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

PAT

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

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

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

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 (A) Hb is rigorously purified from outdated, donated red cells via pasteurization and ultrafiltration, to completely remove blood type antigen and pathogen.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

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

INTRODUCTION

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

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

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

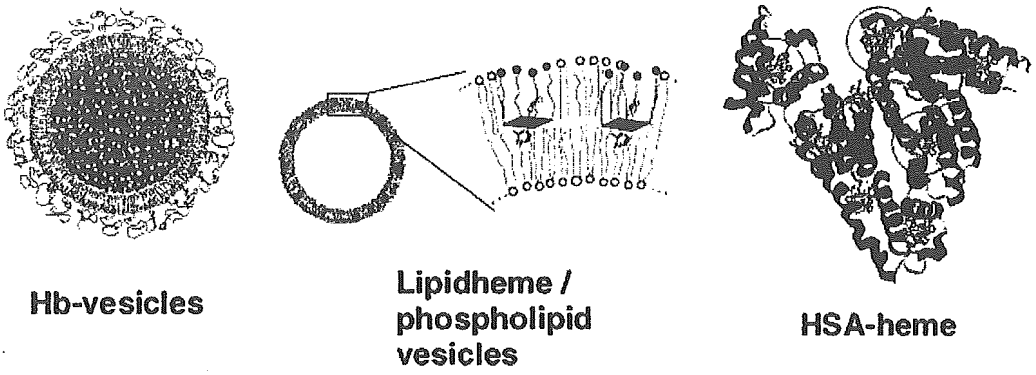


Figure 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme.

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

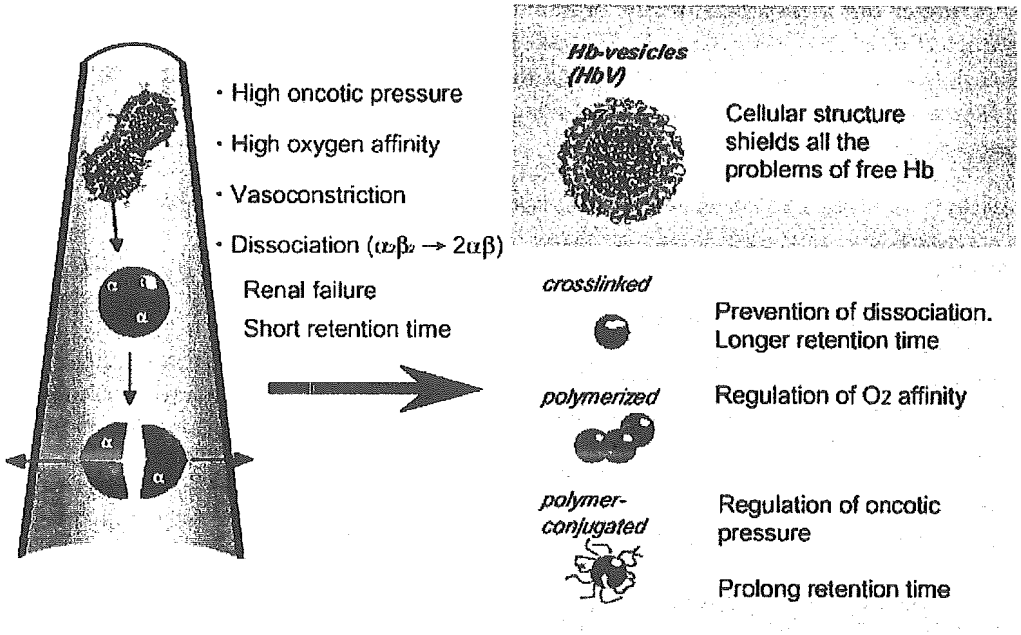


Figure 2. Approaches to solve the problems of utilization of Hb as an O_2-carrier, chemical modification or encapsulation of Hb.

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.

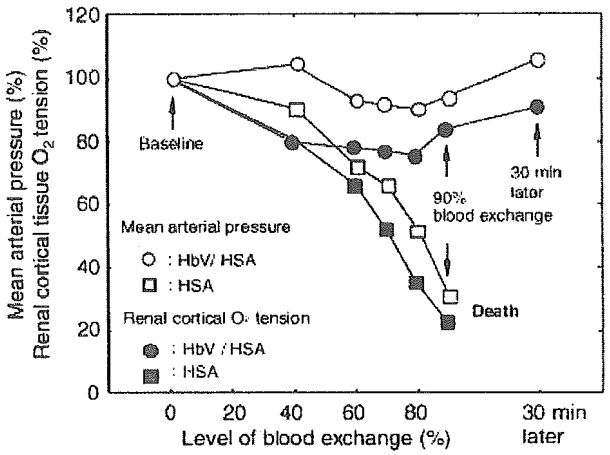


Figure 3. Ninety per cent exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored.

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

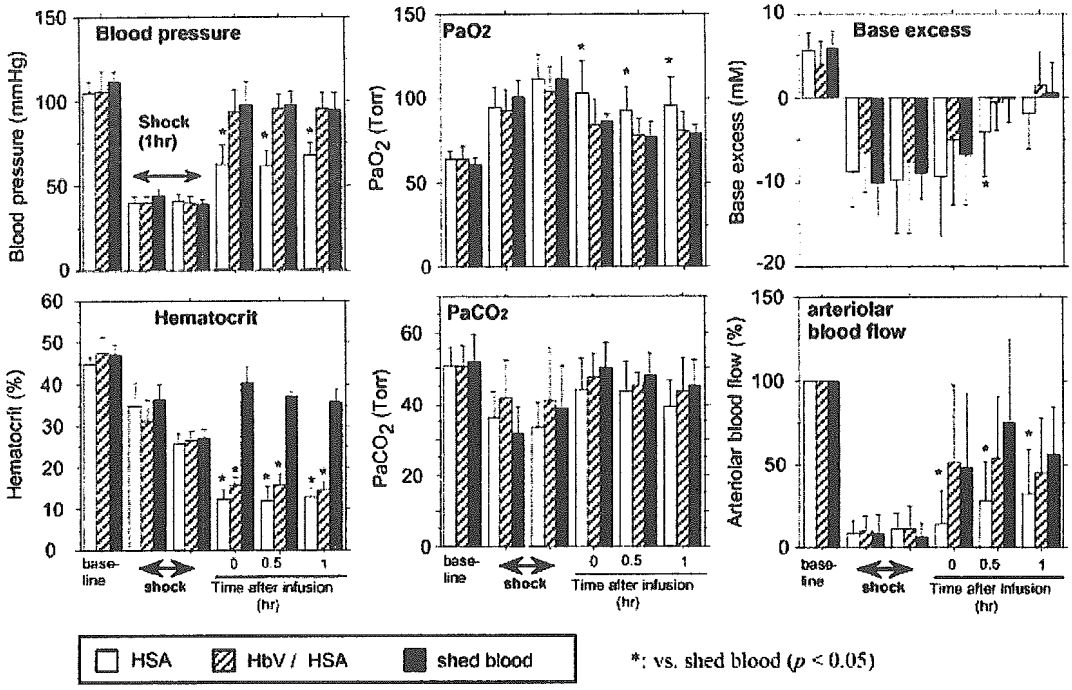


Figure 4. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD.

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

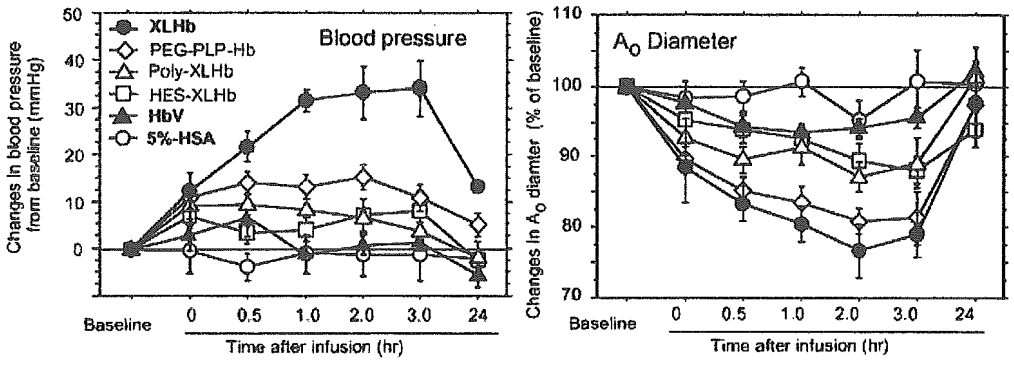


Figure 5. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2-carriers. Mean ± SD.

