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BLOOD SUBSTITUTES

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1. INTRODUCTION: PROBLEMS OF BLOOD TRANSFUSION SYSTEM AND EXPECTATIONS FOR THEIR SUBSTITUTES

Since the discovery of blood type antigen by Landsteinter in 1900, allogeneic blood transfusion has been developed as a routine clinical practice; it has contributed to human health and welfare. Infectious diseases such as hepatitis and HIV are now social problems, but a strict virus test by nucleic acid amplification test (NAT) is extremely effective to detect trace presences of a virus to minimize infection (though it is available in few developed countries). Even so, NAT poses problems such as detection limits during the window period and limited species of viruses for testing. Emergence of new viruses (such as West Nile virus, avian influenza, Ebola, dengue) and a new type of pathogen, prions, also threaten us. The preservation period of donated red blood cells (RBCs) is limited to 3–6 weeks. Platelets can be preserved for only a few days. Immunological responses (such as anaphylaxis and graft versus host disease) and contingencies of blood type incompatibility further limit the utility of blood products. To obviate or minimize homologous transfusion, the transfusion trigger has been reconsidered, and roughly reduced from 10 to 7–8 g/dl. Bloodless surgery and preoperational enhancement of erythropoiesis for storing autologous blood have become common. However, these epoch-making treatments are not always practical for all patients. Some developed countries with aging population are facing a decreasing number of young donors and an increasing number of aged recipients. On the other hand, in some developing countries, establishment of a safe blood donation system is difficult. Under such circumstances, research toward blood substitutes has gathered great attention and has been developed worldwide (1,2). In Japan, for example, the government has given strong support to a spectrum of projects for development of blood substitutes in the wake of two tragedies: the infection of hemophiliac patients, who had received non-pasteurized plasma products, by AIDS; and the Great Hanshin Earthquake disaster. In China, because of the lack of safe transfusion, blood substitute R&D is a national project.

Blood is separable into two fractions after centrifugation: plasma and cells. The roles of all plasma components are well characterized and their substitutes are already established (Table 1). Especially, recombinant human serum albumin (rHSA) will be commercialized soon in Japan. On the other hand, substitutes for cellular components—platelets and RBCs—are challenging (3). In this chapter, we specifically examine artificial oxygen carriers, which are substitutes for RBCs. The requisites for artificial oxygen carriers should be not only effectiveness for tissue oxygenation, but also the following:

1. No blood type antigen and no infection (no pathogens);
2. Stability for long-term storage (e.g., over 2 years) at room temperature for stockpiling for any emergency;

Table 1. Roles of Blood Components and Their Substitutes

Fraction		Components	Substitutes*
Plasma (55 vol%)	Plasma proteins	Albumin (maintenance of blood volume)	Plasma expanders (dextran, hydroxyethyl starch, modified gelatin, recombinant human serum albumin)
		Globulin (antibody)	Antibiotics artificial immunoglobulin
		Fibrinogen coagulation factors	Fibrin adhesive recombinant coagulation factors
	Electrolytes and other solutes	Na^+,K^+,Ca^{2+},Mg^{2+}, Cl^-,HCO_3^-,HPO_4^{2-}, etc.	Electrolyte infusion
		Vitamins, amino acids, glucose, lipids, etc.	Nutrient infusion (triglyceride, amino acids, saccharides)
	Cells (45 vol%)	Platelets	Artificial platelets
		White blood cells	None (antibiotics)
		Red blood cells	Artificial red cells (artificial O_2 carriers, O_2-infusions)

*including the materials under development.

3. Low toxicity and prompt metabolism even after massive infusion;
4. Rheological properties can be adjusted to resemble human blood; and
5. Reasonable production expense and cost performance.

Realization of an artificial oxygen carrier will bring innovative change in transfusion medicine.

2. CHEMICALLY MODIFIED HEMOGLOBIN AS AN OXYGEN CARRIER

Historically, the first attempt in the 1930s of Hb-based O_2 carrier was to simply use stroma-free Hb because Hb in RBCs binds and releases O_2. However, several problems became apparent: impurity of stroma-free Hb' dissociation into dimers that have a short circulation time; renal toxicity; high oncotic pressure; and high O_2 affinity. Since the 1970s, various approaches have been developed to overcome these problems, especially in the United States, because of military use for infusion to combat casualties (Fig. 1).

Materials included intra-molecular crosslinking using dibromosalicyl fumarate (4) or pyridoxal 5'-phosphate, polymerization using glutaraldehyde (2) or oxidized *o*-raffinose (5), and conjugation with water-soluble polymers such as polyethylene glycol (PEG), hydroxyethyl starch (HES), and dextran (2). The source of Hb is mostly human Hb purified from outdated donated human blood. An industrial-scale production of human Hb-based O_2 carriers requires a cooperation with blood banks, the Red Cross, and hospitals to establish a collection system of outdated donated RBCs. However, the amount is limited due to the limited number of blood donors and the fact that the hospitals are trying to use packed RBCs in a well-planned manner to reduce the discarded packed RBCs. Bovine or swine Hb can be a huge source obtainable from the cattle and hogs industries. The absence of heterologous immune reaction and prion protein has to be guaranteed. Recent biotechnology enables production of human Hb from transgenic swine blood. Moreover, a large-scale production of reombinant human Hb mutants as well as recombinant human serum albumin is possible from *E. coli* or yeast that should not include any pathogens from humans and mammals. For all the cases, Hbs should be strictly purified and free of pathogen via rigorous purification procedure such as ultrafiltration, pasteurization, irradiation, and solvent-detergent method (6), because the dose rate is considerably large.

