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

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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 (A) Hb is rigorously purified from outdated, donated red cells via pasteurization and ultrafiltration, to completely remove blood type antigen and pathogen.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.

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

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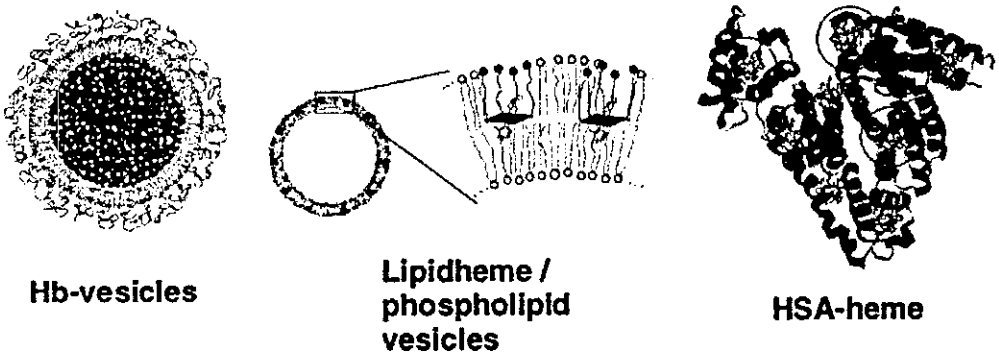

Figure 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme.

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

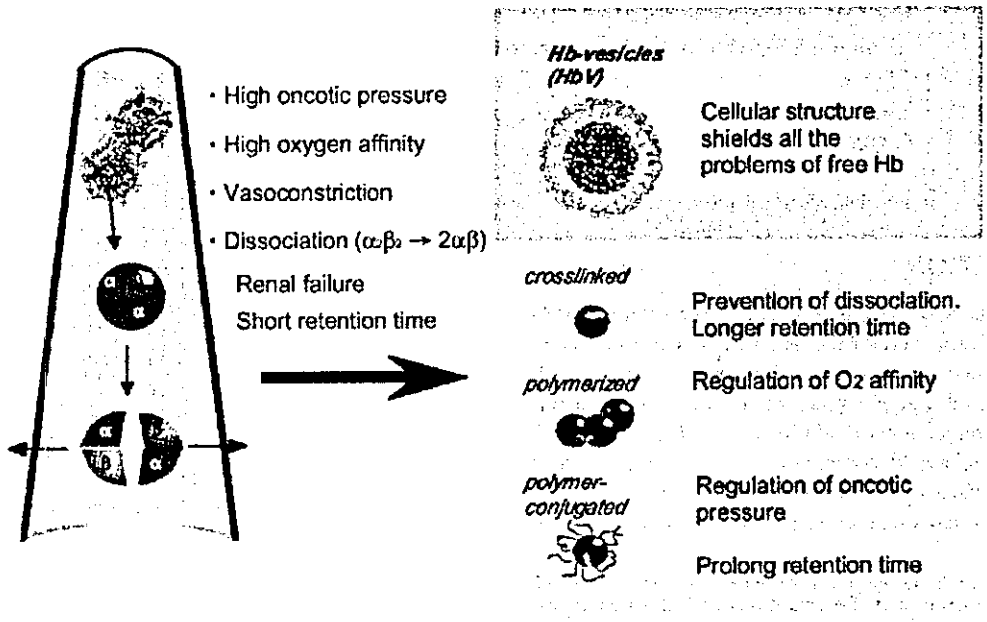

Figure 2. Approaches to solve the problems of utilization of Hb as an O_2-carrier, chemical modification or encapsulation of Hb.

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.

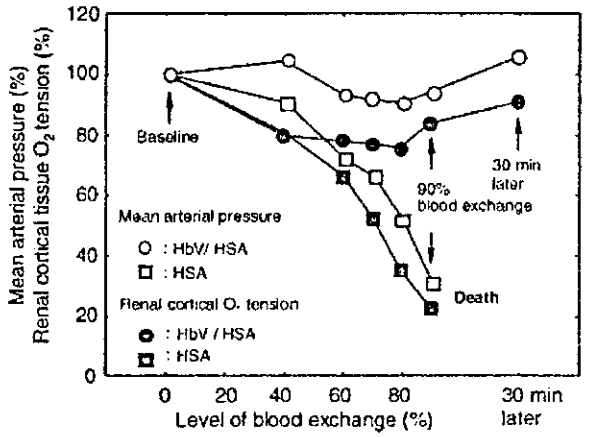

Figure 3. Ninety per cent exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored.

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

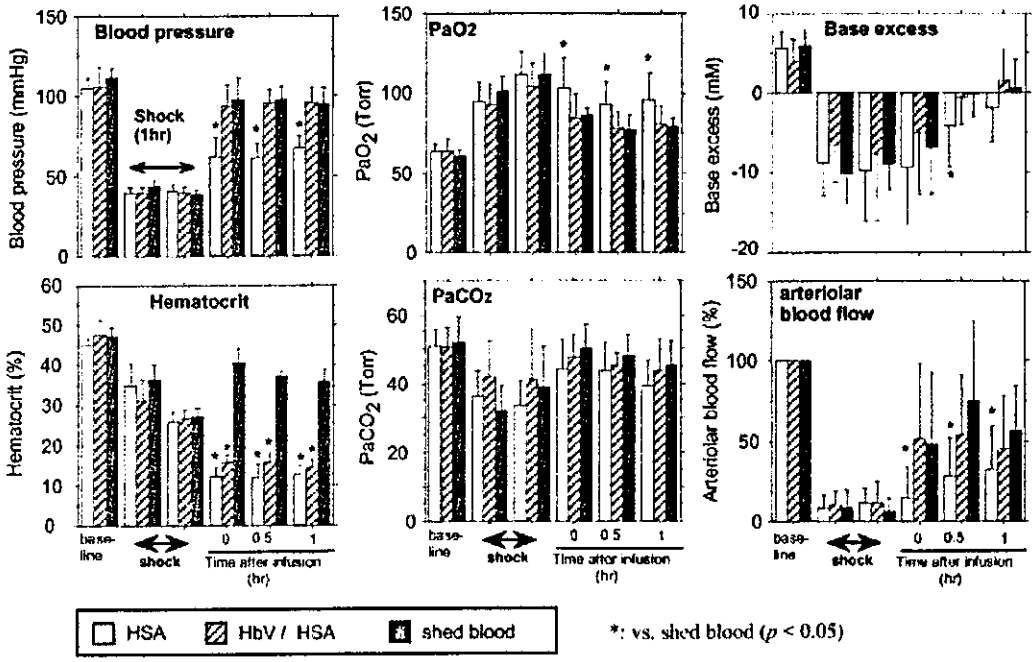

Figure 4. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD.

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

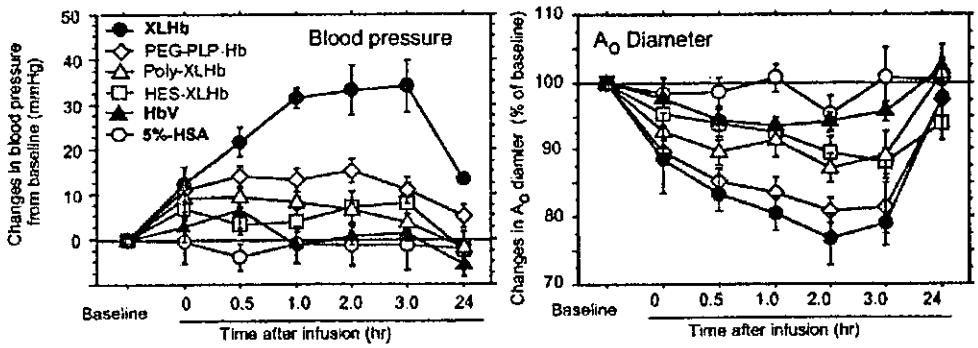

Figure 5. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2-carriers. Mean ± SD.