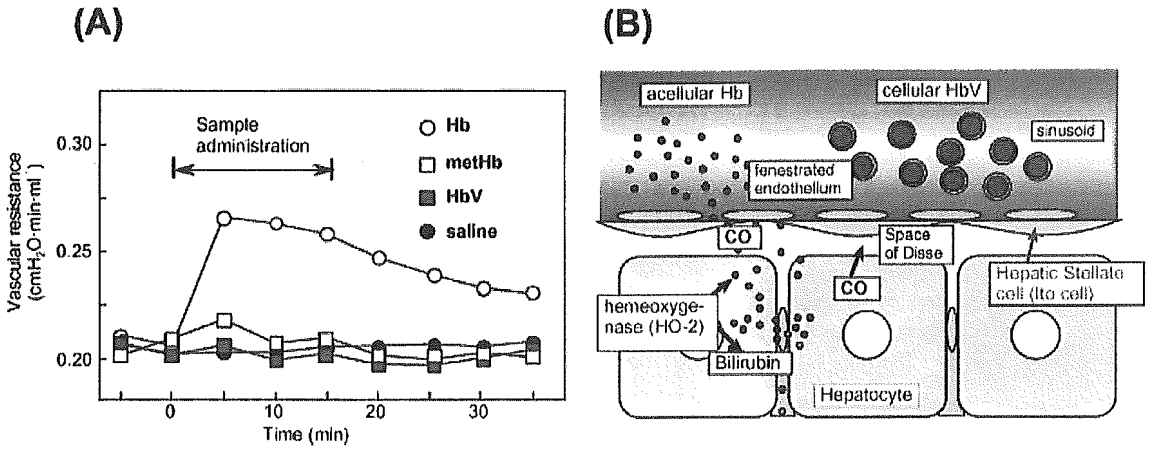


Figure 6. (A) Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. (B) Schematic representation of hepatic microcirculation: the small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolized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

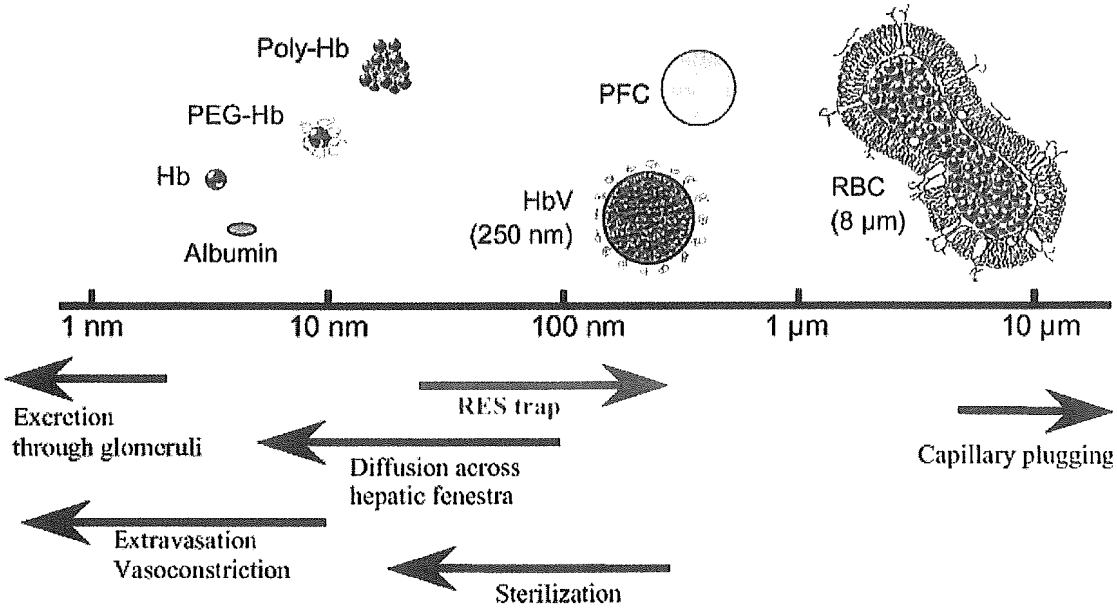


Figure 7. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process.

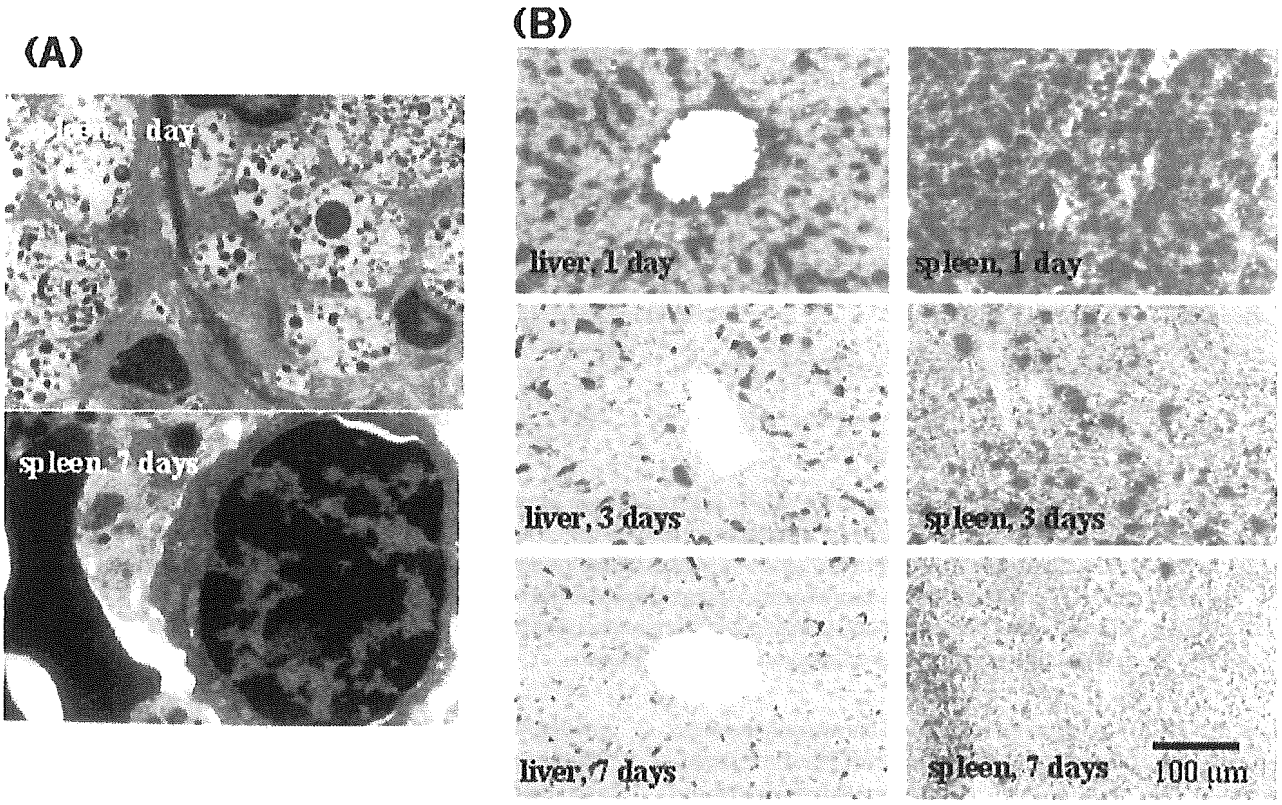


Figure 8. (A) TEM of rat spleen 1 day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. (B) Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. HbV particles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

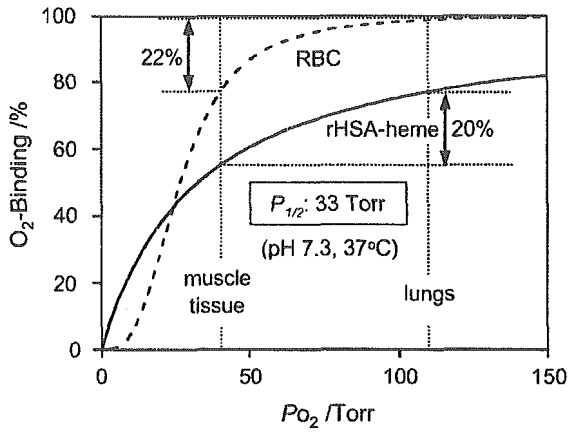


Figure 10. O_2-binding equilibrium curve of albumin-heme.

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

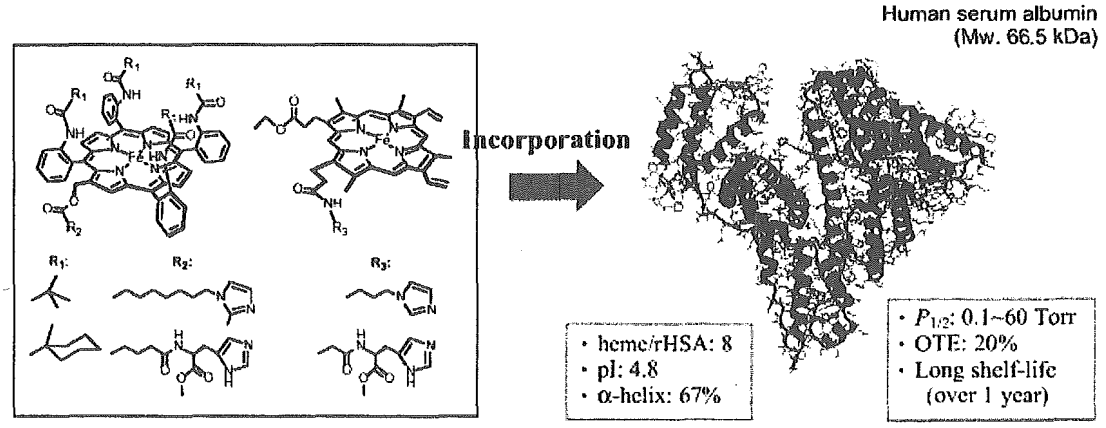


Figure 9. Structure of the albumin-heme molecule.