In some cases of chemically modified Hbs, their structure (acellular structure) is so different from that of RBCs and caused side effects such as vasoconstriction (4). They are presumably attributable to the specific affinity of Hb to endogenous gas molecules, NO and CO, which are important messenger molecules for vasorelaxation. Although many companies have developed chemically modified Hb solutions as a transfusion alternative for elective surgery and trauma, some of them suspended clinical trials because of vasoactive properties. (See CLINICAL TRIALS in another chapter).The fact that myocardial lesion is caused by intramolecular crosslinked Hb (both chemically modified and recombinant Hb mutants) deters further development of these Hb-based O_2 carriers (7). Presently, glutaraldehyde-polymerized bovine or human Hbs and PEG-conjugated Hbs have progressed to the final stages of clinical trials (Table 2).

Oxyglobin™, a polymerized bovine Hb produced by Biopure Co. (Cambridge, MA), is now approved for veterinary use in the United States. This material can be stored in a liquid state at room temperature for years because Hb is stabilized by deoxygenation with addition of *N*-acetylcysteine. A more purified product, Hemopure™, with a narrower molecular weight distribution, is approved in South Africa for treating adult surgical patients who are acutely anemic and for eliminating, reducing, or delaying the need for allogeneic red blood cell transfusion in such patients (8). Because prion proteins are known to cause mad cow

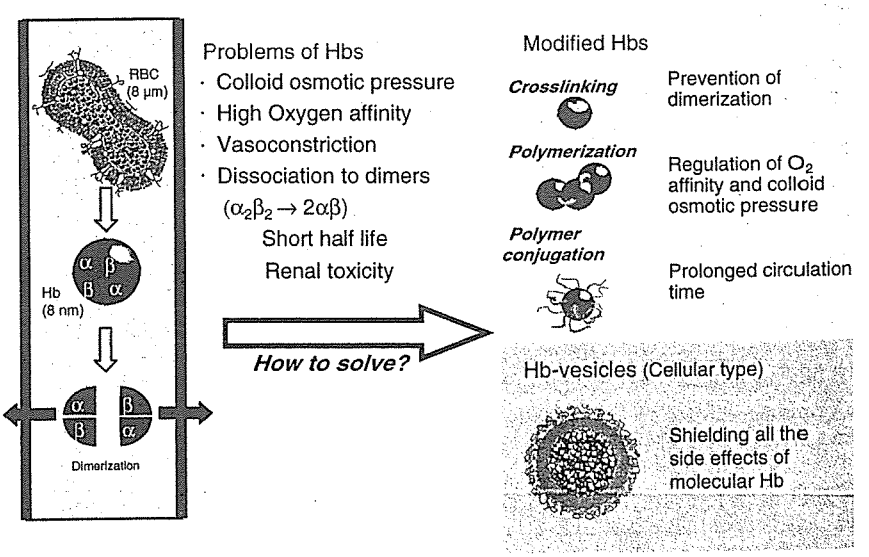

Figure 1. Chemically modified Hbs and encapsulated Hb to solve the side effects of molecular Hbs. (This figure is available in full color at http://www.mrw.interscience.wiley.com/ebe.)

Table 2. Artificial Oxygen Carriers Currently Developed for Clinical Application

Products (Group)	Composition	Indication	Present R&D Situation
PolyHeme (Northfield Labs. Inc.)	Glutaraldehyde-polymerized human Hb	Trauma	Phase III (US)
Hemopure (Biopure Corp.)	Glutaraldehyde-polymerized bovine Hb	Elective surgery	Phase III (US) approved in South Africa
PHP (Curacyte AG)	Pyridoxalated-human Hb, PEG-conjugated	Septic shock	Phase II (US)
Hemospan (Sangart Inc.)	PEG-modified human Hb	Elective surgery	Phase II (Sweden)
Hemolink (Hemosol Corp.)	o-raffinose polymerized human Hb	Elective surgery	Phase III, suspended
Oxygent (Alliance Pharm. Corp.)	Perfluorooctylbromide emulsion	Elective surgery	Phase II (US) suspended, R&D in China
Perftoran (Perftoran)	Perfluorodecalin, perfluoromethylcyclohexilpiperidine, proxanol	Hypovolemia, elective surgery	Approved in Russia
Hb-vesicles (Waseda-Keio-Oxygenix-Nipro)	Phospholipid vesicles encapsulating Hb,		Preclinical
Hemozyme (SynZyme Technol.)	Polynitroxyl human Hb		Preclinical
HemoTech (Hemobiotech Inc.)	Bovine Hb conjugated with o-ATP, o-adenosine and reduced glutathione.		Preclinical
PLA-PEG Hb nanocapsules (McGill Univ.)	Polylactide-PEG copolymer nanocapsules with Hb and enzymes		Preclinical
PolyHb-SOD-CAT (McGill Univ.)	Copolymerized Hb with SOD and catalase		Preclinical
Dex-BTC-Hb (Univ. Henri Poincare-Nancy)	Dextran conjugated Hb		Preclinical
HRC 101 (Hemosol Corp.)	Human Hb and hydroxyethyl starch conjugate		Preclinical
PEG-bHb (Beijing Kaizheng Biotech Corp)	PEG-modified bovine Hb		Preclinical
TRM-645 (Terumo Co.)	Liposome-encapsulated Hb		Preclinical
OxyVita (Oxyvita Inc.)	Zero-link polymer of bovine Hb		Preclinical
Albumin-hemes (Waseda-Nipro Corp.)	Synthetic heme-albumin composite		Preclinical
PHER O_2 (Sanaguine Corp)	Second generation Fluosol		Preclinical

disease (bovine spongiform encephalopathy: BSE), the key is to collect safer bovine blood exclusively from closed herds with well-documented health histories and controlled access.

