of medical care in the 21st century by complementing current blood products for transfusion, and creating a stable supply of safe products. Promotion of the research and development aimed at commercialization of artificial blood has been set as a basic policy of this country (a Resolution at the Health, Labour and Welfare Committee of the House of Representatives, July 24, 2002: a matter concerning promotion of the safety measures for pharmaceuticals and medical devices).

This article will focus upon artificial red blood cells, of which practical application is close to becoming a reality. The following are anticipated from its materialization: (1) feasible blood transfusion without regard to selection of blood group/type in case of an emergency, (2) no need of apprehension of HIV, hepatitis, and other viral or bacterial infections inclusive of unknown viruses, and (3) practicable massive reserves so that accidents in disasters such as earthquakes can be immediately coped with.

## Present Status of Artificial Red Blood Cell Development

Materials such as perfluorocarbon emulsion and modified hemoglobin have been assessed and clinically used as artificial red blood cells, but none has proven to be satisfactory from the viewpoints of function and safety. The hemoglobin vesicles (HbV) comprising a high-concentration hemoglobin encapsulated with phospholipid bilayer, hence analogous to erythrocytes, which are currently under investigation in Japan (Fig. 1), are safest and promising for practical use.[1,2] While effective utilization of hemoglobin from expired donated blood is being put forward at the present stage, use of recombinant hemoglobin will probably be utilized in the future.

Blood group substances, proteins other than hemoglobin, and viruses (if present at all) are completely removed by heating and filtration through the process of hemoglobin purification from erythrocytes. Re-encapsulation with

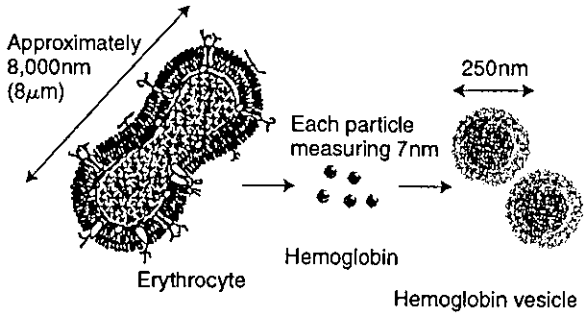


Fig. 1 Assembly of the hemoglobin vesicle with hemoglobin purified from erythrocytes

a stable lipid membrane ensures the preservability of the product in the liquid state for 2 years at room temperature (in contrast to the current erythrocyte preparations which may be stored for 3 weeks with refrigeration after blood drawing). When stored in the form of dry powder, the product can be preserved for a longer period. These are generally thought to be great advantages of the artificial blood product.

The research on the HbV is being pursued as a cooperative study (aided by a Grant-in-Aid for Health-Labour Science Research) mainly by the study group headed by Prof. Emeritus Eishun Tsuchida at the Advanced Research Institute for Science and Engineering, Waseda University, where the author is affiliated, and the study groups headed by Prof. Koichi Kobayashi and Prof. Makoto Suematsu at Keio University School of Medicine. In collaboration with a private enterprise, the project aims at finalization of the pharmaceutical formulation and an early initiation of its clinical trials.

## Evaluation of Function and Safety of Hemoglobin Vesicles (HbV)

The physical and chemical properties of the HbV are specified in Table 1. The colloidal osmotic pressure is practically zero because hemoglobin is encapsulated. It will be likened to a state where erythrocytes are dispersed in physiological saline. In the case where the colloidal osmotic pressure is to be adjusted,