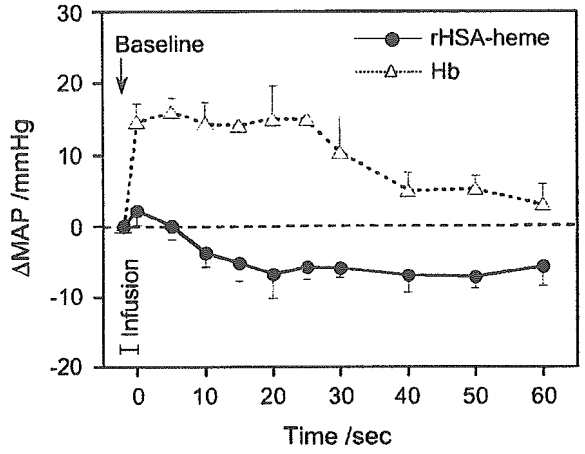


Figure 11. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats ($n=5$). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± SE. Basal value is 90.1 ± 3.0 mmHg.

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.

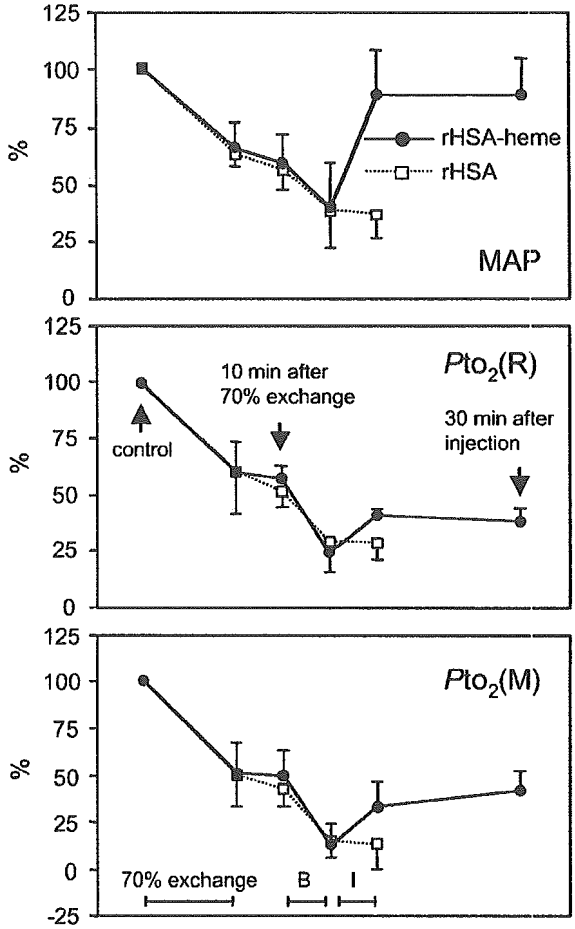


Figure 12. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats ($n=5$). All data are shown as changes from the basal values and expressed as mean ± SE.

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

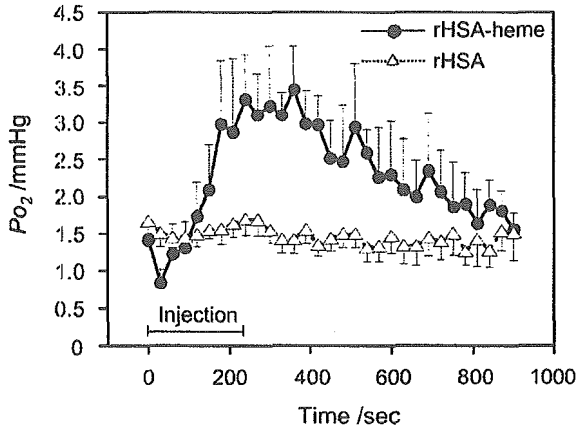


Figure 13. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats ($n=4$ each). All data are shown as changes from the basal values (P_{O_2}) just before the infusion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

This work has been supported by The Ministry of Education, Culture, Sports, Science and Technology and The Ministry of Health, Labor and Welfare.

REFERENCES

1. Tsuchida E (ed.). *Macromolecular Complexes, Dynamic Interactions and Electronic Processes*. VCH: New York, 1991.
2. Ciardelli F, Tsuchida E. Wöhrle D (eds). *Macromolecule-metal Complexes*. VCH: New York, 1996.
3. Matsushita Y, Hasegawa E, Eshima K, Tsuchida E. Synthesis of amphiphilic porphinatoiron complexes having phosphocholine groups. *Chem. Lett.* 1983; 1387–1389.
4. Tsuchida E. Liposome-embedded iron-porphyrins as an artificial oxygen carrier. *Ann. New York Acad. Sci.* 1985; **446**: 429–442.
5. Tsuchida E, Nishide H. Hemoglobin model—artificial oxygen carrier composed of porphinatoiron complexes. *Top. Curr. Chem.* 1986; **132**: 63–99.
6. Komatsu T, Moritake M, Nakagawa A, Tsuchida E. Self-organized lipid-porphyrin bilayer membranes in vesicular form: nanostructure, photophysical properties and dioxygen coordination. *Chem. Eur. J.* 2002; **8**: 5469–5480.
7. Chang TMS. *Blood Substitutes: Principles, Methods, Products, and Clinical Trials*. Karger: Basel, 1997.
8. Riess JG. Oxygen carriers ("blood substitutes")-raison d'etre, chemistry, and some physiology. *Chem. Rev.* 2001; **101**: 2797–2919.
9. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, Rodman G Jr. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock. *JAMA* 1999; **282**: 1857–1864.

10. Tsuchida E. *Blood Substitutes: Present and Future Perspectives*. Elsevier: Amsterdam, 1998.
11. Djordjevich L, Mayoral J, Miller IF, Ivankovich AD. Cardiorespiratory effects of exchange transfusions with synthetic erythrocytes in rats. *Crit. Care Med.* 1987; **15**: 318–323.
12. Takeoka S, Ohgushi T, Yokohama H, Sakai H, Nishide H, Tsuchida E. Preparation conditions of human hemoglobin vesicles covered with lipid membrane. *Artif. Organs Today* 1993; **3**: 129–136.
13. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E. Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif.* 1993; **4**: 563–569.
14. Takeoka S, Ohgushi T, Terase K, Ohmori T, Tsuchida E. Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir* 1996; **12**: 1755–1759.
15. Naito Y, Fukutomi I, Masada Y, Sakai H, Takeoka S, Tsuchida E, Abe H, Hirayama J, Ikebuchi K, Ikeda H. Virus removal from hemoglobin solution using Planova membrane. *J. Artif. Organs* 2002; **5**: 141–145.
16. Fukutomi I, Sakai H, Takeoka S, Nishide H, Tsuchida E, Sakai K. Carbonylation of oxyhemoglobin solution using a membrane oxygenator. *J. Artif. Organs* 2002; **5**: 102–107.
17. Sou K, Endo T, Takeoka S, Tsuchida E. Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjugate Chem.* 2000; **11**: 372–379.
18. Sakai H, Tomiyama K, Sou K, Takeoka S, Tsuchida E. Poly(ethyleneglycol)-conjugation and deoxygenation enable long term preservation of hemoglobin vesicles as oxygen carriers. *Bioconjugate Chem.* 2000; **11**: 425–432.
19. Sou K, Naito Y, Endo T, Takeoka S, Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using the freeze–thawing and extrusion. *Biotechnol. Prog.* 2003; **19**: 1547–1552.
20. Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, Kobayashi K. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. *Crit. Care Med.* 1996; **24**: 1869–1873.
21. Izumi Y, Sakai H, Kose T, Hamada K, Takeoka S, Yoshizu A, Horinouchi H, Kato R, Nishide H, Tsuchida E, Kobayashi K. Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J.* 1997; **43**: 289–297.
22. Kobayashi K, Izumi Y, Yoshizu A, Horinouchi H, Park SI, Sakai H, Takeoka S, Nishide H, Tsuchida E. The oxygen carrying capability of hemoglobin vesicles evaluated in rat exchange transfusion models. *Artif. Cells Blood Substitues Immobilization Biotechnol.* 1997; **25**: 357–366.
23. Sakai H, Takeoka S, Park SI, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, Tsuchida E. Surface modification of hemoglobin vesicles with poly(ethyleneglycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjugate Chem.* 1997; **8**: 23–30.
24. Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, Tsuchida E, Intaglietta M. Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. *J. Biomed. Mater. Res.* 1998; **40**: 66–78.
25. Sakai H, Tsai AG, Rohlfs RJ, Hara H, Takeoka S, Tsuchida E, Intaglietta M. Microvascular responses to hemodilution with Hb-vesicles as red cell substitutes: influences of O_2 affinity. *Am. J. Physiol.* 1999; **276**: H553–H562.
26. Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, Tsuchida E. Systemic and microvascular responses to the hemorrhagic shock and resuscitation with Hb-vesicles. *Am. J. Physiol. Heart Circ. Physiol.* 2002; **283**: H1191–H1199.
27. Erni D, Wettstein R, Schramm S, Contaldo C, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A. Normovolemic hemodilution with hemoglobin-vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am. J. Physiol. Heart Circ. Physiol.* 2003; **284**: H1702–H1709.
28. Contaldo C, Schramm S, Wettstein R, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A, Erni D. Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol.* 2003; **285**: H1140–H1147.
29. Goda N, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M. Distribution of heme oxygenase isoform in rat liver: topographic basis for carbon monoxide-mediated micorvascular relaxation. *J. Clin. Invest.* 1998; **101**: 604–612.
30. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension in conscious hamster model. *Am. J. Physiol. Heart Circ. Physiol.* 2000; **279**: H908–H915.
31. Wakamoto S, Fujihara M, Abe H, Sakai H, Takeoka S, Tsuchida E, Ikeda H, Ikebuchi K. Effects of PEG-modified hemoglobin vesicles on agonist induced platelet aggregation and RANTES release *in vitro*. *Artif. Cells Blood Substitues Immobilization Biotechnol.* 2001; **29**: 191–201.
32. Kyokane T, Norimizu S, Taniai H, Yamaguchi T, Takeoka S, Tsuchida E, Naito M, Nimura Y, Ishimura Y, Suematsu M. Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. *Gastroenterology* 2001; **120**: 1227–1240.
33. Ito T, Fujihara M, Abe H, Yamaguchi M, Wakamoto S, Takeoka S, Sakai H, Tsuchida E, Ikeda H, Ikebuchi K. Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on *N*-formyl-methionyl-leucyl-phenylalanine induced responses of polymorphonuclear neutrophils *in vitro*. *Artif. Cells Blood Substitues Immobilization Biotechnol.* 2001; **29**: 427–438.
34. Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial systems. *Am. J. Pathol.* 2001; **159**: 1079–1088.
35. Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, Tsuchida E. Pretreatment of serum containing Hb-vesicles (oxygen carriers) to avoid their interference in laboratory tests. *Clin. Chem. Lab. Med.* 2003; **41**: 222–231.
36. Sakai H, Horinouchi H, Masada Y, Takeoka S, Kobayashi K, Tsuchida E. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials* 2004; **25**: 4317–4325.
37. Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Kobayashi K, Tsuchida E. Physiologic capacity of reticuloendothelial system for degradation of homoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J. Pharmacol. Exp. Ther.* 2004; **311**: 874–884.
38. Curry S, Mandelkow H, Brick P, Franks N. Crystal structure of human serum albumin complexes with fatty acid reveals an asymmetric distribution of binding sites. *Nature Struct. Biol.* 1998; **5**: 827–835.
39. Sumi A, Ohtani W, Kobayashi K, Ohmura T, Yokoyama K, Nishida M, Suyama T. Purification and physicochemical properties of recombinant human serum albumin. In *Biotechnology of Blood Proteins*, Rivat C, Stoltz JF (eds). John Libbey Eurotext: Montrouge, 1993; vol. 227, 293–298.
40. Komatsu T, Ando K, Kawai N, Nishide H, Tsuchida E. O_2-transport albumin: a new hybrid-haemoprotein incorporating tetraphenylporphinatoiron(II) derivative. *Chem. Lett.* 1995; 813–814.
41. Tsuchida E, Ando K, Maejima H, Kawai N, Komatsu T, Takeoka S, Nishide H. Properties of oxygen binding by albumin-tetraphenylporphyrinatoiron(II) derivative complexes. *Bioconjugate Chem.* 1997; **8**: 534–538.
42. Wu J, Komatsu T, Tsuchida E. Resonance raman studies of O_2-binding to *ortho*-substituted tetraphenyl- and tetranaphthyl-porphinatoiron(II) derivatives with a covalently linked axial imidazole. *J. Chem. Soc., Dalton Trans.* 1998; 2503–2506.
43. Komatsu T, Hamamatsu K, Wu J, Tsuchida E. Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) serivatives. *Bioconjugate Chem.* 1999; **10**: 82–86.
44. Tsuchida E, Komatsu T, Mastukawa Y, Hamamatsu K, Wu J. Human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) derivative as a totally