PolyHeme™ is a glutaraldehyde polymerized human Hb developed by Northfield Laboratories Inc. (Evanston, IL). Even though most chemically modified Hbs show vasoconstriction, it is reported that PolyHeme™ does not induce vasoconstriction (9). Information on this material is more limited in the academic literature than for other products. PolyHeme™ is now undergoing phase III clinical trials designed to evaluate the safety and efficacy of Polyheme when used to treat patients in hemorrhagic shock following traumatic injuries. According to the company, this is the first trial of an Hb-based O_2 carrier in which treatment begins in the pre-hospital setting, such as in an ambulance during transport.

Ajinomoto Co. Inc. (Tokyo) first tested PEG-conjugation to pyridoxalated Hb (PHP™) (10); Curacyte AG (Chapel Hill, NC) is continuously developing that material as an NO scavenger. PHP™ has been demonstrated to reverse the vasodilatation caused by excess NO produced by inducible NO synthase. It resolves the hypotension associated with septic shock. It has completed Phase II clinical studies in distributive shock. Sangart Inc. (San Diego, CA) has developed PEG-modified human Hb (Hemospan™) with unique physicochemical properties: markedly higher O_2 affinity [P_{50} (partial pressure of O_2 at which Hb is half-saturated with O_2) = 6 Torr]; viscosity (2.5 cP); and colloid osmotic pressure (55 Torr). It is effective for microcirculation and targeted O_2 transport to tissues (11). This material is now in clinical phase II trials in Sweden. Even though criticism exists that the O_2 affinity is too high to release O_2 in peripheral tissues, a comparative study of PEG-modified albumin indicated that Hemospan reliably delivers O_2 to tissues with no vasoconstriction or hypertension (12). This reliability suggests that the appropriate physicochemical properties for artificial O_2 carriers should not necessarily be merely equal to those of blood or RBCs (13).

3. IMPORTANCE OF Hb-ENCAPSULATION IN RBC FOR ARTIFICIAL RBC DESIGN

Physicochemical analyses have revealed that the cellular structure of RBCs retards O_2 release and binding of the inside Hb in comparison with a homogeneous Hb solution (14,15). However, nature has selected this cellular structure during evolution. Historically, Barcroft et al. insisted that the reasons for Hb encapsulation in RBCs were (1) a decreased high viscosity of Hb and a high colloidal osmotic pressure, (2) prevention of the removal of Hb from the blood circulation, and (3) preservation of the chemical environment in the cells such as concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes (1). Moreover, during the long development of Hb-based O_2 carriers, numerous side effects of molecular Hb have become apparent, such as the dissociation of tetrameric Hb subunits into two dimers ($\alpha_2\beta_2 \rightarrow \alpha\beta$) that might induce renal toxicity, and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow, and tissue oxygenation at microcirculatory levels (16,17), neurological disturbances, and the malfunctioning of esophageal motor function (18), and heme-mediated oxidative reactions with various active oxygen species (19). These side effects of molecular Hbs imply the importance of the cellular structure or the larger particle dimension of Hb-based O_2 carriers.

Pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed by Chang in 1957 (2) who prepared microcapsules (5 μm) made of nylon, collodion, etc. Toyoda in 1965 (20) and the Kambara-Kimoto group (21) also covered Hb solutions with gelatin, gum Arabic, or silicone, etc. Nevertheless, it was extremely difficult to regulate the particle size that was appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior (22), it was reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. (23). In the US, Naval Research Laboratories showed remarkable progress of LEH (24). What we call Hb-vesicles (HbV) with a high-efficiency production process and their improved properties have been established by Tsuchida's group based on technologies of molecular assembly and precise analysis of pharmacological and physiological aspects (25,26) (Fig. 2).

Liposomes, as molecular assemblies, had been generally accepted as structurally unstable. Many researchers have sought to develop stabilization methods that use polymer chains (27). Polymerization of phospholipids that contain dienoyl groups was studied extensively. For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in phospholipids. This method produces enormously stable liposomes, like rubber balls, which are resistant to freeze-thawing, freeze-drying, and rehydration (1,28). However, the polymerized liposomes were so stable that they were not degraded easily in the macrophages even 30 days after injection. It was concluded that polymerized lipids would not be appropriate for intravenous injection. Selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-lipid) and their composition