Table 1 Specifications of the Hemoglobin Vesicle

| Item | Specification |
|---|---|
| Particle size (nm) | 240~280 |
| $P_{50}$ (torr) | 27~34 |
| Hb (g/dl) | 10.0±0.4 (8.6±0.4*) |
| Total lipid (g/dl) | 5.3 to 5.9 (4.6 to 5.4*) |
| Hb/total lipid (g/g) | 1.6~2.1 |
| PEG-lipid (mol%) | 0.3 |
| metHb (%) | <3 |
| HbCO (%) | <2 |
| Viscosity (cP at 230$s^{-1}$) | 2~3 (3~4*) |
| Crystalloid osmotic pressure (mOsm) | 300 |
| Colloidal osmotic pressure (torr) | 0 (20*) |
| pH (37°C) | 7.4 |
| Endotoxin (EU/ml) | <0.1 |
| Sterility test | None detected |

*After mixing with a 20% recombinant human serum albumin preparation
PEG: Polyethylene glycol

therefore, concomitant use of a substance such as human serum albumin (recombinant) is required. As the particle diameter is strictly adjusted to 250 nm, corresponding to about 1/30 that of the red blood cell, a function of which erythrocytes are devoid may be expected, e.g., passage through a site of infarction.

The affinity for oxygen is adjustable to a desired value by co-encapsulating an allosteric effector such as pyridoxal-5'-phosphate (PLP). The composition and contents of lipids are uniquely devised, and the problems inherent in the conventional vesicles have been resolved, including preservability in the liquid state for 2 years at room temperature,[3] avoidance of hemolysis in bloodstream, an appropriate in-blood retention time (i.e., estimated to be about 3 days in humans), and avoidance of platelet and complement activation.

The following are brief accounts of results of the studies conducted to assess the HbV heretofore obtained. The studies were performed mostly in rats and hamsters, and have sufficiently verified the basic safety and oxygen-carrying effect. A safety study in primates is in progress at present.

In rats with 90% of the total blood volume replaced with albumin solution alone to explore the oxygen-carrying effect, decreases in systemic blood pressure and renal cortical oxygen partial pressure became conspicuous after an approximately 70% exchange, resulting in death. When the exchange was carried out using a system consisting of HbV dispersed in albumin solution, both the systemic blood pressure and renal cortical oxygen partial pressure were maintained even after a 90% exchange.[4] In an 80% exchange transfusion study with HbV dispersions in albumin solution conducted in hamsters, tissue oxygen partial pressure in the subcutaneous microcirculation system, as measured non-invasively, was noted to have decreased to 60–70% of pre-exchange value, yet it was maintained at levels more than 5 times as high as those in controls receiving an exchange with albumin alone.[5] Furthermore, constriction of resistant blood vessels and elevation in blood pressure, which are the case with modified hemoglobin, were not observed at all. These are interpreted as implying that, as HbV is of the size to which the vascular wall is impermeable, HbV has little or no effect on nitrogen monoxide (nitric oxide) being an endothelial-derived relaxation factor (EDRF).[6]

The half-life of the HbV in circulating blood was determined to be approximately 35 hours in rats following administration of 25% $^{99m}$Tc-labeled HbV. It is considered that the administered HbV is captured by Kupffer cells in the liver and macrophages in the spleen, and undergo the same metabolic pathways as those of erythrocytes.

In rats injected with HbV in a dose of 20 ml/kg, exploration of the metabolic process in the reticuloendothelial system and blood biochemical tests disclosed a transient increase in

weights of the liver and spleen, and that HbV taken up by phagocytes disappeared almost completely in a week. There was no evidence of any particular abnormality in hepatic or renal function. Blood lipase level showed a significant elevation transiently, while there was no change in blood amylase level.[7] Serum lipid components, especially cholesterol, rose during the metabolic process, and returned to normal levels 7 days afterwards.