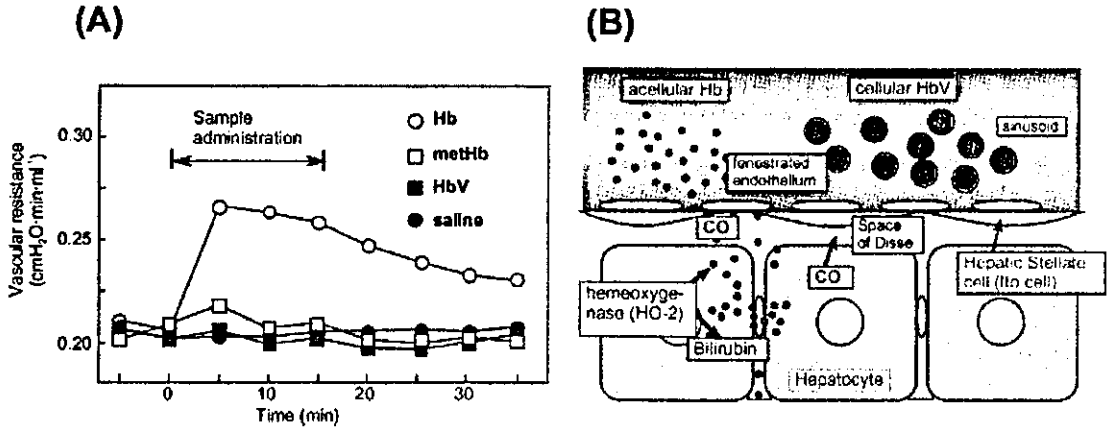

Figure 6. (A) Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. (B) Schematic representation of hepatic microcirculation: the small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolized 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

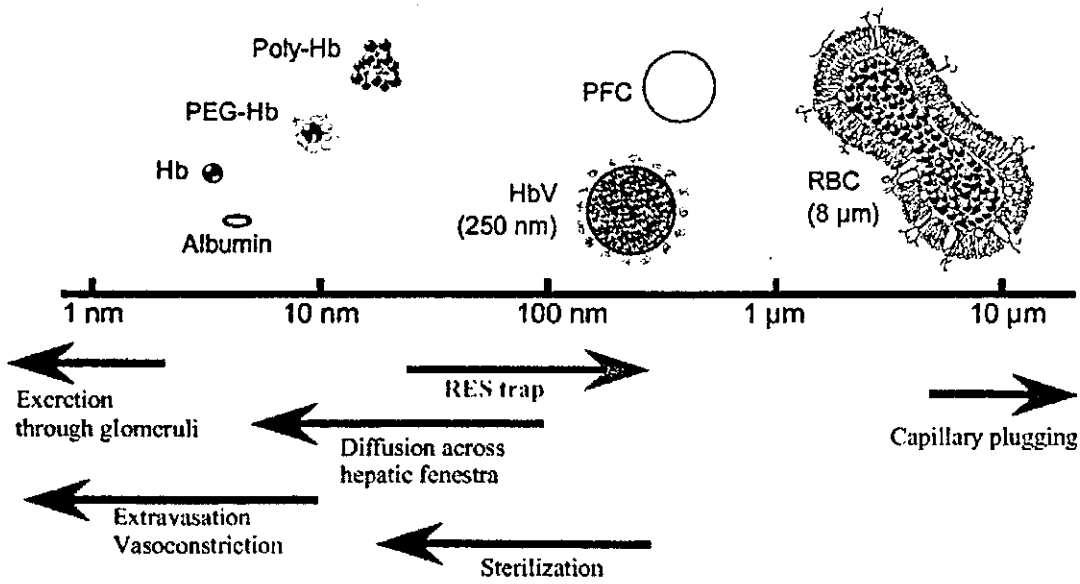

Figure 7. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process.

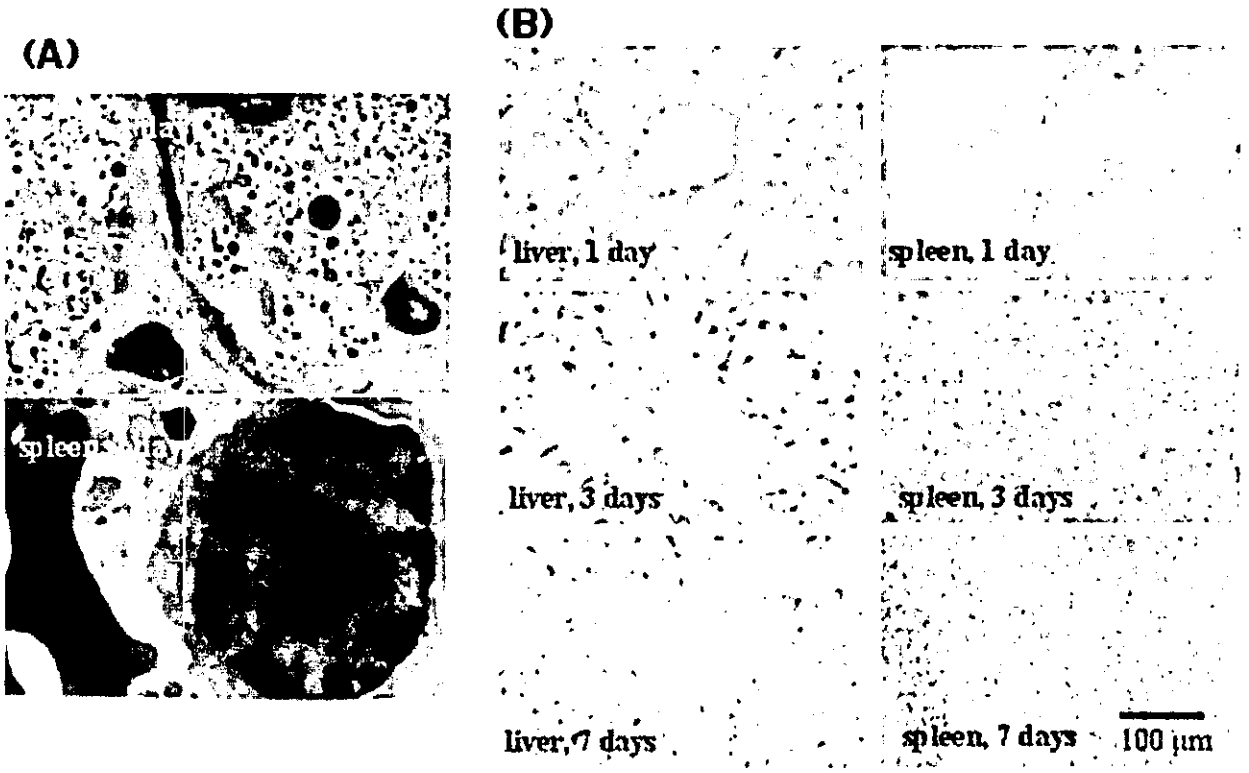

Figure 8. (A) TEM of rat spleen 1 day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. (B) Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. HbV particles disappeared within 7 days.

showed the time course of biodistribution. After HbV 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 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. 8). RBCs and HbV contain a lot of ferric ion with a high electron density, so that they show strong contrast in TEM. However, after 7 days, the HbV structure cannot be observed. There were no abnormalities in the tissues and no irreversible damages to the organs. 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 they have almost disappeared after 7 days in both the spleen and liver. Therefore, 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. A very clear supernatant for accurate analyses can be obtained.[35] This is one advantage of HbV in comparison with acellular Hb solutions. Accordingly, the influence on organ functions by serum clinical laboratory tests after the bolus infusion of HbV at a dose rate of 20 ml/kg was examined. Albumin, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, which reflect the liver function, moves their values within normal range.[36] 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.