synthetic O_2-carrying hemoprotein. *Bioconjugate Chem.* 1999; **10**: 797–802.
45. Komatsu T, Matsukawa Y, Tsuchida E. Kinetics of CO and O_2 binding to human serum albumin-heme hybrid. *Bioconjugate Chem.* 2000; **11**: 772–776.
46. Komatsu T, Matsukawa Y, Tsuchida E. Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(II) derivatives. *Bioconjugate Chem.* 2001; **12**: 71–75.
47. Nakagawa A, Komatsu T, Tsuchida E. Photoreduction of autooxidized albumin-heme hybrid in saline solution: revival of its O_2-binding ability. *Bioconjugate Chem.* 2001; **12**: 648–652.
48. Komatsu T, Okada T, Moritake M, Tsuchida E. O_2-Binding properties of double-sided porphyrinatoiron(II)s with polar substituents and their human serum albumin hybrids. *Bull. Chem. Soc. Jpn* 2001; **74**: 1695–1702.
49. Wu Y, Komatsu T, Tsuchida E. Electrochemical studies of albumin-heme hybrid in aqueous media by modified electrode. *Inorg. Chim. Acta* 2001; **322**: 120–124.
50. Komatsu T, Matsukawa Y, Tsuchida E. Effect of heme structure on O_2-binding properties of human serum albumin-heme hybrids: intramolecular histidine coordination provides a stable O_2-adduct complex. *Bioconjugate Chem.* 2003; **13**: 397–402.
51. Tsuchida E, Komatsu T, Yanagimoto T, Sakai H. Preservation stability and *in vivo* administration of albumin-heme hybrid solution as an entirely synthetic O_2-carrier. *Polym. Adv. Technol.* 2002; **13**: 845–850.
52. Nakagawa A, Komatsu T, Ohmichi N, Tsuchida E. Synthetic dioxygen-carrying hemoprotein: human serum albumin including iron(II) complex of protoporphyrin IX with an axially coordinated histidylglycyl-propionate. *Chem. Lett.* 2003; **32**: 504–505.
53. Tsuchida E, Komatsu T, Matsukawa Y, Nakagawa A, Sakai H, Kobayashi K, Suematsu M. Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging. *J. Biomed. Mater. Res.* 2003; **64A**: 257–261.
54. Tsuchida E, Komatsu T, Hamamatsu K, Matsukawa Y, Tajima A, Yoshizu A, Izumi Y, Kobayashi K. Exchange transfusion of albumin-heme as an artificial O_2-infusion into anesthetized rats: physiological responses, O_2-delivery and reduction of the oxidized hemin sites by red blood cells. *Bioconjugate Chem.* 2000; **11**: 46–50.
55. Komatsu T, Hamamatsu K, Tsuchida E. Cross-linked human serum albumin dimers incorporating sixteen (tetraphenylporphinato)iron(II) derivatives: synthesis, characterization, and O_2-binding property. *Macromolecules* 1999; **32**: 8388–8391.
56. Kobayashi K, Komatsu T, Iwamaru A, Matsukawa Y, Horinouchi H, Watanabe M, Tsuchida E. Oxygenation of hypoxic region in solid tumor by administration of human serum albumin incorporating synthetic hemes. *J. Biomed. Mater. Res.* 2003; **64A**: 48–51.
57. Kobayashi K, Izumi Y, Yamahata T, Sakai H, Takeoka S, Nishide H, Tsuchida E. Efficacy of synthetic oxygen-carrying substances. In *Int. Congr. Ser. 1995, 1102 (Shock: from Molecular and Cellular Level to Whole Body)*. Elsevier: Amsterdam, 1996; 305–310.
58. Horinouchi H, Tajima A, Kobayashi K. Liuid ventilation using artificial oxygen carrier. *Artif. Blood* 2001; **9**: 2–5 (in Japanese).

総　説

完全合成型人工酸素運搬体の開発

Development of Totally Synthetic Artificial Oxygen Carrier

甲斐俊哉，木田善規，福富一平，帆足洋平，片山直久，山本尚志，大川裕紀，弘津一郎，佐藤　誠

Toshiya Kai, Yoshinori Kida, Ippei Fukutomi, Youhei Hoashi, Naohisa Katayama, Hisashi Yamamoto, Hiroki Ohkawa, Ichiro Hirotsu, Makoto Sato

和文抄録

我々は，遺伝子組換えヒト血清アルブミン分子中に合成ヘム誘導体を最大で8分子包接させた，全く新しい完全合成型人工酸素運搬体であるアルブミン－ヘムの開発を進めている．アルブミン－ヘムはカプセル化の必要がなく，それ自身がアルブミン由来の膠質浸透圧を保有しており，また一酸化窒素（NO）捕捉による血圧上昇等の副作用も示さない．アルブミン－ヘムの大量製造方法を既に確立しており，その概要を示すとともに製剤特性をまとめた．またラットを用いた交換輸血試験の結果，アルブミン－ヘムは明らかな酸素運搬効果を示した．

さらに，ヒトヘモグロビンカプセル型人工酸素運搬体（Hb-V）についても，㈱オキシジェニクスとの共同開発を進めている．現在のところ，最も臨床試験に近い段階に進んでいる人工酸素運搬体であるが，献血由来のヘモグロビンを使用しているため，感染の危険性や安定供給の問題が完全には解決されていない．そこで，遺伝子組換えヒトヘモグロビン（rHb）の開発にも着手した．既にrHbの発現を確認しており，現在大量製造方法の検討に着手している．

これらの人工酸素運搬体の製造施設を当社注射剤工場と併設して設置するために準備を進めているところである．

Abstract

Development of an innovative and original artificial oxygen carrier, albumin-heme, which consists of recombinant human serum albumin (rHSA) and synthetic heme derivative, has been promoted in our company. Albumin-heme, in which maximally eight molecules of synthetic heme are incorporated into albumin molecule, has a suitable colloidal osmotic pressure itself and also do not have side effects such as hypertension due to depletion of nitric oxide (NO). Large-scale production process has already been established. Characteristics of albumin-heme are described in this paper. Furthermore, the exchange transfusion experiments in rats revealed that albumin-heme had oxygen carrying properties.

We also have been developing a hemoglobin vesicle as another type of artificial oxygen carrier in collaboration with Oxygenics Inc. This type of oxygen carrier is the most promising preparation close to clinical use. However, some risks of infection and uncertainty of stable blood supply still exist because of use of donated human blood as raw material. So, we decided to develop recombinant human hemoglobin (rHb). A purified rHb has already been obtained by our original expression method. At the present, we have been preparing for large-scale production facilities in our pharmaceutical factory in Japan to produce these artificial oxygen carriers as mentioned above.

