorrhagic shock in an emergency situation for a temporary time or bridging until the packed RBCs are available, (2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion, (3) a priming solution for the circuit of an extracorporeal membrane oxygenator (ECMO), and (4) other potential indications, e.g., so-called O_2 therapeutics to oxygenate ischemic tissues.

One particle of HbV (diameter, ca. 250nm) contains about 30,000 Hb molecules. Since HbV acts as a particle in the blood, not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires the addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates of plasma expanders are human serum albumin (HSA), hydroxyethyl starch, dextran, or gelatin depending on the clinical setting, cost, countries and clinicians. The absence of any infectious disease from humans is the greatest advantage of recombinant human serum albumin (rHSA) and it will soon be approved as an alternative for clinical use in Japan. Moreover, there should be no immunological and hematological abnormalities that are often seen in the use of dextran and hydroxyethyl starch. Aiming at application of HbV suspended in a plasma expander to the above indications, HbV was tested for resuscitation from hemorrhagic shock [97–100] and extreme hemodilution [57,58,101–105] in the Waseda-Keio group and with Prof. Intaglietta at the University of California, San Diego. Moreover, HbV was tested for oxygenation of an ischemic skin flap by Dr. Erni et al. at Inselspital University Hospital, Bern [106,107], and this implies the further application of HbV for other ischemic diseases such as myocardial and brain infarction and stroke. Some of the published results are summarized in this section.

Resuscitation from Hemorrhagic Shock with HbV Suspended in Recombinant Human Serum Albumin [100]

Objective. The ability of the suspension of HbV to restore the systemic condition after hemorrhagic shock was evaluated in anesthetized Wistar rats for 6h after resuscitation.

Methods. The HbV was suspended in a 5g/dl recombinant human serum albumin solution (HbV/rHSA) at an Hb concentration of 8.6g/dl. Forty male

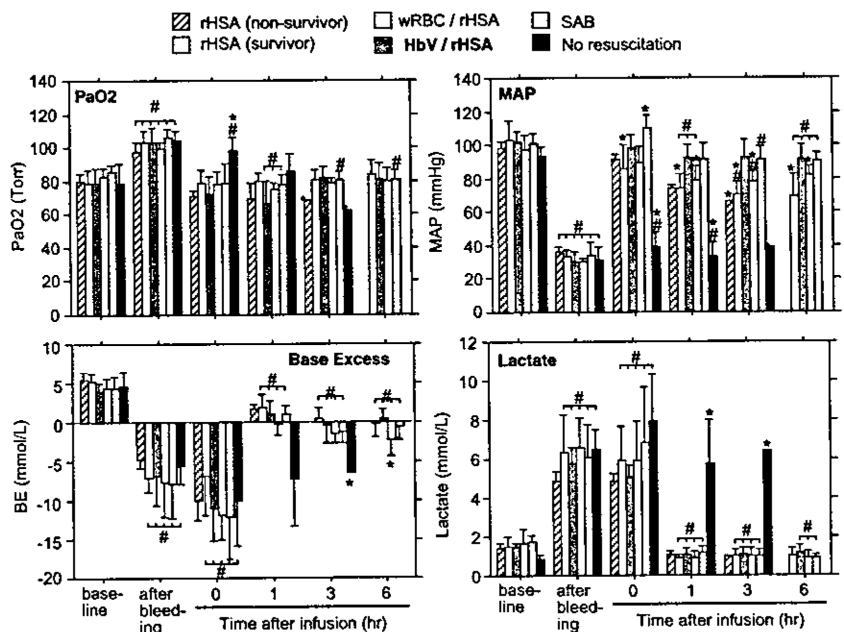

Fig. 6. Changes in mean arterial pressure (MAP) and blood gas parameters during hemorrhagic shock and resuscitation with infusion of HbV suspended in recombinant human serum albumin (HbV/rHSA), shed autologous blood (SAB), washed red blood cells suspended in recombinant human serum albumin (wRBC/rHSA), and recombinant human serum albumin (rHSA) alone. The sham group did not receive a resuscitative fluid after the hemorrhage, and died within 3h. The number of surviving rats was 3 at 1h. In the rHSA group, 2 of the 8 rats died between 1 and 6h. Accordingly, the rHSA group was divided into the rHSA(survivor) group and the rHSA(non-survivor) groups until they died. Therefore, the number of rats (n) for the rHSA(survivor) and rHSA(non-survivor) groups were 6 and 2, respectively. # significantly different from baseline ($p < 0.05$), * significantly different versus the HbV/rHSA group ($p < 0.05$)

Wistar rats were anesthetized with 1.5% sevoflurane inhalation throughout the experiment. Polyethylene catheters were introduced through the right jugular vein into the right atrium for infusion and into the right common carotid artery for blood withdrawal and mean arterial pressure (MAP) monitoring.