A test of daily repeated infusion is required to evaluate the safety of a new drug. The daily repeated infusion of HbV in Wistar rats at a dose rate of 10 ml/kg/day for 14 days, everyday was tested.[37] The total infusion volume (140 ml/kg) was 2.5 times as much as the volume of the whole blood (56 ml/kg), however, all rats tolerated it well and survived. The body weight showed a monotonous but slightly depressed increase in comparison with the saline. However, after 2 weeks there was no significant difference with the saline control group. All the rats seemed very healthy and active. Histopathological examination 1 day after the final infusion of HbV showed significant accumulation of HbV in spleen macrophages, and liver Kupffer cells, and they mostly disappeared after 14 days. There were no irreversible 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 the authors are confident with the safety of HbV.

DESIGN AND PHYSICOCHEMICAL PROPERTIES OF rHSA-HEME

In this study research on totally synthetic O_2-carriers, or so-called albumin-heme that does not require Hb has been conducted. HSA is the most abundant plasma protein in our blood stream, but its crystal structure has not been elucidated for a long time. In 1998, Dr Stephen Curry of the Imperial College London first elucidated the crystal structure of the HSA complexed with seven molecules of myristic acids.[38] He found that the dynamic conformational changes of albumin take place by the binding of fatty acid. However, in Japan, rHSA is now manufactured on a large scale by expression in the yeast *Pichia pastoris*, and it will appear on the market soon.[39] 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, it has been found that synthetic heme derivative is efficiently incorporated into 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, the rHSA-heme hybrid is a synthetic O_2-carrying hemoprotein, and it is believed that its saline solution will become a new class of RBC substitute.[40–51]

Figure 9 summarizes the structure of the rHSA-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 2 years at room temperature. Since the O_2-binding sites of rHSA-heme are iron-porphyrin, 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, rHSA-heme formed a very stable carbonyl complex.

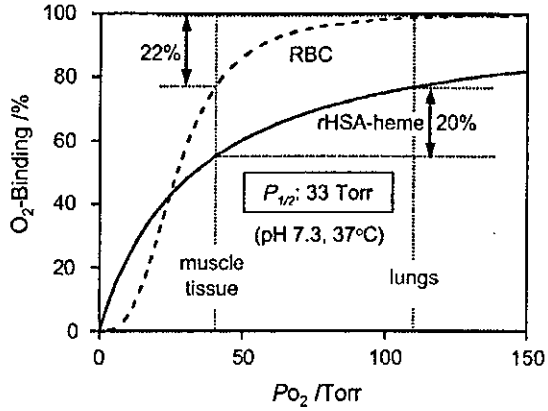

Figure 10. O_2-binding equilibrium curve of albumin-heme.

Figure 10 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 rHSA-heme between the lungs measuring 110 Torr and muscle tissue measuring 40 Torr increases to 22%, which is identical to the 22% efficiency for RBCs. The O_2-binding property of rHSA-heme can be controlled by changing the chemical structure of heme derivatives incorporated. More recently, it has been found that a protoheme derivative is also incorporated into albumin and can bind and release O_2 as well.[52]

IN VIVO SAFETY AND EFFICACY OF rHSA-HEME

Based on these findings, it can be said that rHSA-heme can become an entirely synthetic O_2-carrier, and satisfy the initial clinical requirements for a RBC substitute. However, there is another problem to solve before this material can be used as an O_2-carrier in the circulatory system. This problem is NO scavenging. Of course, rHSA-heme can bind NO, and it may be anticipated that the injection of rHSA-heme also induce hypertensive action. The authors have evaluated the

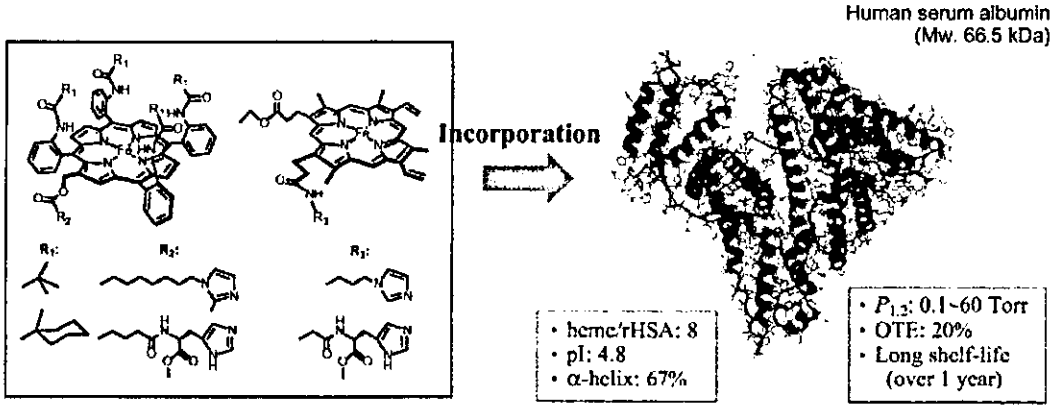

Figure 9. Structure of the albumin-heme molecule.

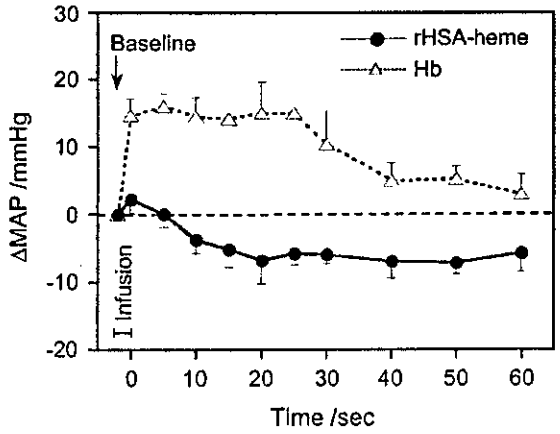

Figure 11. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats ($n=5$). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± SE. Basal value is 90.1 ± 3.0 mmHg.

efficacy and safety of this rHSA-heme solution with animal experiments.

As described earlier, 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.[53] Indeed, only a small change in the mean arterial pressure was observed after the administration of the rHSA-heme solution (Fig. 11). In contrast, the infusion of Hb elicited an acute increase in blood pressure. Why does rHSA-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 muscle 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.

Thus the authors are now evaluating the O_2-transporting ability of this rHSA-heme molecule in the circulatory system with further animal experiments.[54] First, the physiological responses to exchange transfusion with rHSA-heme solution into rats after 70% hemodilution and 40% hemorrhage was determined (Fig. 12). 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. However, 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, HSA dimer, which can incorporate 16 hemes in its hydrophobic domain has been synthesized.[55] The human serum rHSA-heme dimer solution dissolves 1.3-times more O_2 compared to that of RBC and keeps its colloid osmotic pressure at the same level as the physiological value.