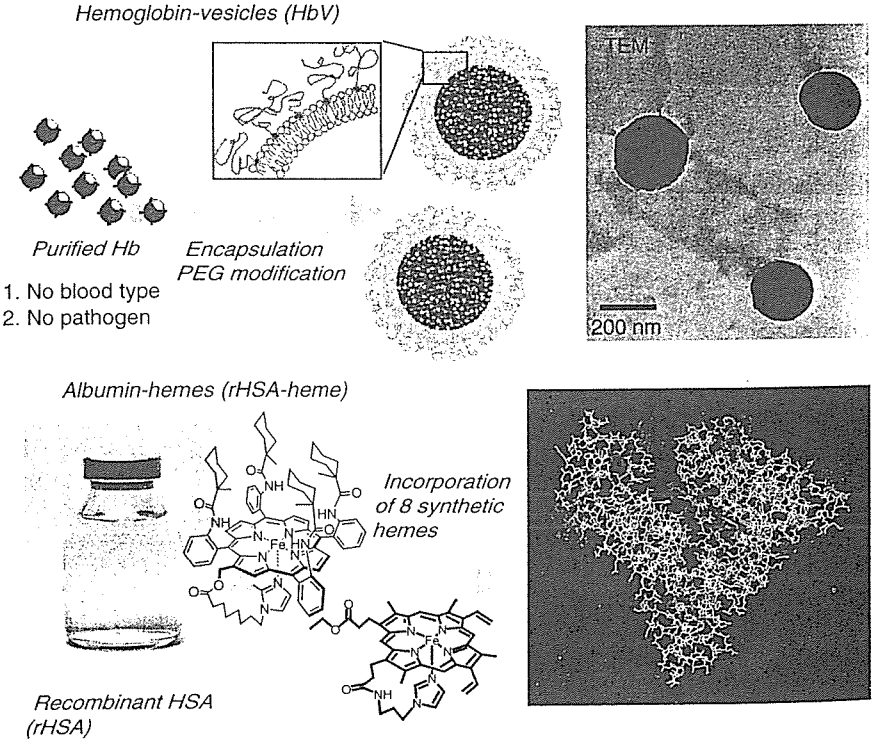

Figure 2. Hemoglobin-vesicles and albumin-hemes as new types of artificial oxygen carriers. (This figure is available in full color at http://www.mrw.interscience.wiley.com/ebe.)

are important to enhance the stability of liposomes without polymerization. Surface modification of liposomes with PEG chains is effective for dispersion stability. Using deoxygenation and PEG-modification, HbV can be stored at room temperature under deoxygenated conditions for two years (29). Moreover, storage does not induce aggregation and metHb formation. Even after injection into blood stream, HbV is homogeneously dispersed in the plasma phase and contributes to tissue oxygenation, as clarified by the microcirculatory observations (30).

One particle of HbV (ca. 250-nm diameter) contains about 30,000 Hb molecules. The HbV acts as a particle in the blood and not as a solute. Therefore, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires addition of a plasma expander for a large substitution of blood while maintaining the blood volume. Candidates of plasma expanders are plasma-derived HSA, hydroxyethyl starch (HES), dextran, or gelatin, depending on the clinical setting, cost, country, and clinician. Recombinant human serum albumin (rHSA) is an alternative that will be approved for clinical use in Japan. The HbV suspended in HSA or rHSA was tested for resuscitation from hemorrhagic shock (31) and extreme hemodilution (30). Moreover, HbV with a high O_2 affinity (low P_{50}) suspended in HES was tested for oxygenation of an ischemic skin flap (32). The results imply the further application of HbV for other ischemic diseases such as myocardial and brain infarction and stroke.

Safety of HbV has been confirmed in terms of blood compatibility (33), no vasoactivity (17), biodistribution of ^{99m}Tc-labeled HbV to reticuloendothelial system (RES) (34) and prompt degradation in RES, even after a massive infusion (35,36). Based on the safety and efficacy of HbV, a joint collaboration partnership of academia, a biotech venture company and a corporation in Japan are seeking clinical trials of HbV within a few years.

4. TOTALLY SYNTHETIC OXYGEN CARRIERS

4.1. Metal Complexes and Heme Derivatives

Minoshima et al. tested the crystalline state of cobalt histidine chelate complex as an O_2 carrier that reversibly binds an O_2 molecule (37). The Kambara and Kimoto group studied heme-derivatives of imidazole complexes. However, the irreversible O_2 binding and the short lifetime of the O_2 complex could not be overcome. Because a heme is inserted into a hydrophobic pocket of a globin macromolecule (such as Hb, myoglobin, neuroglobin), stable O_2 binding requires a hydrophobic environment. Collman et al. in 1973 (38) synthesized a derivative of iron tetraphenyl porphyrin-imidazole complex that makes its O_2 binding site hydrophobic and binds O_2 reversibly in an organic solvent, but not in an aqueous solution because of the spontaneous and irreversible oxidation of heme. Tsuchida et al. in 1983 synthesized an amphiphilic derivative of iron porphyrin that can be inserted into the hydrophobic bilayer membrane of phospholipid vesicles (liposomes) (39). This system represents the first example of an entirely synthetic O_2 carrier that reversibly binds O_2 under physiological conditions.

One role of serum albumin is to provide a hydrophobic binding site to carry nutrients, metabolic wastes, or functional molecules. It was clarified that a synthetic heme derivative can be incorporated efficiently into human serum albumin (HSA) solution, thereby providing a red albumin-heme hybrid (40). In Japan, recombinant human serum albumin (rHSA) is manufactured through expression in *Pichia pastoris* yeast; the Japanese FDA will soon approve it. Combination of the heme derivative, rHSA-heme is a new class of synthetic hemoprotein that requires no blood as a raw material source (41) (Fig. 2). The in vivo tests clarified the efficacy of rHSA-heme for hemodilution and shock resuscitation (42). A physiological colloid osmotic pressure was regulated by 5-wt% HSA concentration in the blood. To increase the O_2 transporting capacity of rHSA-heme, albumin-dimer is effective to reduce the colloid osmotic pressure and to increase the heme content. The dimer can be prepared using intermolecular crosslinking at Cys-34 (43). Surprisingly, this rHSA-heme shows no vasoconstriction or hypertension even though its NO binding properties are similar to those of other modified Hbs of similar molecular size (44,45). This phenomenon is explained by characteristics of negatively charged rHSA molecules, which reduce the permeability across the negatively charged endothelial cell layers, where NO is produced for relaxation of the smooth muscle layer.