Keywords

synthetic oxygen carrier, albumin-heme, synthetic heme, hemoglobin vesicle, recombinant human hemoglobin, recombinant human serum albumin

緒言

米国を中心にヘモグロビン修飾型の人工酸素運搬体（修飾Hb）が開発中であるが，いずれもヒトあるいはウシのヘモグロビンを原料としており，その安全性と安定供給に懸念が残る．

ニプロ㈱医薬品研究所　〒525-0055　滋賀県草津市野路町3023　Pharmaceutical research center, NIPRO CORPORATION, 3023, Nojicho, Kusatsu, Shiga 525-0055, Japan

論文受付 2004年12月2日　論文受理 2005年1月8日

また，これらの修飾Hbは，ヘモグロビン（Hb）濃度の上昇により膠質浸透圧及び粘度が上昇するため，生体に投与できるHb量が限られており，結果として酸素運搬能に限界が認められる．また，天然の赤血球のように細胞膜に覆われていないため，Hbが容易に逸脱し，NO捕捉による血管収縮といったHb自体の毒性の発現やHb機能維持のための解糖系酵素やメトHb還元酵素等の各種酵素系の保持ができないため安定性が悪いといった問題点が依然として指摘されている[1,2]．これに対して日本国内ではリポソームにヘモグロビンを内包した，細胞型人工酸素運搬体の開発が進められている．これらの製剤はヘモグロビンの副作用を低減し，より安全で有効性の高い人工酸素運搬体として最も臨床に近い段階に開発が進んでいる．一方でこれらの人工酸素運搬体は献血由来の原料に依存している点で，国内使用に限定されるとともに安定供給にも不安が残る．

我々は，遺伝子組換えヒト血清アルブミン（rHSA）の高純度，高生体適合性，非感染性，量産性などの特徴に着目し，rHSAに可逆的酸素配位能を有する合成ヘムを包接させた新規の完全合成ヘム蛋白質（アルブミン－ヘム）の開発を行っている．このアルブミン－ヘムは修飾ヘモグロビンで認められるNO捕捉による血管収縮作用は有していない．既に合成ヘムの選定が終了し，ラット，イヌを用いた交換輸血実験で有効な酸素運搬能を確認している．本稿では，アルブミン－ヘムの製剤としての安定性，製造方法，動物での評価結果について報告する．

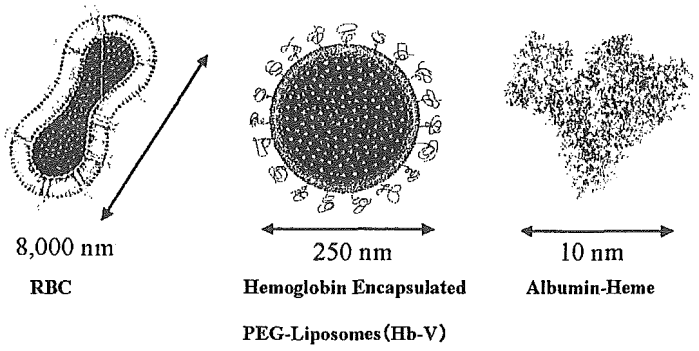


Fig. 1. Two types of artificial oxygen carrier under developing in NIPRO.

一方，Fig. 1.に示すように，ヘモグロビンを封入した細胞型人工酸素運搬体（Hb-V）の開発も併行して進めており，㈱オキシジェニクス，早稲田大学，慶応義塾大学とともにHb-VのGMPに準拠した製造施設の設置を準備中である．さらに内包する献血由来のヘモグロビンを遺伝子組換えヘモグロビン（rHb）に置き換るためにrHbの開発にも着手している． これらの概要についても紹介する．

これらの完全合成型人工酸素運搬体は，原料供給の不安が払拭されるだけでなく，感染の危険性も回避できるため，酸素運搬能を有する薬剤としての全く新しい利用展開も可能となる．本稿で紹介する2種類の完全合成人工酸素運搬体は赤血球より極めて小さいという特長を生かし（Hb-Vで250 nm，アルブミン－ヘムで10 nm），腫瘍の酸素化，虚血部位の酸素化による治療効果の向上についても臨床応用を目指した評価を進めている．これらの市場は赤血球市場（国内約300億円，日米欧で約2,400億円）と同等以上の市場が予測されており，新たな治療手段として今後の期待が大きい．

開発概要

1．アルブミン－ヘム

(1) アルブミン－ヘムの概要

アルブミン－ヘムはFig. 2.に示したように，rHSAの分子中に合成ヘム誘導体を包接した全く新しい概念の人工酸素運搬体である．ヘムの結合サイトはサブドメインⅠb，Ⅱa，Ⅲbなどが推定されており，rHSA 1分子当たり最大8分子のヘムが取り込まれる[3-7]．アルブミン－ヘムのCDスペクトルはrHSA単独の場合とほぼ重なっており，α-helix の含量（約67%）も変わらない．我々のグループは世界に先駆けて開発に成功したrHSAを保有しており[8]，合成ヘムとの組み合わせにより，感染の危険性のない完全合成型の人工酸素運搬体として開発を進めている[9]．

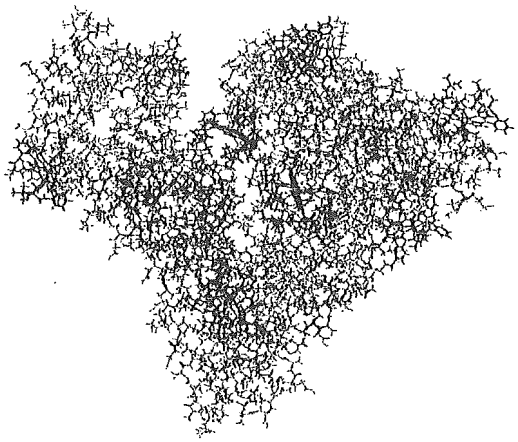


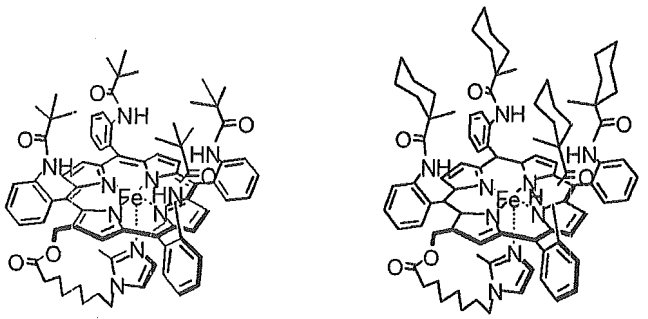
Fig. 2. Stereo view of albumin-heme
Maximally, 8 molecules of synthetic heme can be incorporated into albumin molecule.

酸素配位最小単位分子であるヘム鉄を血漿蛋白質であるアルブミンの内部に包接させた構造のアルブミン－ヘムはカプセル化の必要がなく，そのまま投与できることが最大の利点であり，加えて，膠質浸透圧の調節能を有し，調製が容易，低コストなど，優れた特徴を持つ．また，欧米で開発が進行している修飾Hb製剤は，平滑筋近傍への逸脱とNO捕捉に伴う血管収縮・血圧上昇が問題視されているが[1,2]，アルブミン－ヘム製剤を生体内へ投与しても，そのような副作用は全く観測されない[10]．アルブミン－ヘムもHbと同様にNOを強く結合するものの，アルブミンの等電点が4.8と低いために，血管内皮細胞を覆う基底膜（厚さ50 nm）との間に静電反発を生じ，Hb粒子に比べて

血管外へ漏出しにくいためと考えている．現状は，ヘム誘導体の選定作業を終了し，輸血代替のみならず，腫瘍の酸素化による放射線治療効果の向上，虚血部位の酸素化等の新しい適応症へ向けた評価を行っているところである．

Fig. 3.に選定したヘム誘導体の構造を示した．ポルフィリン骨格を有したピケットフェンス型と呼ばれるポルフィリン誘導体（テトラアミノフェニルポルフィリン誘導体）であるが，分子内に軸塩基として作用する側鎖を有しており，ポルフィリン環の図中下側から第5座配位子として配位することにより，酸素分子の結合力を調整することができるという特徴を有している．種々の誘導体の中から有効性や安全性を中心に評価した結果，図中右に示したシクロヘキサノイルアミノフェニル誘導体を選択した[11]．

(2) 製造方法



Pivalamidophenyl deriv.　　Methylcyclohexanamido-phenyl deriv.

Fig. 3. Molecular structures of synthetic hemes.