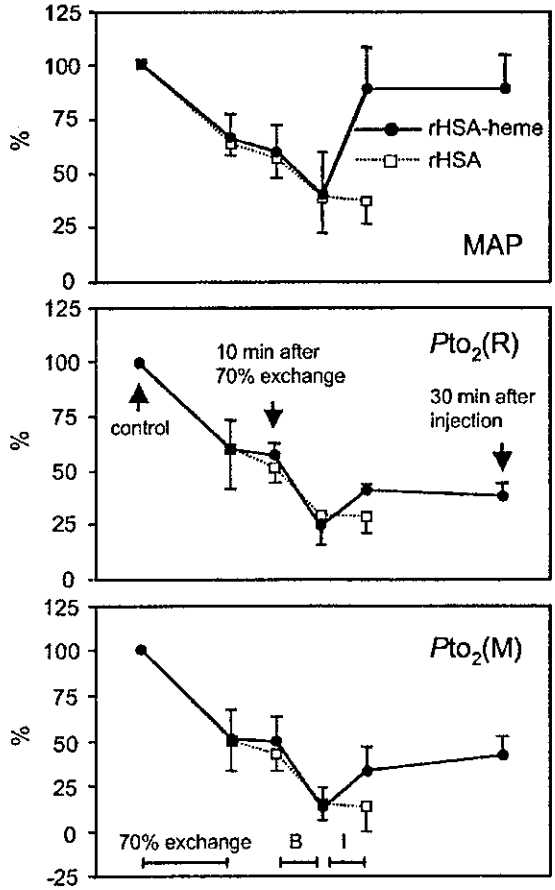

Figure 12. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats ($n=5$). All data are shown as changes from the basal values and expressed as mean ± SE.

POTENTIAL APPLICATIONS OF ARTIFICIAL O_2 CARRIERS

As described earlier the primary application of artificial O_2-carriers would be the resuscitative fluid for hemorrhage. Since some of the characteristics of artificial O_2-carriers overwhelm those of donated blood, there are many potential applications other than blood substitutes.

Tumor oxygenation

Unlike vessels in normal tissues, the development of a vasculature in a tumor lacks regulation 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. The rHSA-heme was injected into the responsible artery that supplies circulation to an implanted tumor (Fig. 13).[56] 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. The findings in animals indicate that tumor tissue O_2-levels can be elevated by the administration of artificial O_2-carriers due to the

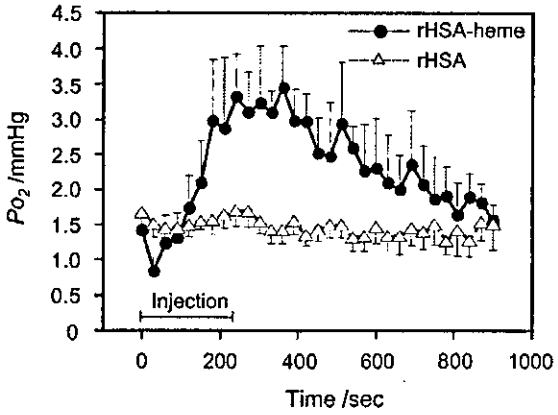

Figure 13. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats ($n=4$ each). All data are shown as changes from the basal values (P_{O_2}) just before the infusion and expressed as mean ± SE.

difference in O_2-transporting properties from RBCs. Whether this increase in tissue O_2 can potentiate cancer treatment is currently under investigation.

Oxygenation of ischemic tissue

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 which point ulceration and gangrene formation become imminent. It is believed that this critical phase can be avoided or delayed by the application of artificial O_2-carriers, which can be designed to flow even through these damaged capillaries.[27,28]

Organ preservation

One of the most important agenda in transplantation medicine is long-term organ preservation and circumvention of ischemia reperfusion injuries. It is believed 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, it may be possible in the future to perform additional organ tests for better compatibility, or even perform genetic modifications during this period. It is believed that through these applications, the concept of organ preservation can be expanded to culture organs, 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.[57] It is believed 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 graft-versus-host disease (GVHD).

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.[58] 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.

FUTURE SCOPE

The research field of the red cell substitutes is moving forward very rapidly, and the paradigm in this field is expanding from red cell substitutes to "O_2 therapeutics". Significant efforts have been made to produce HbV and albumin-heme with a facility of GMP standard, and to start preclinical and finally clinical trials. We look forward to the day that our research will play an effective role in treating patients.

Acknowledgements

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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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hypertension, reduced blood flow and tissue oxygenation in the microcirculatory levels, neurological disturbances, and malfunctioning of esophageal motor function. These side effects of molecular Hb would imply the importance of cellular structure.

The pioneering work was performed by Chang (1957) [2], who started encapsulation of Hb like a RBC and prepared microcapsules (5 μm) made of nylon, collodion, etc. Toyoda (1965) [3] and Kitajima of the Kambara-Kimoto group (1971) [4] also covered Hb solution with gelatin, gum Arabic, or silicone; however, it was very difficult to regulate the particle size appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 that phospholipids assembled to form vesicles in aqueous media [5], and that they encapsulate water-soluble materials in their inner aqueous interior, it was quite reasonable to use such vesicles for Hb encapsulation. Djordjevici and Miller (1977) prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, etc [6]. Hunt, Kondo, Chapman, Gaber, Farmer, Beissinger, Chang, Schmidt, Farmer, Rudolph and others attempted LEH [7–16]. In the United States, Naval Research Laboratories showed the remarkable progress of LEH. Hemoglobin-vesicles (HbV), with a high efficiency production process and improved properties, have been established by Tsuchida's group, based on the technologies of molecular assembly and precise analysis of pharmacological and physiological aspects [1,17] (Fig. 1).

In this chapter we summarize the characteristics of HbV based on the science of molecular assembly and its excellent results.

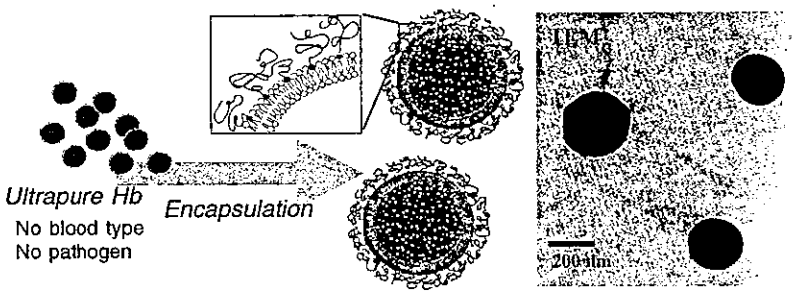

FIG. 1. Hb-vesicles (diameter, ca. 250 nm) are prepared from ultrapure Hb obtained from outdated RBC. The surface of the vesicles is modified with polyethyleneglycol that ensures the dispersion stability during storage and during circulation in the blood stream. Transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles

Preparation and Characteristics of HbV as a Molecular Assembly

Purification of Hb for the Utmost Safety

The primary advantage of using an artificial oxygen carrier should the absence of risk of infectious diseases derived from human blood. Even though strictly inspected RBCs after expiration of limitation period are used as a source of Hb, it is necessary to introduce additional procedures to inactivate and remove viruses in the process of Hb purification in order to guarantee the utmost safety from infection. In our purification process, virus inactivation was performed by pasteurization at 60°C for 12 h, which are the same conditions used for the pasteurization of human serum albumin [18,19]. This process can be introduced by utilizing the stability of carbonylhemoglobin (HbCO). The thermograms of HbCO indicated the denaturation temperature at 78°C, which is much higher than that for oxyhemoglobin (64°C) [20] (Fig. 2).

