Measurements and Main Results. In the ischemic tissue, hemodilution led to an increase in microvascular blood flow to maximally 141%–166% of baseline in all groups (median; $P < 0.01$ vs. baseline, not significant between groups). Tissue oxygen tension was transiently raised to 121 ± 17% after the 30% blood exchange with Dx70 ($P < 0.05$), whereas it was increased after each step of hemodilution with HbV15-Dx70 and HbV30-Dx70, reaching 217 ± 67% ($P < 0.01$) and 164 ± 33% ($P < 0.01$ vs. baseline and other groups), respectively, after the 50% blood exchange. From these results it can be concluded that despite a decrease in total Hb concentration, the oxygenation in the ischemic, hypoxic tissue could be improved with increasing blood exchange with HbV solutions. Furthermore, better oxygenation was obtained with the left-shifted HbVs.

Safety of HbV (In Vitro and In Vivo Tests)

Rheological Property and Oxygen Releasing Behavior

The rheological property of an artificial oxygen carrier is important because the infusion amount should be significantly large and that may affect the blood viscosity and hemodynamics. It has been suggested that the higher viscosity and the resulting higher perfusion pressure would be beneficial to increase the shear stress on the vascular wall for vasorelaxation and to homogeneously transmit the pressure to microvascular networks and thus to supply blood to whole capillaries [108]. PEG-modified HbV suspended in 5% HSA solution was mixed with human blood and the viscosity was measured. The viscosity was similar to that of blood, and the mixtures at various mixing ratios showed a viscosity of 3–4 cP. RBC is the main component to determine blood viscosity and the results indicate no significant interaction between HbV and RBC [39]. To observe the flow pattern of the mixture of HbV and RBC, they were mixed in various volume ratios at [Hb] = 10 g/dl in isotonic saline containing 5% HSA, and the suspension was perfused at the centerline flow velocity of 1 mm/s through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm) exposed to a deoxygenated environment [109]. The mixtures of acellular Hb solution and RBC were also tested. Since HbV was homogeneously dispersed in the HSA solution, increasing the volume of the HbV suspension resulted in a thicker marginal RBC-free layer (Fig. 9).

In the same experimental model, measurement of the O_2 release from the narrow tube was performed using a scanning-grating spectrophotometer with a photon count detector, and the rate of O_2 release was determined based on the visible absorption spectrum in the Q band of Hb [109]. Irrespective of the mixing ratio, the rate of O_2 release from the HbV-RBC mixtures was

