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Hemoglobin-Vesicles (HbV) as Artificial Oxygen Carriers

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

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

Introduction: Importance of Cellular Structure

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

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

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

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

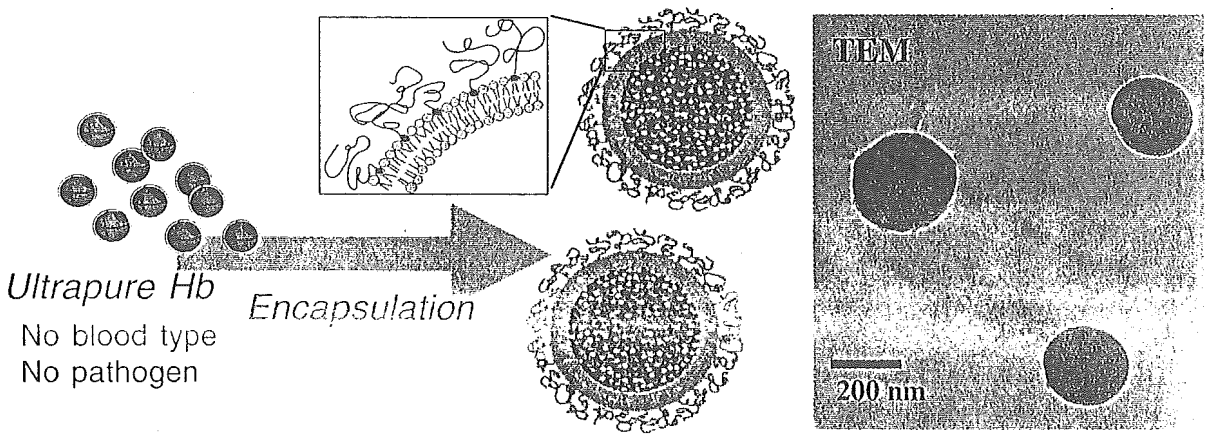

FIG. 1. Hb-vesicles (diameter, ca. 250 nm) are prepared from ultrapure Hb obtained from outdated RBC. The surface of the vesicles is modified with polyethyleneglycol that ensures the dispersion stability during storage and during circulation in the blood stream. Transmission electron micrograph (TEM)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

Preparation and Characteristics of HbV as a Molecular Assembly

Purification of Hb for the Utmost Safety

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

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

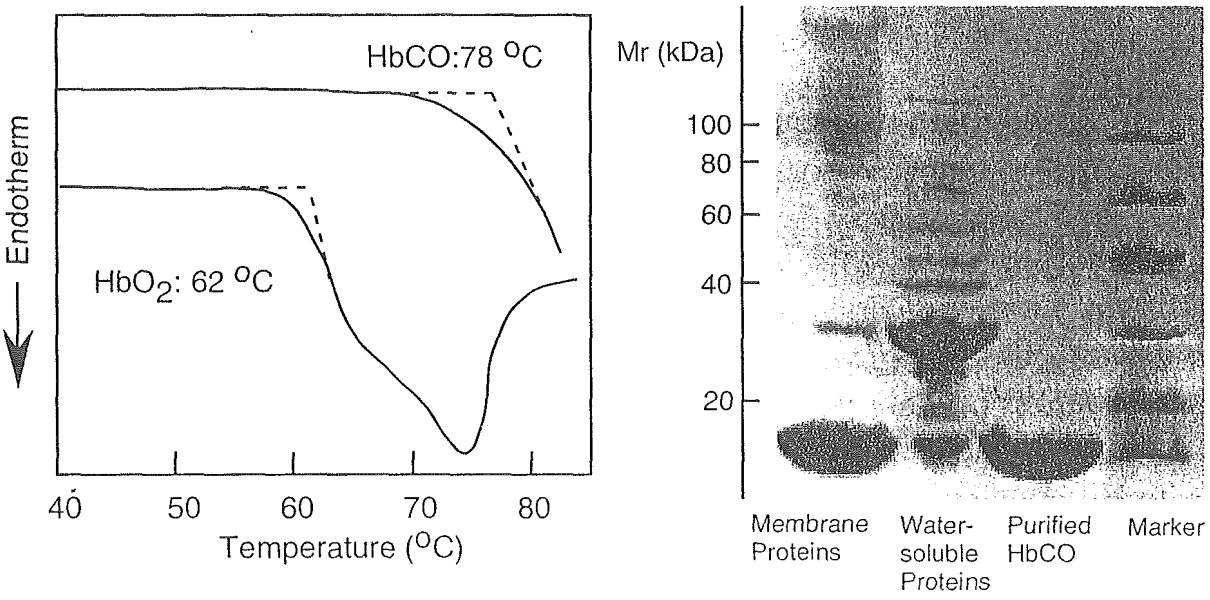

FIG. 2. **Left**, the calorimetric thermograms of HbCO solution showed the decomposition temperature at 78°C, indicating the thermal stability for pasteurization at 60°C. The amount of sample was 60 µl (6.0 g/dl). The heating speed was 1.0°C/min. **Right**, SDS-PAGE of HbCO after the heat treatment (60°C,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