アルブミン－ヘムの製造方法概略をFig. 4.に示した．アルブミン水溶液とヘム誘導体エタノール溶液を至適条件化で精密に混合攪拌することにより，アルブミン分子中にヘム誘導体が包接されたアルブミン－ヘムが出来上がる．包接させるヘム誘導体の数は化学量論的な添加量を変化させることで容易に調整できる．不純物としてのエタノールを除去することを目的として透析（定容量限外濾過）の後，塩濃度の調節並びに濃縮を行い，最終的にアルブミン濃度5％としてアルブミン－ヘム溶液を得る．

なお，製造中のヘム誘導体の安定性を維持するために，アスコルビン酸添加によって予め鉄を還元しておいたヘム誘導体溶液をCOガス通気し，CO結合体として最終アルブミン－ヘム製剤を得る．得られたアルブミン－ヘムの特性をTable 1.に示した．

一方，Fig. 5.に示したようにCO結合体は安定であるが，使用に先立ち，酸素化の操作が必要であるという欠点を有しており，以下に述べるCOを用いない製造方法の検討を併行して進めてきた．

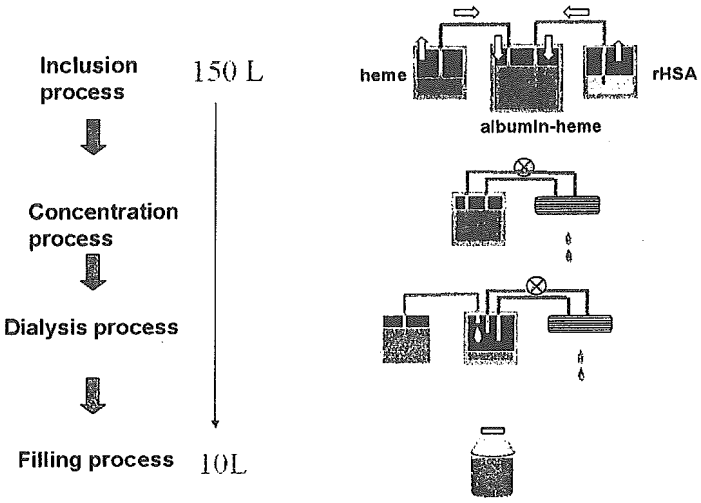


Fig. 4. Outline of manufacturing process of albumin-heme.

Table 1. Physico-chemical characteristics of albumin-heme.

Items	Standard values
[rHSA] (g/dL)	5
[heme] (mM)	3-6
heme/rHSA (mol/mol)	4-8
P_{50} (Torr)	28-38
rHSA pI	4.8
rHSA -helix content (%)	67
Stretching vibration of coordinated O_2 (cm^{-1})	1158
Stretching vibration of Fe-O_2 (cm^{-1})	561
Met-heme (%)	<3
Viscosity (cP at 230s^{-1})	1.1
Specific gravity (g/cm^3)	1.01
Crystal osmotic pressure (mOsm)	300
Colloidal osmotic pressure (Torr)	19
pH (37℃)	7.4
Endotoxin (EU/mL)	<0.2

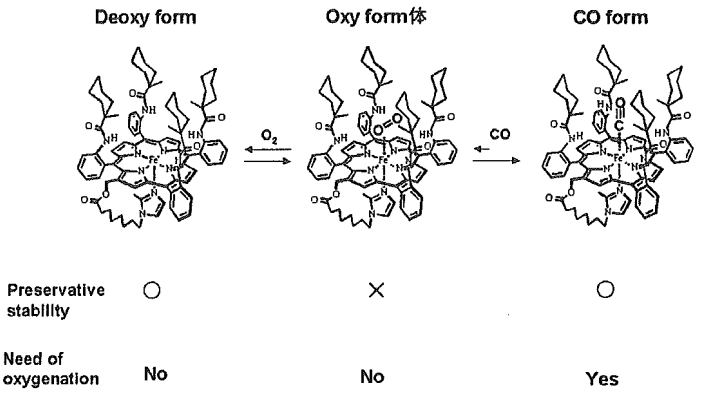


Fig. 5. Comparison of preservative stability and need of prior oxygenation among 3 forms.

(3) COを用いない製造方法

血液透析の際に使用する種々の透析膜（ダイアライザー）を脱酸素工程に利用することを試みた．その結果，トリアセテート製中空糸内にアルブミン－ヘムを流し，密閉ハウジング内の中空糸の外側（本来透析液が灌流するスペース）に加湿したN_2ガスをフローしながら，濃縮された量だけ連続して透析液を補充する定容量限外濾過を行うことによって製造工程を低酸素状態（0.1 Torr未満）で維持することができることを見出し（Fig. 6.），アルブミン－ヘムをデオキシ体として製造することに成功した．デオキシ体の保存安定性を評価した結果をTable 2.に示した．現在のところ，室温保存で6箇月の保存安定性が確認されており，メト化率も初期値と比較して全く変化しないことが明らかとなった．さらに継続して安定性を評価中である．

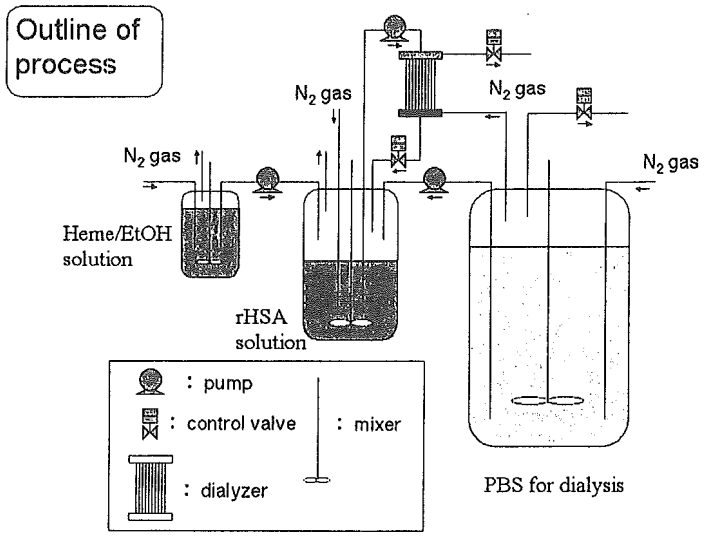


Fig. 6. Manufacturing process of deoxy form under nitrogen atmosphere using blood dialyzer.

Table 2. Stability of deoxy form albumin-heme at room temperature for 6 months.

		Initial	After 6 months
Appearances		good	good
P_{50} (Torr)		34.7	34.7
[rHSA] (%)		5.08	5.35
[Albumin-heme] (mM)		2.75	2.77
Met conversion (%)		1.07	1.08
Viscosity (cP)		1.16	1.16
pH		7.13	7.14
[FeP]/[Alb]		3.3	3.4
UV λ_{max} (nm)	CO	426.5	426.5
	Oxy	424.5	425.0
	Deoxy	443.0	443.5

(4) 具体的製剤例

当社は，無菌充填，無菌の液－粉キット注射製剤等の製造を得意としていることもあり，通常のガラスバイアル充填製剤のみならず，Fig. 7.に示したような凍結乾燥粉末製剤，さらにFig. 8.に示したような凍結乾燥粉末と溶解液のキット製剤としての開発を検討している．災害等に備えた長期備蓄には保管スペースあるいは安定性の維持の面から極めて優れた形態であると思われる．

Fig. 7. Examples of filling product in glass vial
left: solution preparation, right: freeze-dried powder.

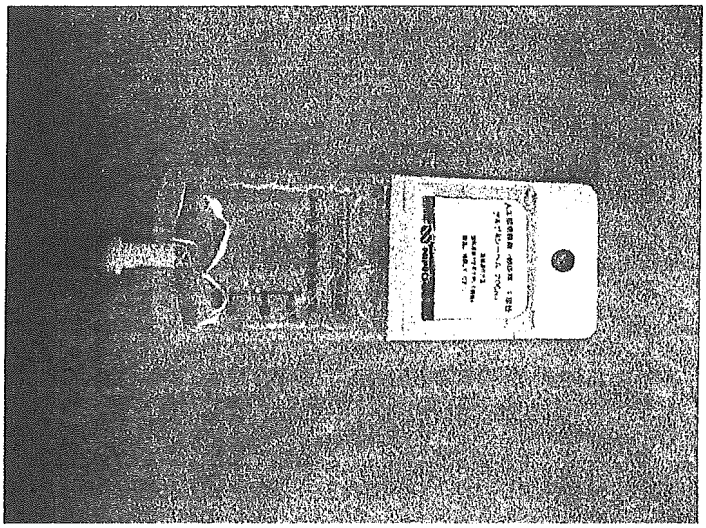


Fig. 8. Example of kit product consists of freeze-dried powder and solvent for injection.

(5) 脱血交換モデルを用いた薬効評価

既に様々な動物評価を行っているが，その中からラットの脱血交換モデルを用いた薬効評価結果を紹介する．

Fig. 9.に実験操作の概略を示した．7～9週齢のWistar系雄性ラット（体重282.3～335.4 g）をウレタン麻酔下に1群5匹として使用した．呼吸数は気管内に挿管したカニューレを熱感知用センサーに接続して測定し，脱血交換開始から呼吸停止までの時間を生存時間とした．血圧は，左大腿動脈内にカテーテルを留置し，圧トランスデューサを介して測定した．被験薬と

の脱血交換輸注は，右総頸動脈より3 mL/kg/min の速度で脱血すると同時に被験薬を右大腿静脈から同速度で投与して行った．ヘマトクリット値は脱血交換開始後10ないし15分間隔で右外頸静脈より採取した血液を用いて測定した．rHSA 及びアルブミン－ヘム投与群のヘマトクリット値から，時間－血液希釈率曲線を作成し，血液希釈率＝100× $e^{-0.0849\times時間}$ （相関係数0.999）の式を得た．本式を用い，生存時間及び平均血圧測定時点の血液希釈率を算出した．なお，直腸温は実験終了まで37.0±1.0℃に維持した．

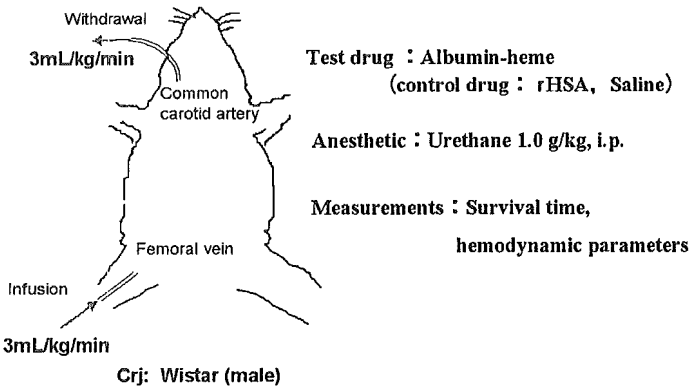


Fig. 9. Rat exchange transfusion model.