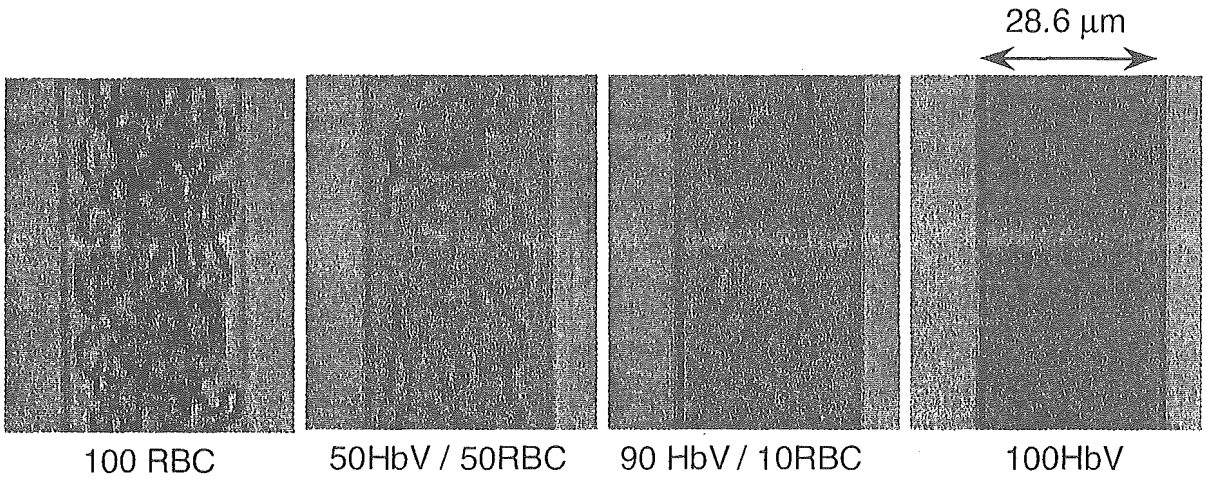

FIG. 9. Flow patterns of the mixture of HbV and RBC suspended in HSA in a narrow tube. HbV particles were homogeneously dispersed in a suspension medium. They tended to distribute in the marginal zone of the flow. The thickness of the RBC-free layer increased with the increasing amount of HbV. The RBC-free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

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

Effects on Hematological Functions

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

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

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

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

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

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

Hypertension and Vasoconstriction in Relation with NO and CO

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

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

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

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

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

Biodistribution and Metabolism of HbV, and Influence on Organ Function

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

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

The influence of HbV on RES, mainly liver and spleen, was studied with carbon clearance measurements and histopathological examination [133]. The HbV suspension was intravenously infused in male Wistar rats (200 g) at dose rates of 10 and 20 ml/kg, and the phagocytic activity was measured by monitoring the rate of carbon clearance at 8 hs, and at 1, 3, 7 and 14 days after infusion. The phagocytic activity transiently decreased one day after infusion by about 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the original level at 7 days, and then returned to the original level at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with hematoxylin/eosin, and anti-human Hb antibody staining showed that HbV was metabolized within 7 days. Hemosiderin was slightly confirmed with Berlin blue staining at 3

and 7 days in the liver and spleen, although they disappeared at 14 days, indicating that the heme metabolism, excretion, or recycling of the iron ion proceeded smoothly and siderosis was minimal. Electron microscopic examination of the spleen and liver tissues clearly demonstrated the vesicular structure of HbV with a diameter of about 1/40 of RBCs in capillaries, and in phagosomes as entrapped in the spleen macrophages and Kupffer cells one day after infusion. The vesicular structure could not be observed at 7 days. Even though infusion of HbV modified the phagocytic activity for two weeks, it does not seem to cause any irreversible damage to the phagocytic organs from the histological point of view.

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

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

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

Summary

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

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

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

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Chapter 44

Hemoglobin Vesicles as a Molecular Assembly: Characteristics of Preparation Process and Performances as Artificial Oxygen Carriers

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IMPORTANCE OF CELLULAR STRUCTURE

Physicochemical analysis has revealed that the cellular structure of RBCs may not be effective for the facilitated O_2 releasing and binding of Hb molecules in comparison with a homogeneous Hb solution (Vandegriff and Olson, 1984; Page *et al.*, 1998; Sakai *et al.*, 2003a); however, nature has selected this cellular structure during its evolution. Historically, Barcroft *et al.* insisted that the reasons for the Hb encapsulation in red blood cells were: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of hemoglobin from the blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes (Tsuchida *et al.*, 1995). Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs) many side effects of molecular Hb have become apparent, such as the dissociation of tetrameric Hb subunits into two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$) that may induce renal toxicity, and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow and tissue oxygenation at microcirculatory levels (Goda *et al.*, 1998; Sakai *et al.*, 2000a), neurological disturbances, and the malfunctioning of the esophageal motor function (Murray *et al.*, 1995). These side effects of molecular Hb would imply the importance of the cellular structure.

The pioneering work of the Hb encapsulation was performed by Chang (1991) using a polymer membrane. After Bangham and Horne (1964) had reported that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it was quite reasonable to use such vesicles for the Hb encapsulation. Djordjevici and Miller (1977) prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid etc. The Naval Research Laboratory showed the remarkable progress of LEH (Rudolph