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

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

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

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

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

Effective Hb Encapsulation

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

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

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

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

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

Regulation of Oxygen Affinity

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

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

however, the easy regulation of O_2 affinity may be useful to meet the requirement of the clinical indications such as oxygenation of ischemic tissues (see section "Improved Oxygenation in Ischemic Hamster Flap Tissue by Hemodilution with HbV").

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

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

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

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

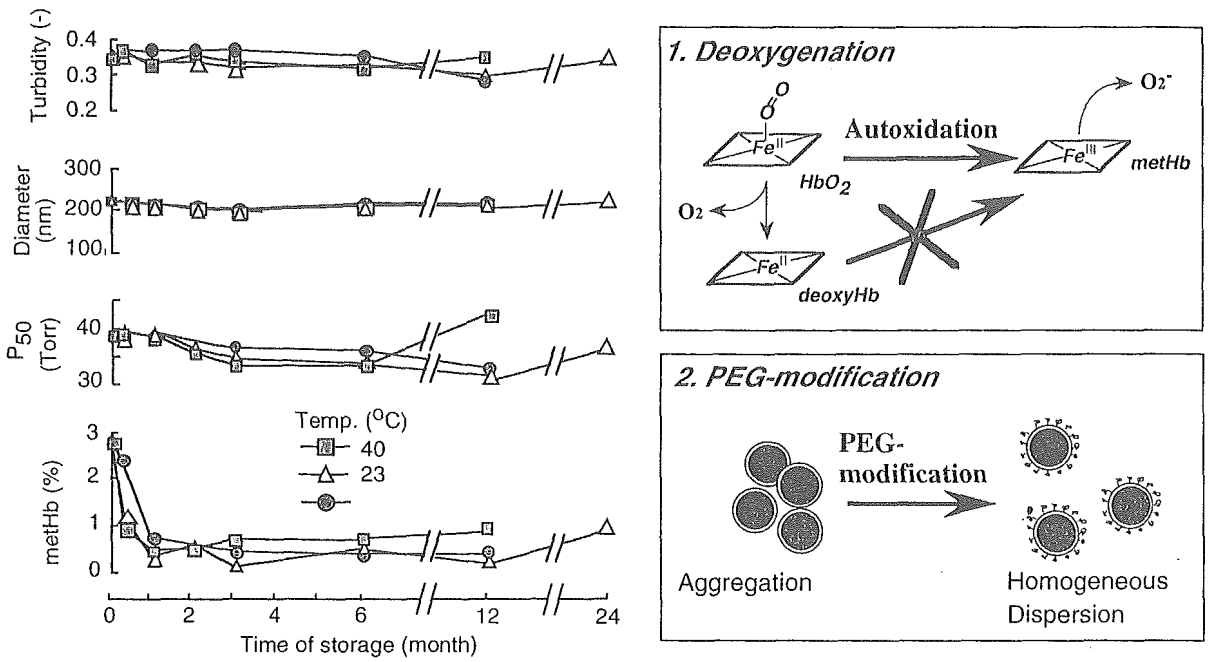

FIG. 3. Stability of HbV during storage for up to 24 months. Deoxygenation is important to prevent autoxidation to metHb, and PEG-modification suppresses aggregation and fusion of HbV to stabilize the dispersion condition. Both techniques are essential for the long-term storage of HbV. A reductant coencapsulated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

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

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

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the appli-

cation of PEG-modified vesicles in other fields. The long-term preservation of oxygen carriers overcomes the limitation of the blood transfusion system and will be of benefit to clinical medicine.