Measurements and Main Results. Shock was induced by 50% blood withdrawal. The rats showed hypotension (MAP = 32 ± 10mmHg) and significant metabolic acidosis and hyperventilation (Fig. 6). After 15min, they received HbV/rHSA, shed autologous blood (SAB), washed homologous red blood cells (wRBC) suspended in rHSA (wRBC/rHSA, [Hb] = 8.6g/dl), or rHSA alone.

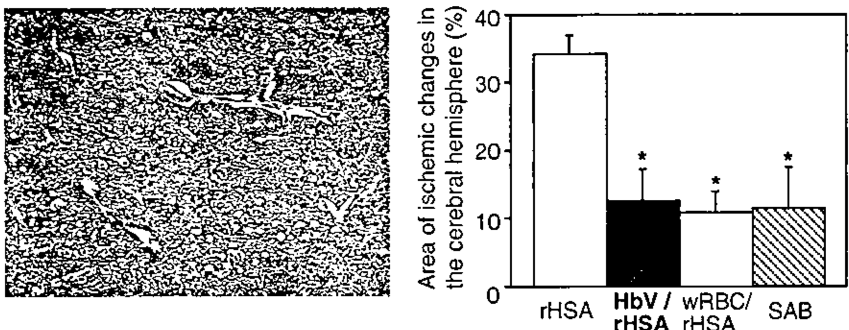

FIG. 7. Resuscitation from hemorrhagic shock with HbV suspended in rHSA in comparison with rHSA, wRBC/rHSA. The cerebral hemisphere on the right side of the rHSA group showed significant ischemic changes, a pyknotic change of the nuclei and an edematous change (34 ± 3% of the total section area) as shown in the left picture, relating to the ligation of the right carotid artery. However, the other groups that were resuscitated with O_2-carrying fluids showed minimal changes (* $p < 0.001$ vs. rHSA; HbV/rHSA, 13 ± 5%; SAB, 11 ± 6%; wRBC/rHSA, 11 ± 3%). The non-resuscitated rats did not show such ischemic changes

The HbV/rHSA group restored MAP to 93 ± 8 mmHg at 1 h, similar to the SAB group (92 ± 9 mmHg), which was significantly higher compared with the rHSA (74 ± 9 mmHg) and wRBC/rHSA (79 ± 8 mmHg) groups. There was no remarkable difference in the blood gas variables between the resuscitated groups; however, two of eight rats in the rHSA group died before 6 h. After 6 h, the rHSA group showed significant ischemic changes in the right cerebral hemisphere relating to the ligation of the right carotid artery followed by cannulation, whereas the HbV/rHSA, SAB, and wRBC/rHSA groups showed less changes (Fig. 7). These results indicate that HbV suspended in recombinant human serum albumin provides restoration from hemorrhagic shock that is comparable with that obtained using shed autologous blood.

90% Exchange Transfusion with HbV Suspended in Human Serum Albumin [58,103,104]

Objective. The effect of surface modification of HbV with poly(ethylene glycol) (Mw. 5 kDa) on hemodynamics and O_2 transport was studied by 90% exchange transfusion with the PEG-modified HbV and unmodified HbV suspended in 5% HSA in anesthetized Wistar rats.

Methods. Male Wistar rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Catheters (PE-20) were introduced into the right jugular vein for infusion (1 ml/min) and the right common carotid

artery for blood withdrawal (1 ml/min) and MAP measurements. MAP and heart rate were monitored through the arterial catheter. Arterial blood samples for gas analyses were also obtained from the arterial catheter. Abdominal aortic blood flow was measured by an ultrasonic pulsed Doppler flow meter as an indicator of cardiac output. The O_2 tension of blood withdrawn from the right atrium was measured as an indicator of mixed venous O_2 tension. These values were employed to calculate O_2 delivery and consumption. Renal cortical and skeletal muscle tissue O_2 tensions were monitored as indicators of tissue perfusion. Unmodified HbV/HSA, HSA alone, and washed rat RBC suspended in 5% HSA containing 10 g/dl of Hb (ratRBC/HSA) were employed as controls.

