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Developmental Trend of Artificial Blood (Artificial Red Blood Cells)

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Abstract: Regarding research on artificial blood, the "Field of Artificial Blood Development" was inaugurated in 1997, supported by the Ministry of Health and Welfare Grant-in-Aid for Health Science Research, for intensive research activities in the three sub-fields, i.e., artificial red blood cells, artificial platelets, and artificial antibodies. Developed by molecular assembling technology, artificial red blood cells, in the form of hemoglobin vesicles comprising hemoglobin encapsulated with a phospholipid bilayer as a highly efficient oxygen carrier, are now under investigation in laboratory animals to verify their function and safety. These vesicles are characterized by a particle size about 1/30 that of erythrocytes, preservability in a liquid state for 2 years at room temperature, and a sufficient retention time in circulating blood without evoking activation of platelet or complements. The hemoglobin vesicles have proven both to possess a high oxygen-carrying capacity in massive exchange transfusion studies in rodents, and to be remarkably safe, based on blood biochemical tests and pathologic findings in load-dosing and repeated-dose studies. Their noticeable safety against active oxygen has also been demonstrated. A joint industry, government, and university research project on artificial red blood cells is in progress with the present objective of developing a complement to transfusion therapy for emergency lifesaving.

Key words: Artificial blood; Artificial red blood cells; Hemoglobin vesicles; Function and safety evaluation

Introduction

We humans and other animals are constantly left exposed to the ferocity of certain viruses, and blood services are substantially affected by those viral entities. In Japan, the "Field of Artificial Blood Development" was inaugurated in 1997 as a Health Science Research – Advanced Frontier Medical Research Project, whereby intensive research activities in the three sub-fields, i.e., artificial red blood cells, artificial platelets, and artificial antibodies, are being pursued. Artificial blood is expected to have a significant influence upon the progress

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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

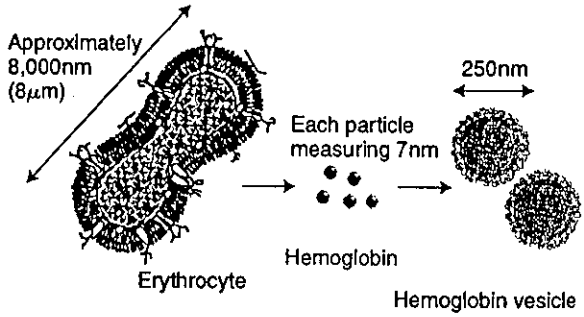

Fig. 1 Assembly of the hemoglobin vesicle with hemoglobin purified from erythrocytes

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,

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(Revised-1)

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

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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, ϕ 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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FIGURE LEGENDS

FIGURE 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 6,000 polymer chains of poly(ethyleneglycol) that ensure the dispersion stability of HbV during storage and during circulation in the blood stream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles.

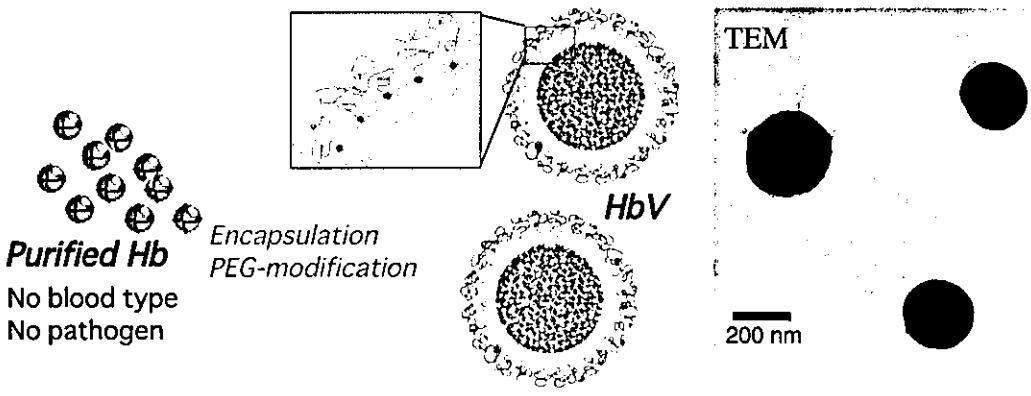

Figure 1
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Albumin-Heme: A Synthetic Heme-Based Oxygen Carrier

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Introduction

The risk of transmission of viral illness by transfused blood has become extremely low and the transfusion of donor blood is currently a routine procedure. However, this level of safety has been achieved at great cost, and hepatitis virus or unknown pathogens cannot be completely excluded by the NAT system. Furthermore, (i) the transfusion of donor blood requires cross-matching and compatibility tests to avoid a hemolytic reaction in the recipient, and (ii) the purified red blood cells (RBC) should be stored in the refrigerator at 4°C. These requirements limit the availability of blood in a disaster or emergency. Under this background, several types of hemoglobin (Hb)-based O_2-carriers have been studied as a RBC substitute or O_2 therapeutic reagent (Chang, 1997; Greenburg, 2004; Squires, 2002; Tsuchida, 1998, Winslow, 1999). Unfortunately, these materials do not fulfill all the requirements of blood replacement compositions. The first concern is the source of human Hb, which is limited by the availability of outdated human blood. Animal blood will raise the anxiety of the transmission of animal pathogens. The Hb products potentially carry risks due to the biological origin of the raw materials. The second problem of the Hb-based O_2-carriers (i.e. modified Hb) are the high colloid osmotic pressure (Keipert, 1988) and its vasoconstriction effect (Abassi, 1997; Moisan, 1998; Schultz,1993). About 50% of the products in advanced clinical trials still increase blood pressure and decrease cardiac output (Squires, 2002). The precise mechanism of this hypertension is controversial, but many researchers suspect that the Hb molecules penetrate the vascular endothelium and capture the endothelial-derived relaxing factor (EDRF), namely NO. Others believe that the excessive delivery of oxygen to arteriolar vascular walls induces autoregulatory vasoconstriction (Guyton, 1964; Rohlfs, 1998; Tsai, 1995; Winslow, 2000).

On the other hand, in our circulatory system, free hemin [iron(III) complex of protoporphyrin IX dissociated from metHb] is captured by hemopexin, which is a