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

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

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

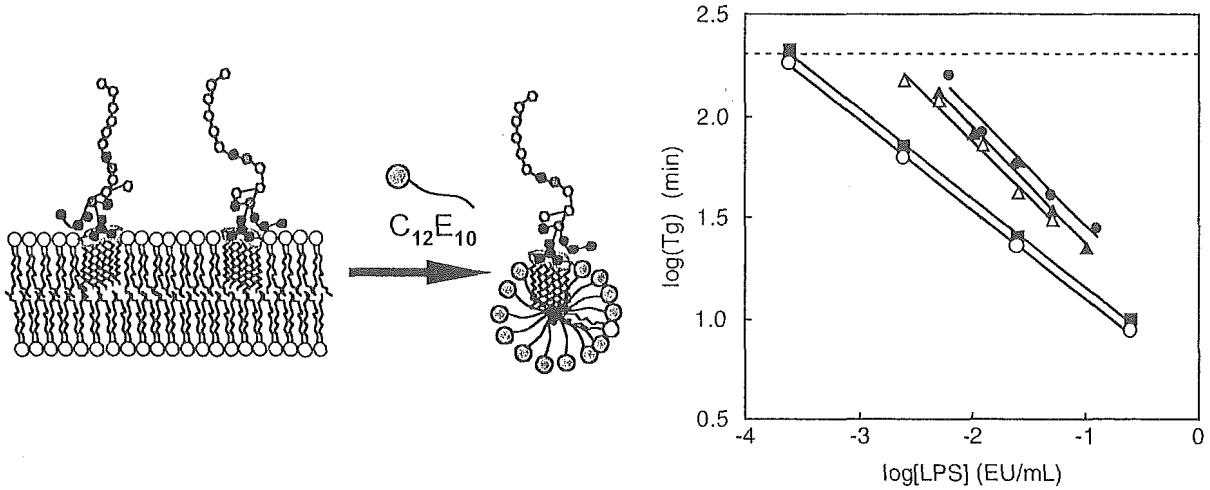

FIG. 4. (*Left*) Solubilization of LPS-contaminated vesicles with a detergent to form mixed micelles. (*Right*) Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations (vol%); 2.0 (●), 0.8 (▲), 0.4 (△), 0.1 (■), 0 (○). [LPS] is the final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

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

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

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

Interaction with Active Oxygen Species

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

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

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

Influence of HbV on Clinical Laboratory Tests and Countermeasures

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

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

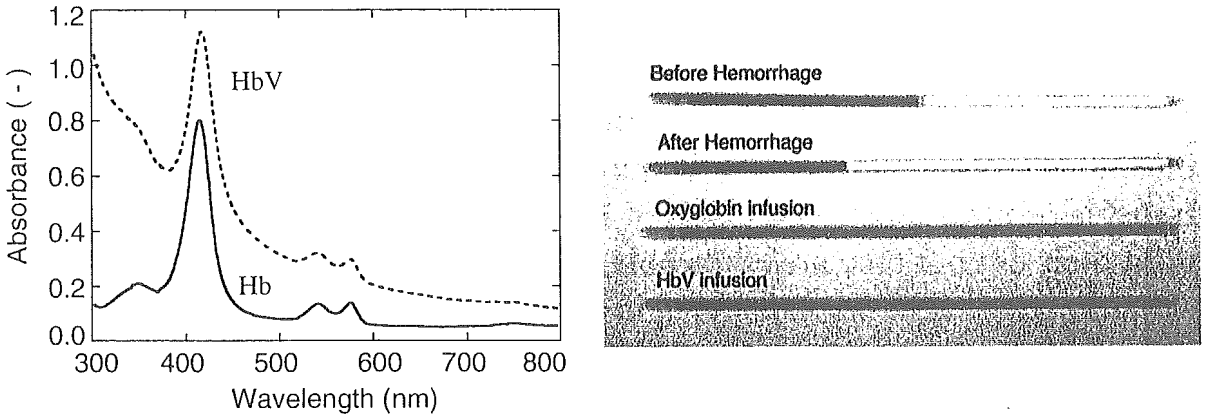

FIG. 5. **Left**, absorption spectrum of Hb and HbV at [Hb] = 0.01 g/dl in a 10 mm cuvette measured using a UV/vis spectrophotometer. The significant turbidity is confirmed especially at shorter wavelength for an HbV suspension. **Right**, glass capillaries for the hematocrit measurements during the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