Measurements and Main Results. Both the PEG-modified HbV/HSA and unmodified HbV/HSA groups showed sustained MAP and blood gas parameters which were comparable with ratRBC/HSA group. Only the HSA group showed the significant decline in these parameters and resulting death within 30 min after completion of exchange. The blood flow in the abdominal aorta increased 1.5 times, and the total peripheral resistance decreased in the PEG-modified HbV/HSA-administered group in comparison with the unmodified HbV/HSA group. As for the blood gas parameters, the base excess and pH remained at higher levels in the PEG-modified HbV/HSA group, and the O_2 tension in mixed venous blood for the PEG-modified HbV/HSA group tended to be maintained at a higher level than that for the unmodified HbV/HSA group. Owing to the physicochemical properties, the PEG modification of HbV reduced the viscosity by the suppression of aggregation and resulted in prompt blood circulation in vivo.

Subcutaneous Microvascular Responses to 80% Exchange Transfusion with PEG-modified and Unmodified HbV [57]

Objective. The function of PEG-modified and unmodified HbV as a blood replacement was tested in the subcutaneous microvasculature of conscious hamsters during severe hemodilution in which 80% of the RBC mass (70 ml/kg) was substituted with suspensions of the vesicles in 5% HSA solution (Fig. 8).

Methods. Conscious male Syrian golden hamsters (60–70 g) with dorsal skinfold preparation were used. Blood withdrawal and sample infusions were simultaneously performed at a rate of 0.3 ml/min. At 30%, 60%, and 80% blood exchange levels, MAP, heart rate, blood gases, and microvascular parameters were measured.

Measurements and Main Results. Both materials yielded normal MAP, heart rate, and blood gas parameters at all levels of exchange, which could not be

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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.

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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].

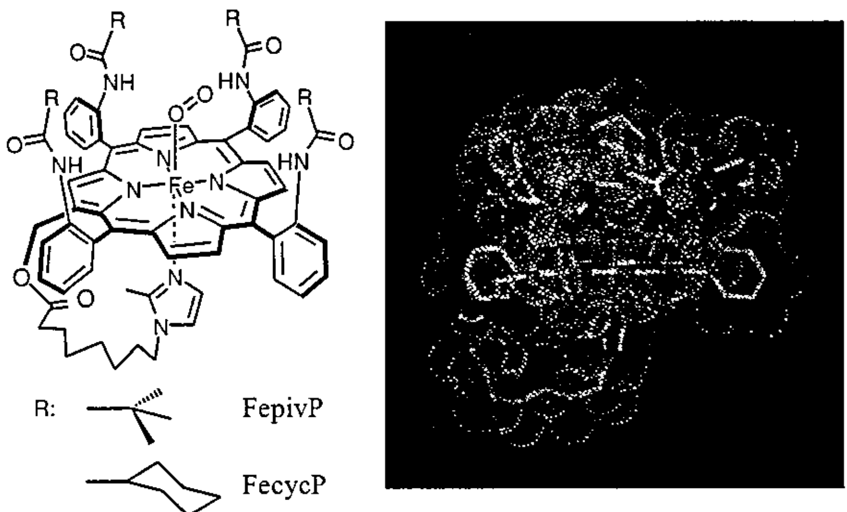

Fig. 1. Structures of the new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) and pivaloylamino substituents (FepivP), and the simulated structure of oxygenated FecycP. The extensible systematic forcefield (ESFF) 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 (pI) 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 pI 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

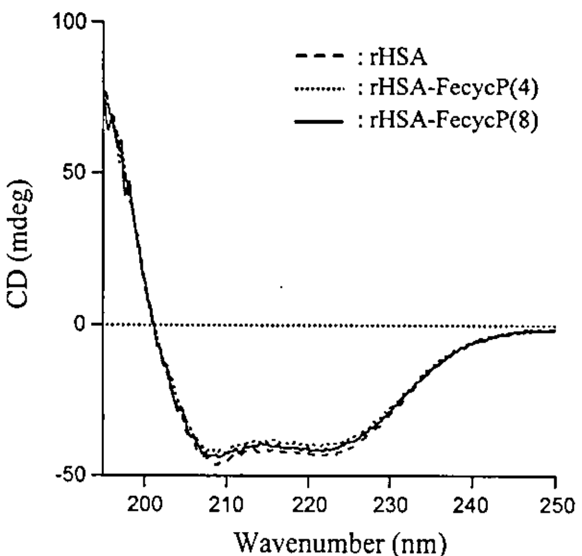

Fig. 2. Circular dichroism (*CD*) spectra of recombinant human serum albumin (*rHSA*) and rHSA-FecycP in water at 25°C

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.