The small molecular dimension of rHSA-heme, which causes no vasoconstriction, will be appropriate to carry O_2 effectively to tissues where RBCs are difficult to reach, such as tumor tissues. The tumor vasculature is highly heterogeneous and is therefore susceptible to hypoxia. In such conditions, tumor cells become resistant to chemotherapy and irradiation. It has been confirmed that injection of rHSA-heme considerably increased the O_2 tension in an implanted tumor in a rat model (46). The succeeding irradiation therapy shows reduced tumor size and improved survival. This therapeutic possibility for cancer therapy is also supported by the trials of chemically modified Hb solutions and perfluorochemicals (47).

rHSA incorporates a protoheme IX into the hydrophobic cavity of the subdomain IB. Introduction of proximal histidine into the heme binding site by site-directed mutagenesis allows O_2 binding to the prosthetic heme group. This albumin-protoheme is a new type of synthetic O_2-carrier (48).

4.2. Perfluorochemicals

Two major discoveries exist in the study of perfluorochemicals (PFC): (1) Clark and Gollan found that mice can survive by breathing an oxygenated PFC liquid (49); (2) Geyer et al. showed that an emulsified PFC can be used to replace the blood of rats completely (50). The former Green Cross Co. (Osaka) produced a PFC solution composed of perfluorodecalin ($C_{10}F_{18}$) and perfluorotripropylamine with a mixture of Pluronic and egg-yolk lecithin as surfactants. The resulting white colored emulsion, Fluosol-DA, was approved in 1978 to undergo clinical trials (51). Because the PFC concentration in the emulsion is only 20–35 vol%, its O_2 carrying capacity is

less than one-tenth that of blood at ambient O_2 pressure. Therefore, patients require inhalation of 100% O_2 gas during an operation. The US FDA approved Fluosol-DA for intracoronary administration only during percutaneous transluminal coronary angioplasty (PTCA). Because of its insufficient O_2 transporting capacity and side effects such as accumulation, pneumonia, and anaphylactic reactions, the company stopped production of Fluosol-DA in 1993.

Riess et al. showed that PFC emulsions from perfluorooctylbromide ($C_8F_{17}Br$) had four-times' higher O_2 solubility than that of Fluosol-DA (52,53). Alliance Pharmaceutical Corp. (San Diego, CA) has extensively developed a so-called second-generation PFC emulsion (Oxygent^{TM}) that is in multi-center international phase II/III trials aimed at its use as a pre-operational or peri-operational infusion for elective surgery to obviate or minimize allogeneic transfusion. In Russia, Perftoran (Moscow) developed PFC emulsion of perfluorodecalin and perfluoromethyl cyclohexylpiperidine. This material is approved in Russia for medical application (54).

5. METABOLISM OF BLOOD SUBSTITUTES AND SIDE EFFECTS

As a dose rate of blood, substitutes would be considerably larger than those of other drugs and the circulation time would be significantly shorter than RBC; their biodistribution, metabolism, excretion, and the side effects have to be characterized. Normally, free Hb released from RBC is rapidly bound to haptoglobin and removed from the circulation by hepatocytes. However, when the Hb concentration exceeds the haptoglobin binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. When the reabsorption capacity of the kidney is exceeded, hemoglobinuria and eventually renal failure occur. The encapsulation of Hb in both RBC and liposomes completely suppresses renal excretion. However, both senescent RBCs and Hb-vesicles in the blood stream are finally captured by phagocytes in the RES (or MPS), that was confirmed by radioisotope-labeling techniques (23,34,35). Particles of Perfluorocarbon emulsions and chemically modified Hbs (such as pyridoxalated polymerized Hb) are also captured by RES (55,56). It has to be clarified whether the accumulation of these materials in phagocytic cells may lead to transient impairment of the function of RES such as elimination of other foreign elements (35). There needs to be a balance between the circulation time of the O_2 carriers and the rates of metabolism and excretion. When their circulation time is too short, they burden on the functions of RES, kidney, and other related organs.

The released heme from Hb-based O_2 carriers should be mainly metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells in the liver and macrophages in the spleen. The resulting bilirubin is excreted in the bile duct. Iron deposition is confirmed as hemosiderin for the chemically modified and encapsulated Hbs. Normally, iron from a heme is stored in the ferritin molecule. This protein has 24 subunits and encloses as many as 4,500 iron atoms in the form of an aggregate of ferric hydroxide (57). Ferritin in the lysosomal membrane may form paracrystalline structures and eventually aggregate in mass with an iron content as high as 50%. These are hemosiderins composed of degraded protein and coalesced iron. Not only infusion of polymerized Hb and Hb-vesicles, but also transfusion of stored RBCs induces hemosiderin deposition in RES. As iron acts as a catalyst for Fenton reaction to produce toxic cytotoxic OH radicals from hydrogen peroxide, the level of hemosiderosis should be carefully monitored.

As for the membrane components of Hb-vesicles and perfluorocarbon emulsions, it was reported that the infused lipid components of liposomes are entrapped in the Kupffer cells, and diacylphosphatidylcholine is metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids and in exhaled air (35). There is no metabolic pathway for inert parfluorocarbon, and this gradually diffuses from the RES to the blood stream and is excreted in exhaled air through the lungs. The PEG chain is widely used for surface modification of both Hb and Hb-vesicles. The chemical crosslinker of PEG-lipid or PEG-Hb is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which is known as an inert macromolecule, should be excreted in the urine through the kidneys (58).