In a rat, 14-day, repeated-dose study (10 ml/kg/day) with an ensuing 14-day recovery phase observation, all animals survived ($n=14$) throughout the study and post-treatment observation periods. During these periods, the rats continued to exhibit uninterrupted weight gain with no appreciable change in blood biochemical parameters except for transient increases in lipids and lipase. These latter parameters returned to normal levels in 14 days.[8]

Removal of endogenous carbon monoxide, overproduction of bilirubin, and depressed bile secretory function in the liver occurred with hemoglobin, whereas with HbV, no such effects were observed in the said metabolic organ.[9,10]

Hemoglobin becomes incapable of binding oxygen when the heme iron is oxidized from bivalent to trivalent on autoxidation or reaction with active oxygen species. The metHb liberates $Fe^{3+}$ ion which induces Fenton reactions, thereby catalyzing generation of hydroxy-radicals. In the case of HbV, on the other hand, it has been demonstrated *in vitro* that reactions with which active oxygen species are associated have no influence external to HbV inasmuch as the hemoglobin is encapsulated with lipid bilayer.[11] Further investigation is needed to elucidate the fate of the iron derived from the HbV metabolized in the reticuloendothelial system.

## Summary and Conclusion

The present objective of artificial red blood cells consists in a complement to transfusion therapy for emergency lifesaving. Upon fulfilling the purpose, artificial red blood cells are relatively rapidly metabolized in metabolizing organs to be replaced with autologous erythrocytes. Moreover, the albumin-heme complex comprising recombinant albumin and a conjugated heme derivative is an oxygen carrier possessing a colloidal osmotic pressure. Its application to new oxygen therapy by taking advantage of its smaller particle diameter than HbV is also anticipated.[2] In addition, cutting-edge research on artificial platelets is also under way by the study group headed by Prof. Yasuo Ikeda at Keio University School of Medicine, with *in vivo* studies already in progress.[12]

Thus, research on artificial red blood cells and artificial platelets in Japan are progressing chiefly with the Grants-in-Aid for Health-Labour Science Research. These efforts may not only contribute to future medical care in Japan, but also lead to a considerable international contribution for many countries where safe blood supplies are falling short. For now, the long-term development is expected to be promoted in the private sector with a view to benefiting all humanity, though profitability will have to be secured.

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**(Revised-1)**

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

Hiromi Sakai[1], Keitaro Sou[1], Shinji Takeoka[1], Koichi Kobayashi[2], Eishun Tsuchida[1]*

[1]*Advanced Research Institute for Science and Engineering, Waseda University Tokyo 169-8555, Japan.*

[2]*Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan*

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* To whom correspondence should be addressed.
Eishun Tsuchida, Professor
Advanced Research Institute for Science and Engineering
Waseda University, Tokyo 169-8555
Phone, +81-3-5286-3120; Fax, +81-3-3205-4740; e-mail, eishun@waseda.jp

## 1. Introduction: Importance of cellular structure

Physicochemical analyses have 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 (Page et al., 1998; Sakai et al., 2003a; Vandegriff & Olson, 1984), 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., 2000c), 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) reported that phospholipids assembled 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 Laboratories showed the remarkable progress of LEH (Rudolph 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 analysis of the pharmacological and physiological aspects (Tsuchida et al., 1998) **(Figure 1).** The *in vivo* studies of HbV have revealed the sufficient $O_2$ transporting efficiency comparable to RBCs (Yoshizu et al., 2004; Sakai et al., 2004a; Izumi et al., 1996, 1997; Kobayashi et al., 1997), the safety in terms of blood compatibility (Wakamoto et al., 2001; Ito et al., 2001), the importance of the particle size and the cellular structure of HbV (Goda et al., 1998; Sakai et al., 2000c), and prompt degradation in the reticuloendothelial system (Sakai et al., 2001, 2004b,d) all of which make us confident about advancing to the further development of HbV. The joint collaboration partnership of academia (Waseda and

Keio Universities), a biotech venture company (Oxygenix, Inc., Tokyo) and a corporation (Nipro Co., Osaka) are aimed at the clinical trials of HbV within a few years.

In this chapter we scientifically summarized 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 Hbs assemble to form a functional nano-particle through secondary binding forces (hydrophobic interaction, Coulombic force, hydrogen bond, van der Waals force).