The virus inactivation efficiency was evaluated by the Hokkaido Red Cross Blood Center [21,22]. The Hb solution spiked with vesicular stomatitis virus (VSV) was treated at 60°C for 1 h under either an air or CO atmosphere. VSV was inactivated at less than 5.8 $\log_{10}$ and less than 6.0 $\log_{10}$ under the air and

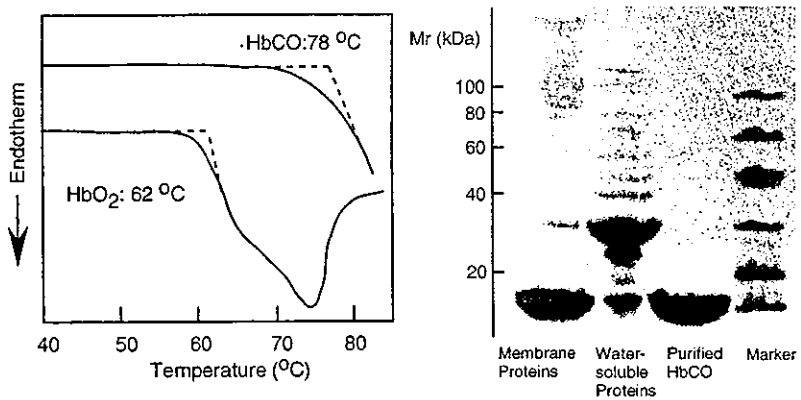

FIG. 2. Left, the calorimetric thermograms of HbCO solution showed the decomposition temperature at 78°C, indicating the thermal stability for pasteurization at 60°C. The amount of sample was 60 μl (6.0 g/dl). The heating speed was 1.0°C/min. Right, SDS-PAGE of HbCO after the heat treatment (60°C, 12 h), indicates the purity and no contamination of other proteins derived from RBC. The concentration of Hb applied to the gel was 10 mg/ml, which was 10 times higher than the maximum concentration described in the instruction manual of PhastSystem, in order to show the absence of other bands in the purified HbCO

CO atmosphere, respectively. Although the methemoglobin (metHb) rate increased after the heat treatment under the air atmosphere, no metHb formation was observed by the treatment under the CO atmosphere. Isoelectric focusing analysis revealed the denaturation of Hb after the heat treatment under the air, while the Hb band was not altered in the carbonylated condition. Some protein bands other than Hb had disappeared on sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) after the heat treatment. During the pasteurization, all the other concomitant proteins are denatured and precipitated. As a result, we obtain ultrapure Hb solution. This high purity is essential to prevent membrane plugging during the next ultrafiltration process.

We tested ultrafiltration of HbCO solution to remove viruses with PLANOVA-35N and -15N (Bemberg Microporous Membrane: BMM; Asahi Kasei, Tokyo, Japan) [23]. The virus removal mechanism is by size exclusion through the capillary pores, and the filtration method is a depth filtration. The unit membrane which has a network structure of capillaries and voids is accumulated to form 150 layers. PLANOVA -35N and -15N have mean pore sizes of 35 nm and 15 nm, respectively. PLANOVA-35N is suitable for removing envelope-type viruses such as HIV, and HCV of which the size ranges from 40 nm to 100 nm, PLANOVA -15N can be used to remove the nonenvelope-type viruses, such as parvoviruses, of which the size is less than 40 nm. However, when the pores of the membrane filter are plugged by impurities, the PLANOVA-35N is sometimes used as a prefilter for PLANOVA-15N. The permeation flux (LMH) and the permeated ratio of HbCO solution ([Hb] = 5.6 g/dl) through PLANOVA-35N at 13°C were 36 (L/m^2/h) and almost 100 (%), respectively. Those through PLANOVA-15N at 13°C were 15 (L/m^2/h) and 95 (%), respectively. The LMH increased to 18 (L/m^2/h) at 25°C. Under the same conditions, a high removal efficiency of a bacteriophage, $\phi \times 174$, (>7.7 log) was confirmed. These results indicate that PLANOVA-15N is effective for the process of virus removal from Hb solutions. We also confirmed the effectiveness of other virus removal ultrafiltration systems such as Viresolve.

Thus, purified HbCO solution can be concentrated to above 40 g/dl very effectively using an ultrafiltration process. After regulation of the electrolyte concentrations, this is supplied for encapsulation procedure. The ligand of the resulting HbV, CO, is converted to O_2 by illuminating the liquid membrane of HbV suspension to a visible light under O_2 flow [24].

Other groups have selected methods to preserve the well-organized enzymatic systems originally present in the RBCs aiming at the prolonged stability of ferrous state of Hb [25,26]. However, this may cause insufficient virus removal or inactivation and cannot guarantee the utmost safety of the resulting artificial oxygen carrier. One advantage of HbV is that any reagent can be coencapsulated in the vesicles. It has been confirmed that coencapsulation of

an appropriate amount of a reductant, such as glutathione or homocysteine, and active oxygen scavengers, such as catalase, effectively retards the metHb formation [27–31].

Effective Hb Encapsulation

The performance of HbV depends on the weight ratio of Hb to lipid ([Hb]/[Lipid]). This value is improved by lowering the number of bilayer membrane (lamellarity) of the vesicle and raising the concentration of Hb in the interior of the vesicle. We studied the optimal conditions for Hb encapsulation using an extrusion method and considering the behaviors of Hb and lipid assemblies as a kind of polymer electrolyte [32–35].

The maximum [Hb]/[Lipid] ratio that would relate to the isoelectric point (pI) of Hb can be obtained at ca. pH 7. The Hb molecule is negatively charged when pH is above 7.0, and the electrostatic repulsion between Hb and the negatively charged bilayer membrane results in lower encapsulation efficiency. However, the lower pH should enhance Hb denaturation by interaction with the lipid bilayer membrane and metHb formation at a lower pH. Therefore, the physiological pH, 7.0–7.4, would be optimal. It was also revealed that the higher ionic strength shields the repulsion between the negatively charged lipid bilayer membranes and increases the lamellarity.

The number of bilayer membranes decreases with increased microviscosity (decreased lipid mobility). Multilamellar vesicles are converted to smaller vesicles with smaller lamellarity during the extrusion procedure. When membrane fluidity is high, deformation of vesicles during extrusion occurs more easily, even for multilamellar vesicles, resulting in larger lamellarity in the final vesicles. Therefore, the use of lipids with higher phase transition temperature is preferred. However, these lipids would make extrusion more difficult, because a higher shear stress (high extrusion pressure) is required. Based on this reasoning, mixed lipids contain 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) as the main component.

Based on the precise analysis of the characterization of the physicochemical properties of the components, the encapsulation efficiency of Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method [36,37]. Mixed lipids (DPPC, cholesterol, 1,5-*O*-dioctadecyl-*N*-succinyl-L-glutamate (DPEA), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5,000)] (PEG-DSPE)] at a molar ratio of 5, 5, 1, and 0.033 were hydrated with a NaOH solution (7.6 mM) to obtain a polydispersed multilamellar vesicle dispersion (50 nm–30 μm in diameter). The polydispersed vesicles were converted to smaller vesicles having an average diameter of ca. 500 nm with a relatively narrow size distribution by freeze-thawing at a lipid concentration of 2 g/dl

and cooling rate of −140°C /min. The lyophilized powder of the freeze-thawed vesicles was rehydrated into a concentrated Hb solution (40 g/dl) and retained the average size and distribution of the original vesicles. The resulting vesicle dispersion smoothly permeated through the membrane filters during extrusion. The average permeation rate of the freeze-thawed vesicles was ca. 30 times faster than that of simple hydrated vesicles. During the extrusion process, Hb solution was encapsulated into the reconstructed vesicles effectively with a diameter of 250 ± 20 nm, and the [Hb]/[lipid] ratio reached 1.7–1.8.