6. NEW CONCEPTS

Development of artificial O_2 carriers was originally initiated with a simple idea and an expectation that the materials that bind or dissolve O_2 can behave like RBCs in the blood stream. However, it was not easy to complete that project. During its long history of development, unexpected side effects were clarified such as capillary plugging, renal toxicity, vasoconstriction, vascular injury, and accumulation. Even after R&D of artificial O_2 carriers for decades, no material is commercially available for clinical use in Europe, Japan, or the US. Recent advanced biotechnology enables *ex vivo* RBC production from hematopoietic stem cells (59). However, problems remain of large-scale production and long-term storage for stockpiling. On the other hand, no doubts exist about a strong demand and expectation of blood-substitute development.

The importance of the sophisticated function of RBCs in concert with vascular physiology has been clarified, and new concepts are proposed in terms of the physicochemical properties of Hb-based artificial O_2 carriers. Historically, it has been regarded that the O_2 affinity should be regulated similarly to RBCs (25–30 Torr). Theoretically, this allows sufficient O_2 unloading during blood microcirculation as can 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 O_2 affinity (increasing P_{50}) increases O_2 unloading. However, small artificial O_2 carriers should release O_2 faster in arterial blood flow (14,15). It has been suggested that faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation. However, this concept is controversial in light of recent findings because an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders. The new concept is that an Hb-based O_2 carriers

with a high O_2 affinity (low P_{50}) should retain O_2 in the upstream artery or arteriole and release O_2 in the capillaries of the targeted tissue. This concept is recently supported by the results of PEG-modified Hbs and Hb-vesicles by the microcirculatory observations (60–62).

Because an infusion of an artificial O_2 carrier results in substitution of a large volume of blood, impact on hemorheology is great. It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases lower viscosity engenders decreased shear stress on the vascular wall, engendering vasoconstriction and reduced functional capillary density (63). Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. In relation to this, solutions of Hb-based O_2 carriers with a higher molecular weight are more viscous and would be appropriate. Moreover, as mentioned above, a larger molecular dimension can reduce the vascular permeability and minimize trapping of NO and CO as vasorelaxation factors.

These new concepts suggest reconsideration of the design of artificial O_2 carriers (13,14). Actually as shown in Table 2, new products are appearing, though they are in the preclinical stage, such as zero-link polymerized Hb (64), Hb-vesicles (65), and HRC 101 with larger molecular dimensions and higher O_2 affinities. The biodegradable polylactide (PLA)-PEG copolymer -nanocapsules (80–100 nm in size) contain Hb and hemolysate-derived enzymes (66). RBCs contain radical scavenging functions by SOD and catalase, and the sophisticated metHb reducing system. Hemozyme, Hemotech, and PolyHb-SOD-CAT have antioxidative properties that would be appropriate for eliminating active oxygen species in ischemia-reperfusion injury (66).

7. ADVANTAGES OF ARTIFICIAL OXYGEN CARRIERS AND CLINICAL INDICATIONS

Advantages of artificial O_2 carriers are the absence of blood-type antigens and infectious viruses, and stability for a long-term storage that overwhelm RBC transfusion. Easy manipulation of physicochemical properties enables tailor-made O_2 carriers that suit the clinical indications. The shorter half-lives of the HBOCs in the blood stream (2–3 days) limit their use, but they are applicable for a shorter periods of use, as: (1) resuscitative fluids for hemorrhagic shock during a pre-hospital emergency situation for temporary use or bridging until packed RBCs are available; (2) fluids for pre-operative hemodilution or peri-operative O_2 supply fluids for a hemorrhage in an elective surgery to obviate or delay allogeneic transfusion; (3) a priming solution for the circuit of an extracorporeal membrane oxygenator (ECMO); (4) O_2 therapeutics to oxygenate ischemic tissues; or (5) *ex vivo* oxygenation of harvested cell cultures, reconstructed tissues, and organs for transplantation (Table 3).

Clinicians and patients await the realization of safe and functional artificial O_2 carriers and their new clinical applications in the near future. This development might require continuous interdisciplinary cooperation to overcome not only emerging problems in preclinical and clinical tests, but also the dogmas of classical blood substitutes and modern transfusion medicine.

Table 3. Expected Clinical Indication of Artificial O_2 Carriers

Transfusion Alternative	Other Expected Applications
Resuscitative fluid for shock in an emergency.	Oxygenation of local ischemic disease (brain or myocardial infarction)
Hemodilution for autologous blood preservation for elective surgery.	Tumor oxygenation for photosensitization
Prime for circuit of extracorporeal membrane oxygenator (ECMO)	Oxygenation fluid of cultured cells for tissue reconstruction
Chronic anemia	Perfusion of organs for transplantation
Infusion to rate blood type patients	
Infusion to patients who do not accept transfusion (e.g., fear of infection, religious reason)	

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BONE, MECHANICAL TESTING OF

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1. INTRODUCTION

Bone is a complex heterogeneous material that in the human body serves the function of support, movement and protection, body mineral homeostasis, and hematopoesis. The study of bone brings together the fields of medicine and engineering in addition to the basic sciences of chemistry, biology, and physics to find ways of preventing and treating disease. As a material whose normal function and operation is integral to the daily life of the human being, bone has been the subject of countless research studies covering topics as diverse as the treatment of fractures to replacing pieces with artificial materials to building bone de novo on the lab bench. It has a hierarchical structural

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Performances of PEG-modified hemoglobin-vesicles as artificial oxygen carriers in microcirculation

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Abstract. Hemoglobin-Vesicles (HbV; diameter, 250 nm) are artificial O_2 carriers encapsulating purified and concentrated human Hb solution in phospholipid vesicles (liposomes), and their safety and efficacy, as a transfusion alternative, have been studied. In this paper, we summarized the characteristics of HbV that have been clarified by the microcirculatory observations.