## 2. Virus inactivation and removal during Hb purification for the utmost safety

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 to additionally introduce procedures to inactivate and remove viruses in the process of Hb purification from outdated RBC in order to guarantee the utmost safety from infection, based on our unforgettable tragedy of the 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 hrs, the same condition 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 carbonylhemolgobin (HbCO). The thermograms of HbCO indicated a denaturation temperature at 78 °C, which is much higher than that for oxyhemoglobin (64 °C) (Sakai et al., 2002b).

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 hr 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 the pasteurization, all the other concomitant proteins are denatured and precipitated. As a result, we obtain an ultrapure Hb solution. This high purity is essential for preventing membrane plugging during the subsequent ultrafiltration process to remove virus. 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$^{TM}$-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 nonenvelope-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$^2$/hr) and almost 100 (%), respectively. Those through P15N were 15 (L/m$^2$/hr) and 95 (%), respectively. Under the same conditions, a high removal efficiency of a bacteriophage, $\phi$ x174, (>7.7log) 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 coencapsulated in the vesicles. It has been confirmed that coencapsulation 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; Teramura et al., 2003; Sakai et al., 2000c; 2004e). 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 at a clinical setting.

**3. Encapsulation of conc. Hb in phospholipid vesicles as a molecular assembly.**

The performance of Hb-vesicles depends on the weight ratio of Hb to lipid ([Hb]/[Lipids]), that is, 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,b, 1996).

The maximum [Hb]/[Lipids] ratio can be obtained at ca. pH 7, that would relate 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 should enhance 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, 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 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 would make extrusion more difficult, because a higher shear rate (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 the Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method (Sakai et al., 1996; Sou et al., 2003). Mixed lipids (DPPC, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol)$_{5,000}$] 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 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 ca. 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 [Hb]/[lipid] ratio reached to 1.7 – 1.8. This improvement of the Hb encapsulation procedure is a breakthrough for the scalability for commercialization.

**4. Regulation of oxygen affinity**

The $O_2$ affinity of purified Hb (expressed as $P_{50}$, $O_2$ tension at which Hb is half-saturated with $O_2$) is about 14 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 should be regulated similar 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 the levels of $O_2$ saturation in accordance to 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 that 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 mice 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 Torr, or higher. Pyridoxal 5'-phosphate (PLP) is coencapsulated in HbV as an allosteric effector to regulate $P_{50}$ (Sakai et al., 2000a). 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 Torr by coencapsulating 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

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 the 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 been yet completely decided, however, the easy regulation of the $O_2$ affinity may be useful to meet the requirement of the clinical indications such as oxygenation of ischemic tissues (Contaldo et al., 2003).

## 5. Stabilized Hb-vesicles for long term storage

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 to preserve the deoxygenated Hbs in the liquid state (Kerwin et al., 1999), using the well-known intrinsic characteristic of Hb that the Hb 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 stabilized in order to prevent intervesicular aggregation, fusion, and leakage of the encapsulated Hb.

The 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 (Klibanov et al., 1990). 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 (Yoshioka, 1991). The PEG-modified HbV has shown an improved blood circulation and tissue oxygenation due to the absence of HbV aggregate formation and viscosity elevation (Sakai et al., 1997, 1998) and prolonged circulation persistence in vivo (Sou et al., 2003). However, little attention has been paid to the ability of the PEG modification for the long-term preservation of vesicles or liposomes in the liquid state. We studied the possibility of the long-term preservation of Hb-vesicles by the combination of two technologies, e.g., surface modification of HbV with PEG chains and deoxygenation during the storage for 2 years (Sakai et al., 2000b). The samples stored at 4 and 23 °C showed a stable dispersion state for 2 years, though the sample stored at 40 °C underwent precipitation and decomposition of the vesicular components, a decrease in pH, and 4% leakage of the total Hb after 1 year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent 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 that consumed the residual $O_2$ (thiol groups in homocysteines reacted with oxygen to generate disulfide and active oxygen species) and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the $O_2$ partial pressure, and no increase in the metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. These results clearly indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years.