Regulation of Oxygen Affinity

Oxygen affinity of purified Hb (expressed as P_{50}, O_2 tension at which Hb is half-saturated with O_2) is about 5 Torr, and Hb strongly binds O_2 and does not release O_2 at 40 Torr (partial pressure of mixed venous blood). Historically, it has been regarded that the O_2 affinity of an Hb-based O_2 carrier (HBOC) should be regulated similarly to that of RBC, namely about 25–30 Torr, using an allosteric effector, or by a direct chemical modification of the Hb molecules. Theoretically, this allows sufficient O_2 unloading during blood microcirculation, as could be evaluated by the arterio-venous difference in O_2 saturation in accordance with an O_2 equilibrium curve. It has been expected that decreasing the O_2 affinity (increasing P_{50}) results in an increase in the O_2 unloading. This expectation is supported by the result that the RBC with a high P_{50} shows an enhanced O_2 release for improved exercise capacity in a mouse model [38].

If this theory is correct, P_{50} of Hb in HbV should be equivalent to that of human RBCs, i.e., 28 Torr, or higher. Pyridoxal 5′-phosphate (PLP) is coencapsulated in HbV as an allosteric effector to regulate P_{50} [39]. The main binding site of PLP is the *N*-terminal of the α- and β-chains and β-82 lysine within the β-cleft, which is part of the binding site of natural allosteric effector, 2,3-diphosphoglyceric acid (2,3-DPG) [40]. The bound PLP retards the dissociation of the ionic linkage between β-chains of Hb during conversion of deoxy to oxyHb in the same manner as does 2,3-DPG. Thus, oxygen affinity of Hb decreases in the presence of PLP. The P_{50} of HbV can be regulated to 5–150 Torr by coencapsulating the appropriate amount of PLP or inositol hexaphosphate as an allosteric effector [41]. Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was coencapsulated, and P_{50} was regulated to 18 Torr. When the molar ratio PLP/Hb was 3/1, P_{50} was regulated to 32 Torr. The O_2 affinities of HbV can be regulated quite easily without changing other physical parameters, whereas in the case of the other modified Hb solutions their chemical structures determine their O_2 affinities, thus regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not yet been completely decided; however, the easy regulation of O_2 affinity may be useful to meet the requirement of the clinical indications such as oxygenation of ischemic tissues (see section "Improved Oxygenation in Ischemic Hamster Flap Tissue by Hemodilution with HbV").

Surface Modification of HbV and Its Stability During Long-Term Storage

Since Hb autoxidizes to form metHb and loses its oxygen-binding ability during storage as well as in blood circulation, the prevention of metHb formation is required. The conventional long-term preservation methods are to store modified Hbs in a frozen state or to store them as a freeze-dried powder with some cryoprotective or lyoprotective agents such as saccharides or polyols [42,43]. Some groups have recently reported a method to preserve deoxygenated Hbs in a liquid state [44], using the well-known intrinsic characteristic of Hb that the Hb oxidation rate in a solution is dependent on the oxygen partial pressure and deoxyHb essentially is not autoxidized at ambient temperature [45,46].

In the case of HbV, not only the inside Hb, but also the cellular structure has to be physically stabilized in order to prevent intervesicular aggregation, fusion, and leakage of encapsulated Hb and other reagents. Phospholipid vesicles are molecular assemblies and generally regarded as unstable capsules which require some reinforcement. We studied the γ-ray polymerization in the bilayer membranes of phospholipids bearing dienoyl groups, and the resulting polymerized phospholipid membrane significantly stabilized HbV [47–49]. The polymerized vesicles preserved the particle diameter and function of the inside Hb even after 10 repeated freeze-thawings and freeze-dryings and rehydrations [50,51]. However, the relatively slow rate of metabolism of the polyphospholipid in the reticuloendothelial systems is considered to be a problem. Cryoprotection and lyoprotection of the HbV were performed by the addition of saccharides such as trehalose [52] or glycolipid [53]. However, preservation of the oxygen carriers in a liquid state may be more useful for infusion in emergency situations than the time-consuming procedures such as redissolving the Hb powder or thawing several hundred milliliters of the frozen Hb solution stored in a freezer.

Surface modification of phospholipid vesicles with the poly(ethylene glycol) (PEG)-conjugated lipid is a well-known method to prolong the circulation time of the vesicles in vivo for drug delivery systems [54,55]. For HbV, the surface of HbV was also modified with PEG chains to improve its dispersion state of the vesicles when mixed with blood components [56]. The PEG-modified HbV has shown an improved blood circulation and tissue oxygenation due to the absence of HbV aggregate formation and viscosity ele-

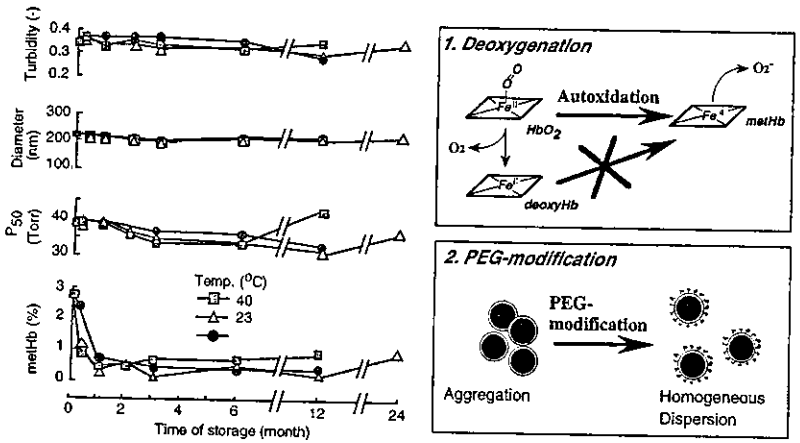

FIG. 3. Stability of HbV during storage for up to 24 months. Deoxygenation is important to prevent autoxidation to metHb, and PEG-modification suppresses aggregation and fusion of HbV to stabilize the dispersion condition. Both techniques are essential for the long-term storage of HbV. A reductant coencapsulated in HbV not only consumed a *trace amount of oxygen* but also gradually reduced metHb

vation [57,58] and prolonged circulation persistence in vivo [59]. However, little attention has been paid to the ability of PEG modification for the long-term preservation of vesicles or liposomes in the liquid state [60,61].

We studied the possibility of the long-term preservation of HbV by the combination of two technologies, i.e., surface modification of HbV with PEG chains and deoxygenation during storage for 2 years [62] (Fig. 3). The samples stored at 4° and 23°C showed a stable dispersion state for 2 years, although the sample stored at 40°C showed the precipitation and decomposition of vesicular components, a decrease in pH, and 4% leakage of total Hb after 1 year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent the aggregation and fusion due to their steric hindrance. The original metHb content (ca. 3%) before the preservation gradually decreased to less than 1% in all the samples after 1 month due to the presence of homocysteine inside the vesicles which consumed the residual oxygen and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the partial pressure of oxygen, and no increase in metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. These results indicate the possibility that HbV suspension can be stored at room temperature for at least 2 years.

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the application of PEG-modified vesicles in other fields. The long-term preservation of oxygen carriers overcomes the limitation of the blood transfusion system and will be of benefit to clinical medicine.

Interaction of Lipopolysaccharide (LPS) with HbV and Quantitative Measurement of LPS

The production process of HbV has to be guaranteed with a good manufacturing practice (GMP) standard as a biological product regarding the strict regulation of impurity and viral and bacterial contamination. It is required to strictly monitor the content of the lipopolysaccharide (LPS), known as an endotoxin, a component of the outer membrane of gram-negative bacteria possessing a large variety of biological influences on numerous mammalian cells and tissues. Endotoxin is an extremely potent toxin with a lethal dose (LD_{50}) of 3 mg/kg and 1 mg/kg in rats and dogs, respectively [63,64]. The U.S. Food and Drug Administration (FDA) has established a guideline on human maximum endotoxin dose permissible for parenteral products (5 EU/kg) [65] that may include Hb-based oxygen carriers. This limit is based on the endotoxin activity (Endotoxin Unit: EU; 1 EU = 100 pg), and can be measured via the *Limulus* amebocyte lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS [66]. In general, the LAL method has advantages over rabbit pyrogen testing, because the LAL method requires a smaller sample, and the assays can easily be repeated [67]. Since the volume of oxygen carriers to be infused for shock resuscitation or acute hemodilution is estimated to be less than 20 ml/kg, the specific endotoxin limits per millimeter should be 0.25 EU/ml (= 5/20), similar to that for water for injection (0.25 EU/ml).