Keywords: Blood substitutes, liposome, microcirculation, EDRF, oxygenation

1. Introduction

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed for use as a transfusion alternative and some of them are now in the process of clinical trials [1]. The advantages of the HBOCs are the absence of blood-type antigenicity and infectious pathogens, and stability for long-term storage when compared with the RBC transfusion [2–4]. A phospholipid vesicle or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) has been developed as an O_2 carrier [2,5–9]. The cellular structure of the HbV (particle diameter, ca. 250 nm) has characteristics similar to those of natural RBCs, since both have lipid bilayer membranes that prevent the direct contact of Hb with the components of blood and the endothelial lining [10]. The reasons for the Hb encapsulation in RBCs should be: (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. Moreover, during the long history of the development of HBOCs, many side effects of molecular Hb have become apparent. These side effects of molecular Hb would imply the importance of the cellular structure.

Our *in vivo* studies of HbV have revealed the sufficient O_2 transporting efficiency comparable to RBCs [11–14], the safety in terms of blood compatibility [15], and prompt degradation in the reticuloendothelial system [16–19], all of which make us confident about advancing to the further development of HbV.

In this paper, we focus on the performances of our polyethylene-glycol (PEG)-modified HbV from the viewpoint of hemorheology and microcirculation.

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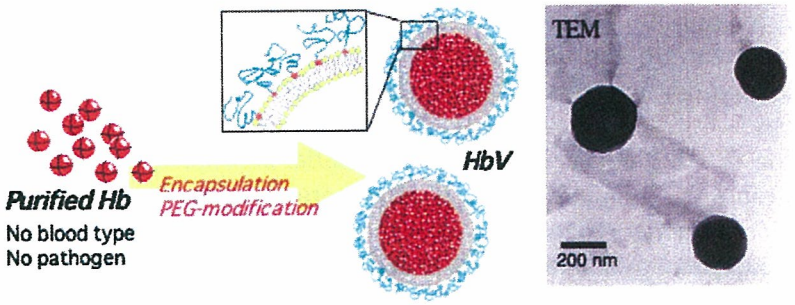

Fig. 1. Hemoglobin-vesicles (HbV) encapsulate the ultrapurified and concentrated human Hb solution (35 g/dl) with phospholipid bilayer membrane, and the surface is modified with polyethylene glycol chains. The well-regulated particle size (about 250 nm) was confirmed by TEM. One particle contains about 30,000 Hb molecules and about 1500 PEG chains were fixed on the surface.

2. Impact of PEG-modification of HbV

The rheological property of an HBOC is important because the infusion amount should be significantly large and that may affect the blood viscosity and hemodynamics. One HbV contains about 30,000 Hb molecules so that the suspension of HbV does not have colloid osmotic pressure (COP) (Fig. 1). The HbV suspended in 5 g/dl human serum albumin (HSA) at [Hb] = 10 g/dl shows comparable COP and viscosity to the blood.

We tested he function of PEG-modified and unmodified HbV as a blood replacement in the subcutaneous microvasculature of awake hamsters during severe hemodilution in which 80% of the red blood cell mass (70 ml/kg) was substituted with suspensions of the vesicles in 5% HSA solution [20,21]. Both materials yielded normal mean arterial pressure, heart rate, and blood gas parameters, which could not be achieved with albumin alone. Subcutaneous microvascular studies showed that PEG-modified HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, and O_2 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. Even though it was confirmed *in vitro* that the aggregates dissociated reversibly at higher shear rates, it is unlikely that they would dissociate in vessels where the flow rate or shear stress was low. Aggregation and decreased flow rate may constitute a vicious circle that reinforces negative effects on blood flow. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. From this result, PEG modification is important for HbV in microvascular blood flow.

3. Interaction with NO and CO

As clinical trials of the chemically modified Hbs are extended to include larger numbers of individuals, it becomes apparent that the principal side effect consistently reported in the administration of acellular Hb solutions is hypertension presumably because of vasoconstriction. Hypertension, a well-defined reaction of the acellular intramolecularly cross-linked Hb (XLHb), was proposed to be beneficial in the treatment of hypotension concomitant to hemorrhagic shock [22]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [23,24]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [25,26].

We analyzed the relationship between the constriction of resistance vessel and hypertension after administration of acellular Hb and the extent to which the effect is dependent on the size of acellular Hb molecules modified by polymerization, polymer conjugation, and cellular liposome encapsulation [8,27]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheter into jugular vein, mean arterial pressure, and heart rate were monitored through jugular arterial catheter, and microvascular responses were monitored by an intravital microscopy. The Hb products included intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethylstarch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb) and HbV. Their molecular diameters were 7, 22, 68 and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 ± 13 mmHg higher 3 hrs after infusion. There was a simultaneous decrease in diameter of A_0 vessels (79 ± 8% of basal value), which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of HBOCs of greater molecular size resulted in lesser vasoconstriction and hypertension with HbV showing the smallest changes. Infusion of HSA was used as control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of HBOCs. Since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different hemoglobin molecules.