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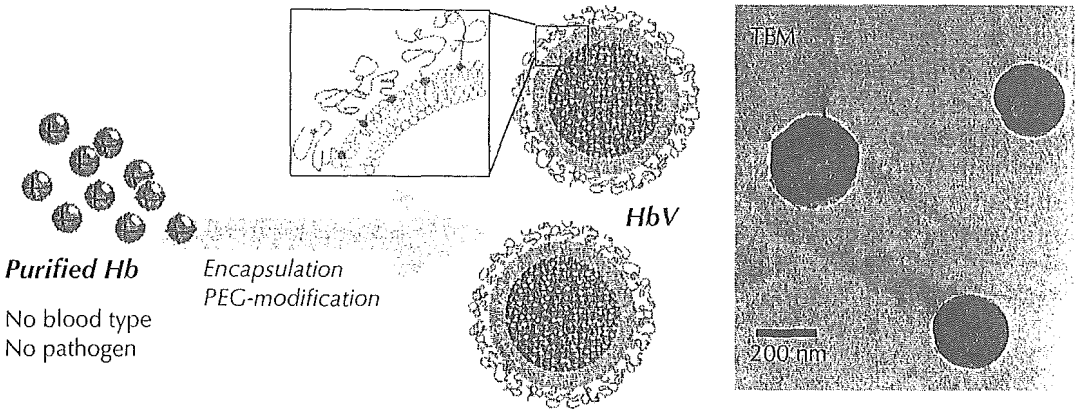

Figure 44.1 Hb vesicles (HbV, diameter *ca.* 250 nm) are prepared from ultra-pure Hb obtained from outdated RBC. One particle contains about 30 000 Hb molecules. The surface of one HbV is modified with about 6000 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles. See color plate 22.

et al., 1991). What we call Hb vesicles (HbV) with a high-efficiency production process and improved properties have been established by Tsuchida's group based on the nano-technologies of molecular assembly and precise analyses of the pharmacological and physiological aspects (Tsuchida, 1998; Figure 44.1). The *in vivo* studies of HbV have revealed O_2 transporting efficiency comparable to that of RBCs (Izumi *et al.*, 1996, 1997; Kobayashi *et al.*, 1997; Sakai *et al.*, 2004a; Yoshizu *et al.*, 2004), safety in terms of blood compatibility (Ito *et al.*, 2001; Wakamoto *et al.*, 2001), the importance of the particle size and the cellular structure of HbV (Goda *et al.*, 1998; Sakai *et al.*, 2000a), and prompt degradation in the reticuloendothelial system (Sakai *et al.*, 2001, 2004b, 2004c, 2004d), all of which make us confident about advancing to the further development of HbV. The joint collaborative partnership of academia (Waseda and Keio Universities), a biotechnology venture company (Oxygenix, Inc., Tokyo) and a corporation (Nipro Co., Osaka) is aiming for clinical trials of HbV within a few years.

In this chapter we scientifically summarize the characteristics of the preparation process of HbV based on the sciences of molecular assembly to induce their excellent performances. It should be emphasized that the components of HbV, lipids and Hb assemble to form a functional nanoparticle through secondary binding forces (hydrophobic interaction, Coulombic force, hydrogen bond, van der Waals force).

PREPARATION OF HEMOGLOBIN VESICLES

Virus inactivation and removal during hemoglobin purification

The primary advantage of artificial O_2 carriers should be no fear of infectious disease derived from human blood. In Japan, the donated blood is strictly inspected by the nucleic acid amplification test (NAT). However, it is necessary also to introduce procedures to inactivate and remove viruses during the process of Hb purification from outdated RBC in order to guarantee the utmost safety from infection, based on the unforgettable tragedy of HIV transmission due to the distribution of non-pasteurized plasma-derived products. In our purification process, virus inactivation was performed by pasteurization at 60°C for 10 hours – the same conditions for the pasteurization of human serum albumin (Sakai *et al.*, 1993; Fukutomi *et al.*, 2002). This process can be introduced by utilizing the stability of carbonylhemoglobin (HbCO). The thermograms of HbCO indicated a denaturation temperature of 78°C, which is much higher than that for oxyhemoglobin (64°C) (Sakai *et al.*, 2002a).