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

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

Efficacy of HbV as Oxygen Carriers in Vivo

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

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

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

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

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

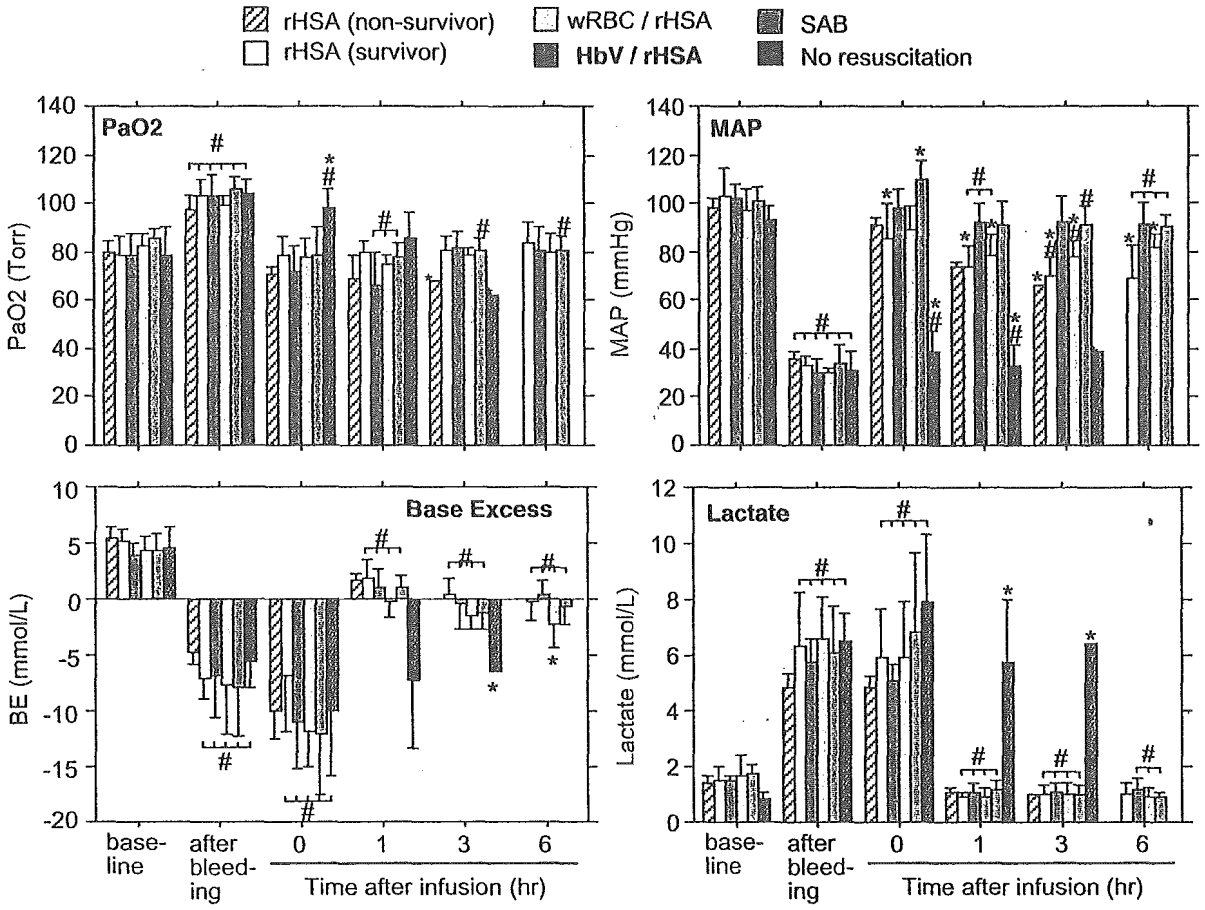

FIG. 6. Changes in mean arterial pressure (MAP) and blood gas parameters during hemorrhagic shock and resuscitation with infusion of HbV suspended in recombinant human serum albumin (HbV/rHSA), shed autologous blood (SAB), washed red blood cells suspended in recombinant human serum albumin (wRBC/rHSA),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