Fig. 10.に最大血液希釈率および平均血圧の推移を示した．図には示していないが，ヘマトクリット値はrHSA及びアルブミン－ヘムの脱血交換輸注により経時的に低下し，群間に差は認められなかった．アルブミン－ヘム投与群（生存時間45.46±1.51分）は，生理食塩液投与群（生存時間 21.48±0.71分）及びrHSA単独投与群（生存時間 32.07±1.15分）に比べて有意な延命効果を示し，最大血液希釈率を有意に上昇させた．

アルブミン－ヘム投与群の平均血圧は，生理食塩液投与群およびrHSA投与群に比べ有意に高値を示し，血圧維持効果が認められた．

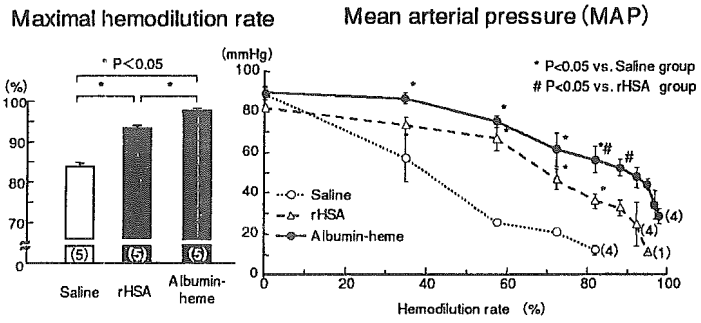


Fig. 10. Mean arterial pressure and hemodilution rate during exchange transfusion by albumin-heme, rHSA and saline in urethanized rats. The data represent means ± SEM of 4-5 rats. Figures in parenthesis show No. of animal survived.

以上の結果から，酸素結合能を有するアルブミン－ヘムは，酸素結合能を持たないrHSAと比較し，血液希釈に伴う呼吸・循環器系変化を遅延させ，明らかな延命効果を示したことから，生体内で酸素運搬能を発揮しているものと考えられた．

2．遺伝子組換えヘモグロビン（rHb）

早稲田大学，慶應義塾大学の研究グループは，ポリエチレングリコールで表面修飾を施した生体適合性の高いリポソーム中にヒト血液由来のヘモグロビンを内封した細胞型の人工酸素運搬体（Hb-V）の開発に永年に亘って取り組んでいる．このリポソーム封入型人工酸素運搬体は，非カプセル型の持つ問題点を克服できるものとして既に多くの成果が報告され[12-18]，実現の可能性という意味において他を一歩リードした製剤であり，早期の臨床治験開始が望まれている．当社も，㈱オキシジェニクス（早稲田大学，慶應義塾大学のリポソーム封入型人工酸素運搬体を開発することを目的として設立されたバイオベンチャー企業）とともにこのリポソーム封入型人工酸素運搬体の開発を共同で行っている．

しかし，ヒト血液（献血）から抽出した精製ヘモグロビンを使用する点で，感染の危険性や供給量の問題は完全には解決されておらず，ヘモグロビンを遺伝子組換え体に置き換えることも今後の展開として極めて重要であると考えている．

rHbの開発に関しては，既に幾つかの事例が報告されている．いずれもHbの二量体への解離を防止するための架橋体であるが，非細胞型（非カプセル化）製剤であるため，修飾Hbで認められる副作用を回避出来ずに開発が中止となっている．Baxter 社がHemeAssist™として開発した分子内架橋Hbは，2本のHb α 鎖99番目のLys間をフマル酸架橋したものである．しかし，外傷に対する輸血代替療法において，従来法よりも死亡率が高くなったため，臨床試験が中止された[19]．さらに遺伝子組換え技術を用いて培養菌体に発現させたrHbは，Somatogen Inc. 社によってOptro™として具体化された．ヒトHbのアミノ酸配列の一部を変換して二量体への解離防止と適当な酸素親和性が実現されている．臨床第Ⅰ相試験では，投与直後に一過性の血圧上昇が起こり，軽い嚥下障害，吐き気や嘔吐などが訴えられ，食道や胃腸に変形が認められた[20]．これらの症状はすべてHb分子が血管平滑筋部位まで拡散し，血管弛緩因子であるNOを捕捉する作用に関連しているものと考えられている．

一方，我々の今日に至る様々な共同研究の結果，熊本大学の組換え蛋白質製造技術とグループ（㈱バイファ）の組換え蛋白質開発・製造ノウハウを融合させることで，全く新たに遺伝子組換えヘモグロビンを開発する目処が得られた．カプセル化技術は早稲田大学と慶應義塾大学の永年の共同研究から，既にその製造技術が確立されており，これらの技術をそのまま応用することでリポソーム封入型遺伝子組換えヘモグロビン（rHb-V）が実現に近づいたことになる．

(1) rHbの発現方法

熊本大学で開発された大腸菌における高発現・高コピーベクターであるpBEXベクター（特願2003-1885）をFig. 11.に示した．このベクターに予め合成しておいたヘモグロビンα鎖とβ鎖の遺伝子を組み込み，大腸菌で4量体のヘモグロビンとして発現させることに成功した．

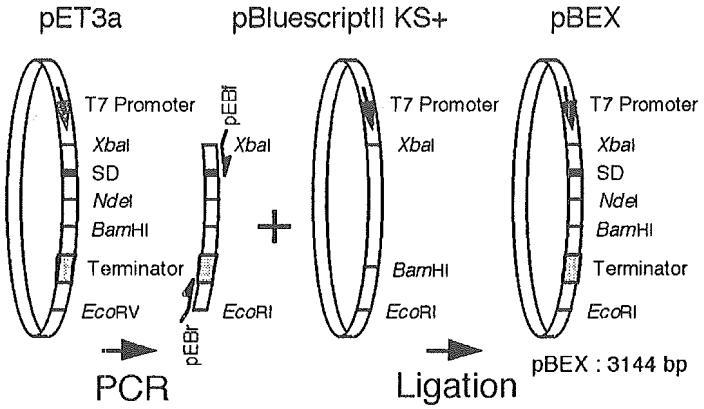


Fig. 11. New pBEX vector with superior character for high expression and copy in colon bacillus.

大腸菌の場合は，ヒトとコドンの使用頻度が異なるため，蛋白質のcDNAをそのまま用いても大量発現できない場合がある．そこで，あらかじめ大腸菌のコドン使用頻度にあわせて最適化した遺伝子を試験管内で全合成した．この方法により，既に多くのヘム蛋白質の大量発現に成功している．ヘモグロビンの場合，α鎖，β鎖に対応する遺伝子（Fig. 12.）をそれぞれ別個に合成し，ベクターにつなぎ込む（Fig. 13.）．これらのベクターは強力なT7プロモーターを共通に有しているため，rHbの大量発現が可能となる．

培養条件をTable 3.に示したが，この条件で培地1 L当たり，g単位のrHbの発現が可能となっている．

Table 3. List of culture condition employed

Host :Colon Bacillus strain BL21Gold (DE3)
Culture medium:Terrific Broth
Culture temperature:30℃
Revolution of shaking:120rpm
Additive:hemin or aminolevulinic acid

(2) rHb発現の確認

Fig. 14.及び15.に，得られたrHbのnative-PAGE及びマススペクトルの測定結果をそれぞれ示した．これらの結果から発現した蛋白質が確かにヘモグロビンと同一の分子量であるとともにα鎖とβ鎖が1：1の比率からなる四量体であることが示唆された．現在，構造の詳細検討並びに特性解析を行うとともに，大量製造へ向けた準備を進めている段階である．

Fig. 16.には，秋田県大館市で拡張中の当社の注射剤製造工場の概観を示した．現在，この無菌製剤工場に隣接して人工酸素運搬体の製造工場を設置する作業を進めている．

おわりに

以上述べた人工酸素運搬体は，当社ニプロ，㈱オキシジェニクス，早稲田大学，慶應義塾大学及び熊本大学との共同研究で事業化に向けた開発が進められており，30年来蓄積された大学での基盤技術研究の成果が現実の医薬品（人工酸素運搬体）として輸血代替治療やその他様々な治療分野で使用される日がすぐそこに迫ってきている．

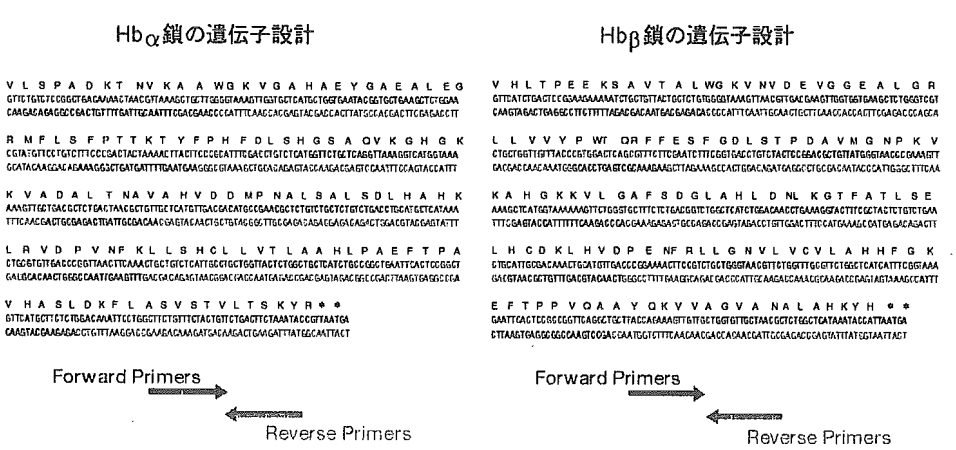


Fig. 12. Synthetic cDNA of hemoglobin α and β chain with codon sequence adapted to colon bacillus.