The virus inactivation efficiency was evaluated by the Hokkaido Red Cross Blood Center (Abe *et al.*, 2001; Huang *et al.*, 2002). The Hb solution spiked with vesicular stomatitis virus (VSV) was treated at 60°C for 1 hour under a CO atmosphere. VSV was inactivated at > 6.0 $\log_{10}$ without metHb

formation and denaturation. Some protein bands other than Hb disappeared on SDS-PAGE and IEF after the heat treatment. During pasteurization, all the other concomitant proteins are denatured and precipitated. As a result, we obtain an ultra-pure Hb solution. This high purity is essential for preventing membrane plugging during the subsequent ultrafiltration process to remove virus. The FDA requires two orthogonal steps of not only virus inactivation but also virus removal.

We tested the ultrafiltration of the HbCO solution to remove viruses with PLANOVA™-35N and -15N (P35N, P15N, Bemberg Microporous Membrane: BMM; Asahi Kasei Co.) (Naito *et al.*, 2002). The virus removal mechanism is by size exclusion through the capillary pores, and a depth filtration. The unit membrane, which has a network structure of capillaries and voids, is accumulated to form 150 layers. P35N and P15N have mean pore sizes of 35 nm and 15 nm, respectively. P35N is suitable for removing envelope-type viruses of which the size ranges from 40 nm to 100 nm, such as HIV, HCV etc., and P15N can be used to remove the non-envelope-type viruses with size of less than 40 nm, such as parvoviruses. The permeation flux and the permeated ratio of the HbCO solution ([Hb] = 5.6 g/dl) through P35N at 13°C were 36 (l/m^2 per hour) and almost 100 (per cent), respectively. Those through P15N were 15 (l/m^2 per hour) and 95 (per cent), respectively. Under the same conditions, a high removal efficiency of a bacteriophage, $\phi \times 174$, (>7.7 log) was confirmed. These results indicate that P15N is effective for the process of virus removal from Hb solution. We also confirmed the effectiveness of other virus removal ultrafiltration systems such as Viresolve (Millipore Co.).

The obtained purified HbCO solution can be very effectively concentrated to above 40 g/dl using an ultrafiltration process. After regulation of the electrolyte concentrations, this is supplied for the encapsulation procedure. The ligand of the resulting HbV, CO, is converted to O_2 by illuminating the liquid membrane of the HbV suspension with a visible light under flowing O_2 (Chung *et al.*, 1995).

Other groups have selected the way to preserve the well-organized but relatively unstable enzymatic systems originally present in RBCs, aiming at the prolonged stability of the ferrous state of Hb (Ogata *et al.*, 1997). The enzymatic system can partly be preserved with the compensation of insufficient virus removal or inactivation, but this cannot guarantee the utmost safety of the resulting HBOCs. One advantage of HbV is that any reagent can be co-encapsulated in the vesicles. It has been confirmed that co-encapsulation of the appropriate amount of a reductant (such as glutathione or homocysteine) and active oxygen scavengers (such as catalase) effectively retards the metHb formation (Takeoka *et al.*, 1997, 2002; Sakai *et al.*, 2000a; 2004d; Teramura *et al.*, 2003). However, our recent idea is that the metHb formation may not be a serious problem in the emergency situation because HbV will be infused to bridge to the blood transfusion in a clinical setting.

Encapsulation of concentrated Hb in HbV

The performance of Hb vesicles depends on the weight ratio of Hb to lipid ([Hb]/[lipids]), that is, the ability to carry more Hb with fewer vehicles made of lipids. This value is improved by lowering the number of bilayer membranes (lamellarity) of the vesicle and raising the concentration of Hb in the interior of the vesicle. We studied the optimal conditions for the Hb encapsulation using the extrusion method, and considered the behaviors of the Hb and lipid assemblies as a kind of polymer electrolyte (Takeoka *et al.*, 1993, 1994a, 1994b, 1996).