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the application of PEG-modified vesicles in other fields. The long-term preservation of $O_2$ carriers overcomes the limitation of the blood transfusion system and will contribute to benefiting clinical medicine.

**6: Sterility of Hb-vesicles: 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 impurities and viral and bacterial contamination. It is strictly required to 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. The U.S. FDA has established a guideline for the human maximal endotoxin dose permissible for parenteral products (5 EU/kg) that may include HBOCs. 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 (Levin & Bang, 1964). Since the volume of $O_2$ carriers to be infused for shock resuscitation or acute hemodilution is estimated to be less than 20 mL/kg, the specific endotoxin limits per ml should be 0.25 EU/mL (= 5/20), similar to that for water for injection (0.25 EU/mL).

Bacterial LPS is a gigantic amphiphilic macromolecule, therefore, it hydrophobically interacts with protein and biomembranes. Hb strongly interacts with LPS showing synergistic toxicity. The constituent of endotoxin that causes LAL gelation is a glycophospholipid-designated lipid-A. 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 (Richardson et al., 1983). 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 (Cliff et al., 1995; Harmon et al., 1997).

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 a Toxinometer® (Sakai et al., 2004c). 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 HbV production system. 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 0.2 EU/mL at [Hb] = 10 g/dL. Moreover, the utilization of the histidine-immobilized agarose gel (Pyrosep®) 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. 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.

## 7. Performances of Hb-vesicles as $O_2$ carriers *in vivo.*

The advantages of the HbV and other 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 the HBOCs in the blood stream (2 - 3 days) limit their use but they are applicable for a shorter period of use, such as (i) a resuscitative fluid for hemorrhagic shock during an emergency situation for a temporary time or bridging until the packed RBCs are available, (ii) a fluid for preoperative hemodilution or perioperative $O_2$ supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion, (iii) a priming solution for the circuit of an extacorporeal membrane oxygenator (ECMO)

(Yamazaki et al., 2004), and (iv) other potential indications, i.e., 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 and not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires an addition of a plasma expander for a large substitution of blood to maintain the blood volume. The candidates of plasma expanders are HSA, hydroxyethyl starch, dextran, or gelatin depending on the clinical setting, cost, countries and clinicians. Recombinant human serum albumin (rHSA) will be the alternative. The absence of any infectious disease from humans is the greatest advantage of rHSA that will be soon approved for clinical use in Japan. Moreover, there should be no immunological and hematological abnormalities that are often seen when using dextran and hydroxyethyl starch. Aimed at the application of HbV suspended in a plasma expander for the above indications, HbV was tested for resuscitation from hemorrhagic shock (Yoshizu et al., 2004; Sakai et al., 2002a, 2004a) and extreme hemodilution (Izumi et al., 1997; Kobayashi et al., 1997; Sakai et al., 1997, 1998, 1999) in collaboration with Waseda- Keio and Prof. Intaglietta at UCSD. Moreover, HbV with a high $O_2$ affinity (low $P_{50}$) suspended in HES or dextran was tested for oxygenation of an ischemic skin flap by Dr. Erni et al. at the Inselspital University Hospital, Berne (Erni et al.2003; Contaldo et al., 2003) and the results imply the further application of HbV for other ischemic diseases such as myocardial and brain infarction and stroke.

## CONCLUSION

Based on the above establishment of the HbV production system and the potential clinical applications of HbV, significant efforts have been made in the joint collaboration partnership of Waseda-Keio-Oxygenix-Nipro to produce HbV with a facility of GMP standard, and to start preclinical and finally clinical trials. Since the combination of recombinant Hb (rHb)-vesicles suspended in recombinant albumin (rHSA) would be the most ideal "artificial red blood cells", this project has recently initiated for the next generation HbV (Kai et al., 2004).

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