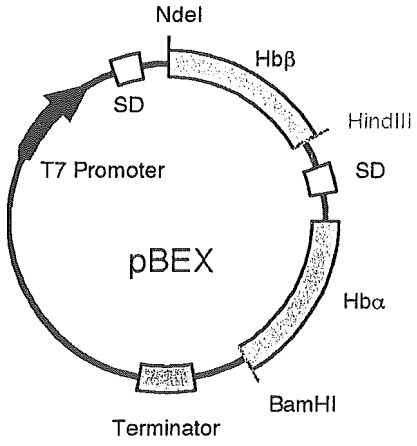


Fig. 13. Our original pBEX vector for co-expression of α and β chain in colon bacillus.

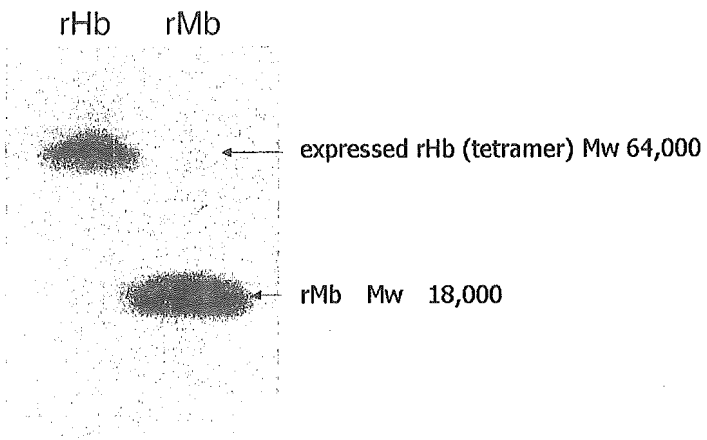


Fig. 14. Identification of expressed rHb (tetramer) by native-PAGE.

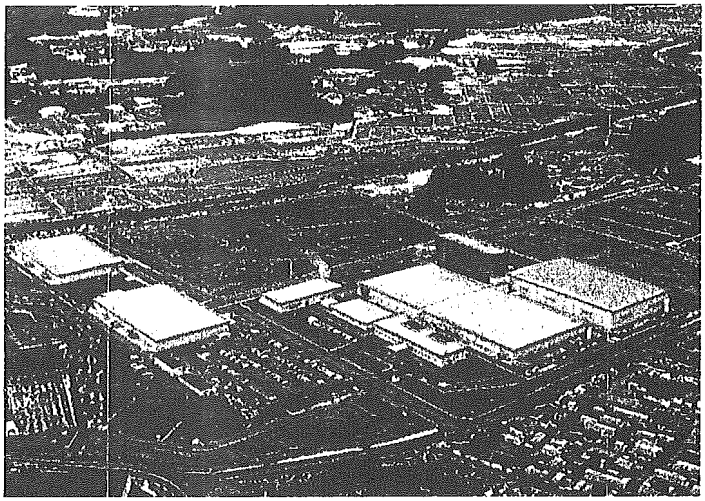


Fig. 16. External appearance of pharmaceutical factory in NIPRO
New facilities for the production of artificial oxygen carriers will be constructed in this factory.

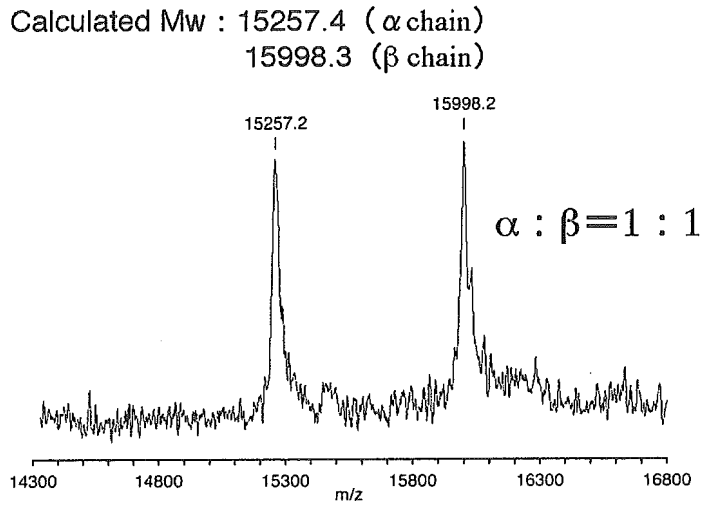


Fig. 15. MALDI-TOF-MS spectrum of expressed rHb.

参考文献

1. Tsuchida E. Is there a need for blood substitutes in the new millennium and what should we expect in the way of safety and efficacy?. Artif Cell Blood Substit Immobil Biotechnol 2000;28:v-xii.
2. Chang TMS. Blood Substitutes: Principles, Methods, Products, and Clinical Trials. Basel, Karger 1997.
3. Tsuchida E, Komatsu T, Matsukawa Y, Hamamatsu K, Wu J. Human serum albumin incorporating tetrakis(o-pivalamido)phenylporphinato-iron(Ⅱ) derivative as a totally synthetic O_2-carrying hemoprotein. Bioconjugate Chem 1999;10:797-802.
4. Komatsu T, Hamamatsu K, Tshchida E. Cross-linked human serum albumin dimers incorporating sixteen (tetraphenylporphinato)iron(Ⅱ) derivatives: synthesis, characterization, and O_2-binding property. Macromolecules 1999;32:8388-8391.
5. Komatsu T, Matsukawa Y, Tsuchida E. Kinetics of CO and O_2 binding to human serum albumin-heme hybrid. Bioconjugate Chem 2000;11:772-776.
6. Komatsu T, Matsukawa Y, Tsuchida E. Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(Ⅱ) derivatives. Bioconjugate Chem 2001;12:71-75.
7. Komatsu T, Okada T, Moritake M, Tsuchida E. O_2-binding properties of double-sided porphyrinatoiron(Ⅱ)s with polar substituents and their human serum albumin hybrids. Bull Chem Soc Jpn 2001;74:1695-1702.
8. 小林薫，リコンビナントアルブミンの開発．人工血液 2000;8:1-5.
9. 甲斐俊哉，土田英俊，小林紘一．アルブミンヘムの開発と酸素輸液への応用．人工血液2002;3:86.
10. Tsuchida E, Komatsu T, Matsukawa Y, Nakagawa A, Sakai H, Kobayashi K, Suematsu M. Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging. J Biomed Mater Res 2003;64A:257-261.
11. Komatsu T, Matsukawa Y, Tsuchida E. Effect of heme structure on O_2-binding properties of human serum albumin-heme hybrids: intramolecular histidine coordination provides a stable O_2-adduct complex. Bioconjugate Chem 2002;13:397-402.
12. Takeoka S, Sakai H, Terase K, Nishide H, Tsuchida E. Characteristics of Hb-vesicles and encapsulation procedure. Artif Cells Blood Substit Immobil Biotechnol 1994;22:861-866.
13. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E. Purification of concentrated hemoglobin using

organic solvent and heat treatment. Protein Expr Purif 1993;4:563-569.
14. Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E. Physical properties of hemoglobin vesicles as red cell substitutes. Biotechnol Prog 1996;12:119-125.
15. Naito Y, Fukutomi I, Masada Y, Sakai H, Takeoka S, Tsuchida E, Abe H, Hirayama J, Ikebuchi K, Ikeda H. Virus removal from hemoglobin solution using Planova membrane. J Artif Organs 2002;5:141-145.
16. Fukutomi I, Sakai H, Takeoka S, Tsuchida E, Sakai K. Carbonylation of oxyhemoglobin solution ($HbO_2 \rightarrow HbCO$) using a membrane oxygenator. J Artif Organs 2002;5:102-107.
17. Sou K, Endo T, Takeoka S, Tsuchida E. Poly(ethylene glycol)-modification of the phospholipids vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. Bioconjugate Chem 2000;11:372-379.
18. Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjugate Chem 2000;11:425-432.
19. Przybelski RJ, Daily EK, Kisicki JC, Mattia-Goldberg C, Bounds MJ, Colburn WA. Phase I study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution. Crit Care Med 1996;24:1993-2000.
20. Murray JA, Ledlow A, Launspach J, Evans D, Loveday M, Conklin JL. The effects of recombinant human hemoglobin on esophageal motor functions in humans. Gastroenterology 1995;109:1241-1248.

Reprint

人工血液　Vol.13, No.1, pp.29-33, 2005

ヒト赤血球由来ヘモグロビンによるヘモグロビン小胞体の開発と酸素輸液としての医療応用

高木　智史，大村　光代，太田　勝次，須賀　裕子，松浦　昭宏

日本血液代替物学会
The Society of Blood Substitutes, Japan