Bacterial LPS is an amphiphilic macromolecule; therefore, it hydrophobically interacts with protein and biomembranes [68]. Hb strongly interacts with LPS showing synergistic toxicity [69,70]. The constituent of endotoxin that causes LAL gelation is a glycophospholipid-designated lipid-A [71]. Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A such as its LAL gelation activity [72]. Using isothermal titration calorimetry (ITC), we quantitatively clarified for the first time that LPS from the *Salmonella minnesota* wild type (smooth form) was inserted into the phospholipid vesicles with an enthalpy change (ΔH) of −80 kcal/mol and the maximum incorporation of 7.6 mol% on the outer surface of the vesicles [73]. To our knowledge, the ΔH value of PEG_{5000}-DSPE (MW of PEG = 5 kDa) for the same phospholipid vesicles is only −13 kcal/mol. This comparison indicates that LPS inserted into the bilayer membrane is thermodynamically more stabi-

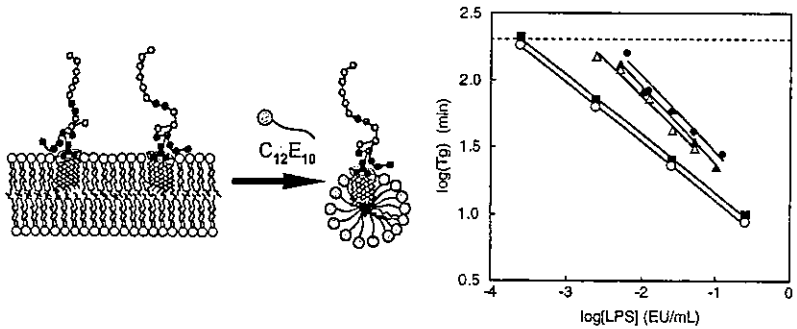

FIG. 4. (*Left*) Solubilization of LPS-contaminated vesicles with a detergent to form mixed micelles. (*Right*) Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations (vol%); 2.0 (●), 0.8 (▲), 0.4 (△), 0.1 (■), 0 (○). [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dl. For example, [LPS] should be multiplied with 400 at $[C_{12}E_{10}]$ = 0.1 wt%. The *broken line* indicates the gelation time limit for Toxinometer (200 min)

lized than PEG_{5000}-DSPE. The large difference in ΔH is probably due not only to the hydrophobic interactions by the eight alkyl chains of LPS but also to the hydrogen bonding of the amide bonds into the interface of the hydrophobic and hydrophilic regions that contribute to the interaction of LPS with the phospholipid bilayer membrane. As for the interaction between Hb and LPS, the ITC analysis was recently reported by Jurgens et al. [74], who clarified that 3–5 LPS molecules bind to one Hb molecule. As a consequence, the researchers who study HbV or other phospholipid vesicles for delivering other functional molecules encountered a problem in measuring the LPS content for the quality control of these materials [75–77].

Considering this background information, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) to release the LPS entrapped in the vesicles as a pretreatment for the succeeding LAL assay of the kinetic-turbidimetric gel clotting analysis using Toxinometer (Wako Pure Chem. Ind. Ltd., Tokyo, Japan) (detecting wavelength, 660 nm) [73] (Fig. 4). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS higher than 0.1 EU/ml in the HbV suspension. This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer is routinely used in our production system of HbV. Significant attention is paid to the quality control of HbV for preclinical studies, and all the HbV prepared under sterile conditions showed an LPS content less than 0.1 EU/ml at [Hb] = 10 g/dl.

Moreover, the utilization of the histidine-immobilized agarose gel (Pyrosep; Wako Pure Chem. Ind. Ltd., Tokyo, Japan) effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element [78]. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/ml. The pretreatment with $C_{12}E_{10}$ would be applicable not only to HbV but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules.

Interaction with Active Oxygen Species

It has been pointed out that that heme-mediated reactions of chemically modified Hbs such as ligand coordinations and redox reactions could cause organ dysfunction and/or tissue damage. Especially, redox reactions may affect the physiological protection against reactive oxygen species [79]. The oxidation of oxyHb by H_2O_2 is known to generate ferrylHb and metHb accompanied by heme degradation and the release of free iron. Furthermore, during the autoxidation of oxyHb to metHb, reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical are generated to damage not only the remaining oxyHb but also living cells and organs. Especially, ferrylHb is known to be a potent oxidant which catalyzes the peroxidation of lipids comprising the biomembrane and other biomaterials [80,81]. In normal human plasma, the concentration of H_2O_2 is 4–5 μM [82] and elevates to 100–600 μM under inflammatory [83] or ischemia/reperfusion conditions [84]. In fact, ferrylHb can be found both in the RBCs [85] and in the endothelial cells model after hypoxia reoxygenation [86,87]. Several *in vitro* studies suggest that free radicals or degradation products catalyzed by ferrylHb could damage the endothelial cells in the presence of acellular-type Hb modifications. Hb-mediated cytotoxicity via ferrylHb is one of the important safety issues of HBOCs [88].

On the other hand, in the cellular-type HbV, reactive oxygen species generated within the HbV during metHb formation were completely consumed by Hb. Although such a reaction leads to Hb oxidation, no reactive oxygen species have been detected outside the vesicles. However, reaction of Hb inside the vesicle with exogenous H_2O_2 is one of the important safety issues to be clarified and compared with a free Hb solution. We confirmed that during the reaction of the Hb solution with H_2O_2, metHb and ferrylHb are produced, and H_2O_2 is decomposed by the catalase-like reaction of Hb [89]. The aggregation of discolored Hb products due to heme degradation is accompanied by the release of iron (ferric ion). On the other hand, the concentrated Hb within the vesicles reacts with H_2O_2 that permeated across the bilayer membrane, and the same products as the Hb solution were formed inside the vesicles. However, there is no turbidity change, no particle diameter change of the HbV,

and no peroxidation of lipids comprising the vesicles after the reaction with H_2O_2. Furthermore, no free iron is detected outside the vesicle, though ferric ion is released from the denatured Hb inside the vesicle, indicating the barrier effect of the bilayer membrane against the permeation of ferric ion. When vesicles composed of egg yoke lecithin (EYL) as unsaturated lipids are added to the mixture of Hb and H_2O_2, the lipid peroxidation is caused by ferrylHb and hydroxyl radical generated from reaction of the ferric iron with H_2O_2. However, no lipid peroxidation is observed in the case of the HbV dispersion because the saturated lipid membrane of the HbV should prevent the interaction of the ferrylHb or ferric iron with the EYL. These results indicate the high safety of the Hb vesicles which enclose the reactive Hb products in the reaction with H_2O_2.

Influence of HbV on Clinical Laboratory Tests and Countermeasures

One of the remaining issues of the chemically modified acellular Hbs is the interference during clinical laboratory tests by the presence of Hbs in the serum. This topic has been extensively discussed in the field of clinical chemistry and laboratory medicine [90–94]. Even though clinical laboratory assays of blood serum components play an important role in the diagnosis and the care of many peri- or postoperative and traumatic patients, both hemolysis and lipemia are well known to cause interference in many colorimetric and spectrophotometric methods in routine automated assays. Accordingly, the presence of HBOCs interferes with the measurements due to the strong optical absorbances attributed to the Hb species (400–600 nm in wavelength). An appropriate pretreatment or calculation to subtract the deviation should be required to obtain accurate concentrations of the analytes. HbV particles (diameter, 250 nm), which possess both the Hb absorption and light scattering, show strong interference in various measurements [95]. It is important to clarify the interference of the HbV suspension in clinical laboratory tests performed on serum and to establish a pretreatment method to avoid such interference (Fig. 5).