The maximum ([Hb]/[lipids]) ratio can be obtained at ~pH 7, which relates to the isoelectric point (pI) of Hb. The Hb molecule is negatively charged when the 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 enhances the Hb denaturation by too much interaction with the lipid bilayer membrane and metHb formation at a lower pH. Therefore, the physiological pH, 7.0–7.4, is 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 increasing the microviscosity (decreased lipid mobility). Multilamellar vesicles are converted to smaller vesicles with a smaller lamellarity during the extrusion procedure. When the membrane fluidity is high, deformation of the vesicles during extrusion occurs more easily even for multilamellar vesicles, resulting in larger lamellarity in the final vesicles. Therefore, the use of lipids with a higher phase transition temperature is preferred. However, these lipids make extrusion more difficult, because a higher shear rate (high

extrusion pressure) is required. Based on this reasoning, mixed lipids contain dipalmitoylphosphatidylcholine (DPPC) as the main component.

Based on the precise analysis of the characterization of the physicochemical properties of the components, the encapsulation efficiency of the Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method (Sakai *et al.*, 1996; Sou *et al.*, 2003a). Mixed lipids (DPPC, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[mono-methoxy poly(ethylene glycol)$_{5000}$]) at a molar ratio of 5:5:1:0.033 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 ~500 nm and with a relatively narrow size distribution by freeze-thawing at a lipid concentration of 2 g/dl and a 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 size and size 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 ~30 times faster than that of the simple hydrated vesicles. During the extrusion process, the Hb solution was effectively encapsulated into the reconstructed vesicles with a diameter of 250 ± 20 nm, and the Hb/lipid ratio reached 1.7–1.8. This improvement of the Hb encapsulation procedure is a breakthrough for the scalability for commercialization.

REGULATION OF OXYGEN AFFINITY

The O_2 affinity of purified Hb (expressed as *P*50, the O_2 tension at which Hb is half-saturated with O_2) is about 14 mmHg, and Hb strongly binds O_2 and does not release O_2 at 40 mmHg (the partial pressure of mixed venous blood). Historically, it has been considered that the O_2 affinity should be regulated to a level similar to that of RBC, namely about 25–30 mmHg, 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 can be evaluated by the arteriovenous difference in the levels of O_2 saturation in accordance with an O_2 equilibrium curve. It has been supposed that decreasing the O_2 affinity (increasing *P*50) will result in an increase in the O_2 unloading, which is supported by the result that RBC with a high *P*50 shows an enhanced O_2 release for improved exercise capacity in a mouse model (Shirasawa *et al.*, 2003).

If this theory is correct, the *P*50 of Hb in HbV should be equivalent to that of human red blood cells, i.e., 28 mmHg, or higher. Pyridoxal 5′-phosphate (PLP) is co-encapsulated in HbV as an allosteric effector to regulate *P*50 (Sakai *et al.*, 2000b). 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). The bound PLP retards the dissociation of the ionic linkage between the β-chains of Hb during conversion of deoxy to oxyHb in the same manner as does 2,3-DPG. Thus the O_2 affinity of Hb decreases in the presence of PLP. The *P*50 of HbV can be regulated to 5–150 mmHg by co-encapsulating the appropriate amount of PLP or inositol hexaphosphate as an allosteric effector (Wang *et al.*, 1992). Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was co-encapsulated, and *P*50 was regulated to 18 mmHg. When the molar ratio PLP/Hb was 3/1, *P*50 was regulated to 32 mmHg. The O_2 affinities of HbV can be regulated quite easily without changing the other physical parameters, whereas in the case of the other modified Hb solutions their chemical structures determine their O_2 affinities and thus regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not been yet completely decided; however, the easy regulation of the O_2 affinity may be useful in meeting the requirement for clinical indications such as oxygenation of ischemic tissues (Contaldo *et al.*, 2003).

STORAGE STABILITY

Since Hb autoxidizes to form metHb and loses its O_2-binding ability during storage as well as during blood circulation, the prevention of metHb formation is required. Some groups have reported a method of preserving the deoxygenated Hb in the liquid state (Kerwin *et al.*, 1999), using the well-known intrinsic characteristic of Hb that its oxidation rate in a solution is dependent on the O_2 partial pressure and deoxyHb is essentially not autoxidized at ambient temperature (Levy *et al.*, 1988). For the HbV, not only the inside Hb but also the cellular structure has to be physically