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

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

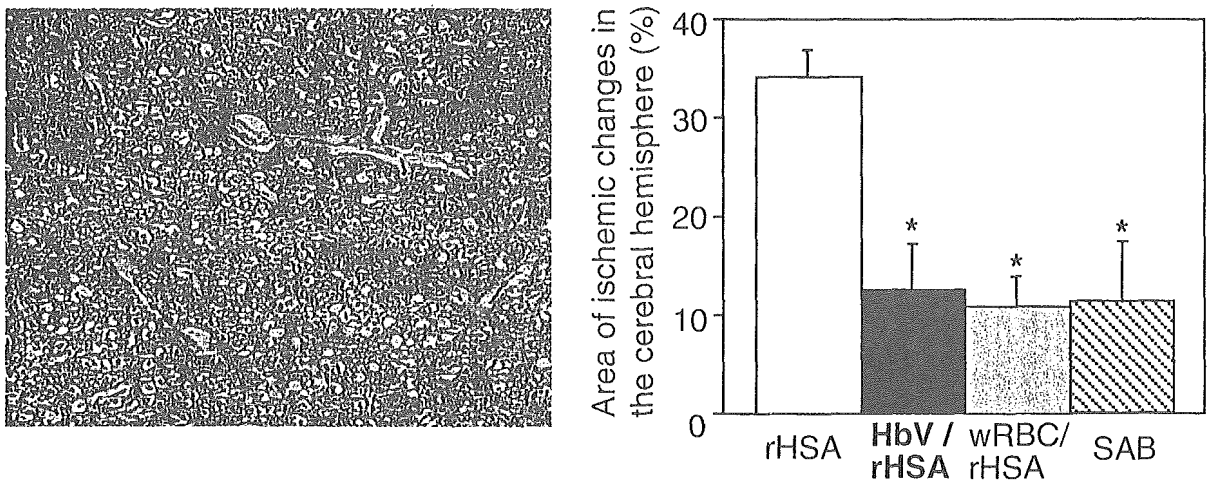

FIG. 7. Resuscitation from hemorrhagic shock with HbV suspended in rHSA in comparison with rHSA, wRBC/rHSA. The cerebral hemisphere on the right side of the rHSA group showed significant ischemic changes, a pyknotic change of the nuclei and an edematous change (34 ± 3% of the total section area) as shown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

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

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

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

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

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

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

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

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

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

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

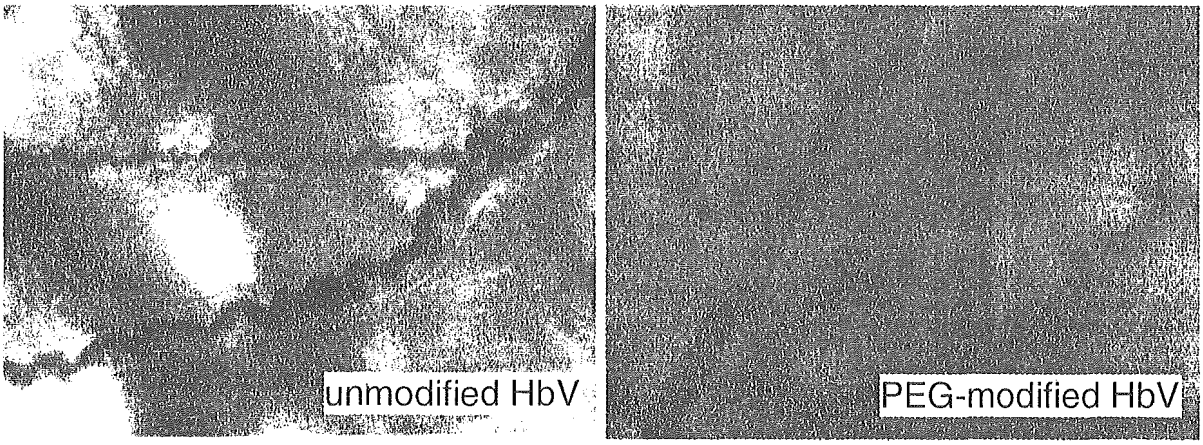

FIG. 8. Micrographs of microvasculature after 80% exchange transfusion with unmodified HbV or PEG-modified HbV suspended in 5% HSA. A high contrast was obtained by illumination with a wavelength range of around 420 nm, being absorbed at the Soret band of Hb molecules in HbV and RBC. **Left**, the aggregated vesicles block and chains in collecting venules were observed. **Right**, the microvasculature of postcapillaries is blackened owing to the homogeneous dispersion of PEG-modified HbV particles in the plasma phase. This is effective for better blood flow in the microcirculation

achieved with HSA alone. Subcutaneous microvascular studies showed that PEG-modified HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, and oxygen tension) relative to unmodified HbV/HSA. PEG-modified HbV was homogeneously dispersed in the plasma phase while the unmodified HbV showed aggregation in venules and capillaries. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. However, the microvascular perfusion with PEG-modified HbV/HSA was lower than the blood perfused one.

Improved Oxygenation in Ischemic Hamster Flap Tissue by Hemodilution with HbV [107]

Objective. The aim of this study was to test the influence of oxygen affinity of HbVs and level of blood exchange on the oxygenation in collateralized, ischemic, and hypoxic hamster flap tissue during normovolemic hemodilution.

Methods. Microhemodynamics were investigated with intravital microscopy. Tissue oxygen tension was measured with Clark-type microprobes. HbVs with a P_{50} of 15 Torr (HbV_{15}) and 30 Torr (HbV_{30}) were suspended in 6% Dextran 70 (Dx70). The Hb concentration of the solutions was 7.5 g/dl. A stepwise replacement of 15%, 30%, and 50% of total blood volume was performed, which resulted in a gradual decrease in total Hb concentration.