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

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

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

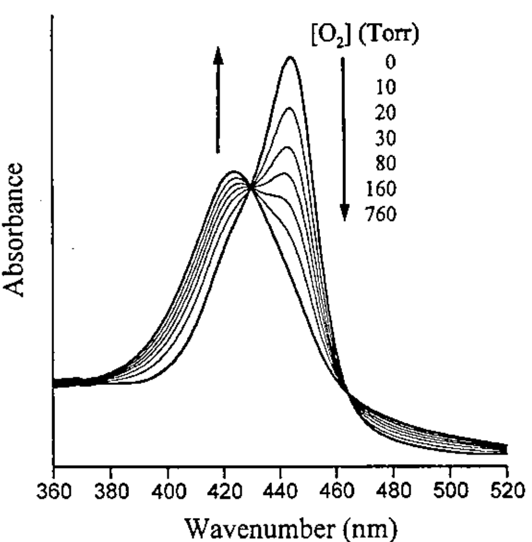

FIG. 3. Ultraviolet-visible. Absorption spectral changes of rHSA-FecycP(4) dependent on the O_2 partial pressure in phosphate-buffered solution (pH 7.3) at 37°C. The number in parenthesis is molar ratio of FecycP and rHSA

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

	$k_{on}[M^{-1}s^{-1}]$		$k_{off}[s^{-1}]$	
Substance	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].

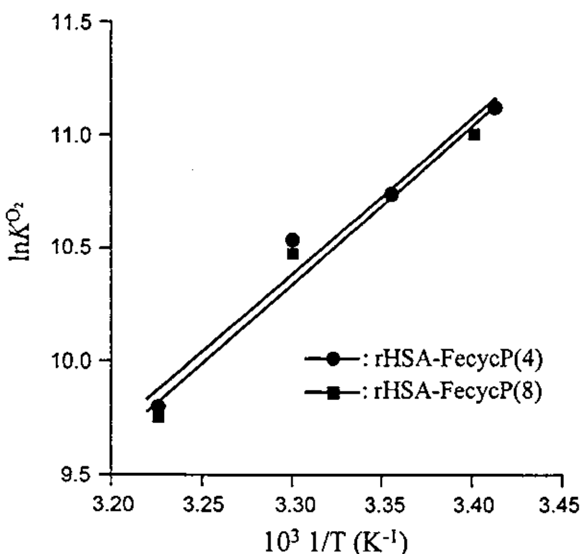

Fig. 4. Van't Hoff plots of O_2-binding affinity of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

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	k_{on}($M^{-1}s^{-1}$) 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.

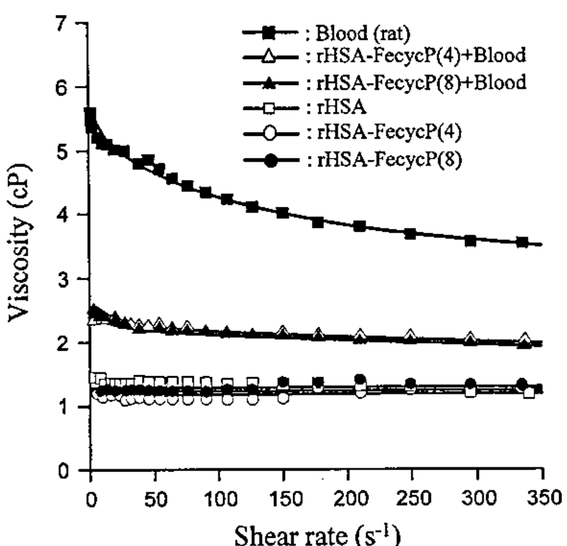

Fig. 5. Viscosity of rHSA-FecycP solution with whole blood at 37°C

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