The HbV suspension, acellular Hb solution ([Hb] = 10 g/dl) or saline, was mixed with a pooled human serum at various ratios up to 50 vol% ([Hb] = 5 g/dl), and the magnitude of the interference effect of HbV and Hb on 30 analytes was studied. The mixture of the HbV suspension and serum was ultracentrifuged (50,000 g, 20 min) to remove the HbV particles as precipitate, and the supernatant was analyzed and compared with the saline control group. The HbV particles were also removed by centrifugation (2,700 g, 30 min) in the presence of dextran (Mw 200 kDa). The HbV suspension showed considerable interference effects in most analytes. The majority of these

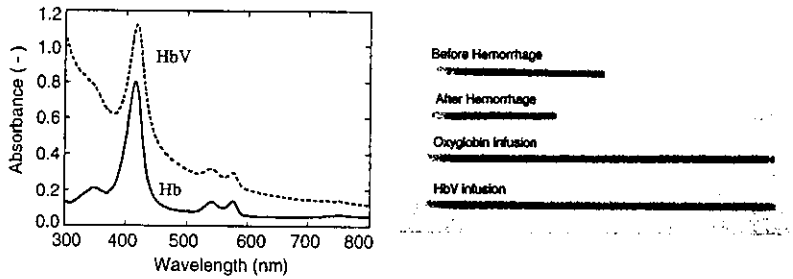

FIG. 5. Left, absorption spectrum of Hb and HbV at [Hb] = 0.01 g/dl in a 10 mm cuvette measured using a UV/vis spectrophotometer. The significant turbidity is confirmed especially at shorter wavelength for an HbV suspension. Right, glass capillaries for the hematocrit measurements during the resuscitation of hemorrhagic shocked hamsters. The centrifuged glass capillary containing blood after the infusion of glutaraldehyde-polymerized bovine Hb showed a red colored supernatant due to the presence of Hb components. In the case of HbV infusion, HbV does not precipitate at the normal centrifugation condition (10,000 g, 5 min), therefore, the supernatant is red and turbid

effects were more serious than those of the acellular Hb solution. These findings are thought to be due to the light absorption of Hb in HbV and/or the light scattering generated in the suspension that interferes with the colorimetric and turbidimetric measurements. The components of HbV may also interfere with the chemical reactions of the studied assays. However, removal of the HbV from the supernatant diminished the interference in most of the assays. This pretreatment of plasma allows accurate measurements of total protein, albumin, globulin, AST, ALT, LDH, ALP, γ-GTP, bilirubin, creatinine, urea nitrogen, uric acid, amylase, lipase, creatinine phosphokinase, total cholesterol, free cholesterol, β-lipoprotein, HDL-cholesterol, total lipid, free fatty acid, phospholipid, and electrolytes (Na, K, Cl, Ca, inorganic phosphate, Mg, Fe, and Cu). Neutral fat was not measured accurately due to the sedimentation by ultracentrifugation.

This pretreatment may be applicable to the removal of perfluorocarbon particles as another type of O_2 carrier, because it is reported that the particles form aggregation in the presence of dextran [96]. We currently utilize the pretreatment of ultracentrifugation for the safety study of HbV in animal tests. Addition of Dex may be an alternative way to facilitate the precipitation of HbV at a lower centrifugal force that is more convenient for a clinical situation, because not all clinical laboratories are equipped with an ultracentrifuge. In this research, we studied the major analytes only. Further research is necessary to clarify the interference for the other measurements of analytes including hydrophobic or amphiphilic drugs or biological components that may interact with the hydrophobic bilayer membrane of HbV.

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. 250 nm) 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 6 h after resuscitation.

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

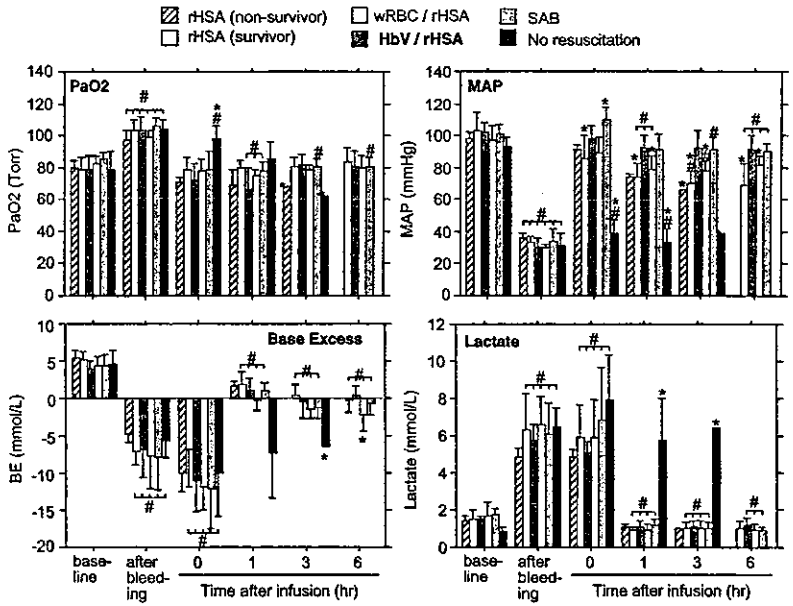

FIG. 6. Changes in mean arterial pressure (MAP) and blood gas parameters during hemorrhagic shock and resuscitation with infusion of HbV suspended in recombinant human serum albumin (HbV/rHSA), shed autologous blood (SAB), washed red blood cells suspended in recombinant human serum albumin (wRBC/rHSA), and recombinant human serum albumin (rHSA) alone. The sham group did not receive a resuscitative fluid after the hemorrhage, and died within 3 h. The number of surviving rats was 3 at 1 h. In the rHSA group, 2 of the 8 rats died between 1 and 6 h. 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 ± 10 mmHg) and significant metabolic acidosis and hyperventilation (Fig. 6). After 15 min, they received HbV/rHSA, shed autologous blood (SAB), washed homologous red blood cells (wRBC) suspended in rHSA (wRBC/rHSA, [Hb] = 8.6 g/dl), or rHSA alone.

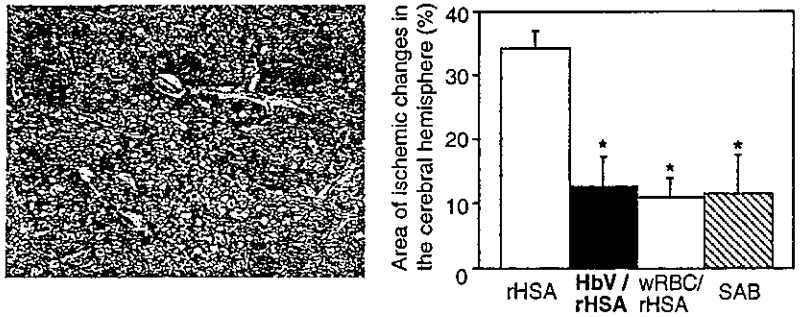

FIG. 7. Resuscitation from hemorrhagic shock with HbV suspended in rHSA in comparison with rHSA, wRBC/rHSA. The cerebral hemisphere on the right side of the rHSA group showed significant ischemic changes, a pyknotic change of the nuclei and an edematous change (34 ± 3% of the total section area) as shown 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