The liver is a major organ that detoxifies excess amount of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXa and CO. Under normal conditions, liver contains at least two OH isozymes for physiologic degradation of the heme: HO-1 and HO-2. One of the important roles of the HO reaction is to generate CO that serves as an endogenous regulator that is necessary for maintaining microvascular blood flow [28]. Since Hb strongly binds with CO (about 200 times stronger than O_2), it is necessary to confirm the effects of HbV in hepatic microcirculation in comparison with stroma free Hb solution. Suematsu et al. studied the perfusion of a rat liver with an acellular Hb solution and HbV, and found out that the Hb solution increased vascular resistance by 30% [29]. The smaller acellular Hb molecules (7 nm) extravasate across the fenestrated endothelium with a pore size of about 100 nm, and reach to the space of Disse. Heme is excessively metabolized by hemeoxygenase-2 to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb in the space of Disse rapidly binds CO, resulting in the vasoconstriction and the increase in vascular resistance. On the other hand, Hb-vesicle (250 nm) is large enough to maintain in the sinusoid, and the vascular resistance is maintained.

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

4. Oxygen releasing behavior of HbV and oxygen therapeutics

We measured the O_2 release from HbV perfused through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm), that was exposed to a deoxygenated environment [30] (Fig. 2). The addition of HbV to RBC did not influence on the O_2-releasing rate. On the other hand, the addition of 50-vol% acellular Hb solution to RBC significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of the O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 7 nm) that affects their

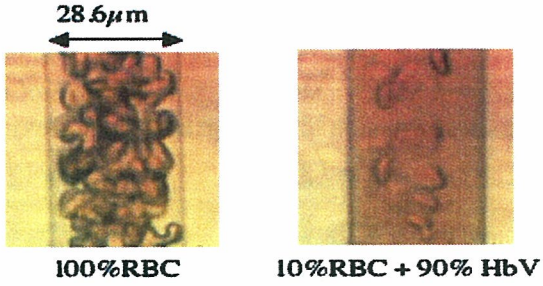

Fig. 2. Flow patterns of RBCs mixed with HbVs suspended in human serum albumin in a narrow tube (diameter, 28.6 μm) [30]. RBCs tended to flow in the centerline, while the HbV particles were homogeneously dispersed in a suspension medium. The individual particles could not be seen at this magnification. However, semitransparent elements were seen in the suspension medium, indicating the presence of HbV. This experimental model, developed by Maeda et al., was used to analyze the O_2 releasing behavior of HbV and RBC. [Hb] = 10 g/dl; centerline flow velocity, 1 mm/s.

diffusion for the facilitated O_2 transport. It has been suggested that the faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation [31]. However, this concept is controversial regarding the recent findings since an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [24,32]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of NO as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

One characteristic of HbV is that the O_2 affinity (P_{50}) of Hb can be easily regulated by the amount of coencapsulated allosteric effector, pyridoxal 5′-phosphate [21]. It has been clarified by Erni et al. that oxygenation of an ischemic skin flap, where one branch of feeding arteriole was ligated, was improved by infusion of HbV with a high O_2 affinity (low P_{50}) [33,34]. To clarify the underlying mechanism of ischemic tissue oxygenation, we prepared two HbVs with different P_{50}s (8 and 29 mmHg, termed HbV_8 and HbV_{29}, respectively), and observed their O_2 releasing behavior from an occluded arteriole in a hamster skinfold window model [35]. Conscious hamsters received HbV_8 or HbV_{29} at the dose rate of 7 ml/kg bw. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator and the reduction of the intra arteriolar O_2 tension (pO_2) 100 μm down from the occlusion was measured by the phosphorescence quenching of pre-infused Pd-porphyrin. The baseline arteriolar pO_2 (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV_8 infusion showed slightly slower rate of pO_2 reduction in comparison with that after HbV_{29} infusion. The arteriolar O_2 content was calculated at each reducing pO_2 in combination with the O_2 equilibrium curves of HbVs, and it was clarified that HbV_8 showed significantly slower rate of O_2 release in comparison with HbV_{29} and was a primary source of O_2 (maximum fraction, 0.55) overwhelming RBCs when the pO_2 was reduced (e.g., <10 mmHg) in spite of a small dosage of HbV.

Accordingly, the result of improved oxygenation of the ischemic skin flap, observed by Erni et al., could be explained by low P_{50} HbVs retaining O_2 in the upstream vessels and delivering it to the ischemic tissue via collateral arterioles, even when these may have significantly slower blood flow. Moreover, an advantage of small HBOCs including HbV is that they are homogeneously dispersed in the plasma phase and therefore can deliver O_2 more homogeneously to the periphery than RBCs because microvascular Hct is heterogeneous particularly in pathological states. In such conditions HbV with a higher O_2 affinity (lower P_{50}) should show a slower O_2 unloading which would be effective for oxygenating ischemic tissues. This result supports the possible utilization of HBOCs with lower P_{50} for oxygenation of ischemic tissues.

In summary, observation of microcirculation is important for the development of HBOCs because it is the site where oxygen is unloaded to the target tissues. From the international collaborative evaluation studies of HbV, we have clarified the rheological property, advantages of the cellular structure, and the performances of HbV not only as a transfusion alternative but also for oxygen therapeutics.

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Hemoglobin-Vesicles as a Transfusion Alternative

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Abstract: Phospholipid vesicles or liposomes encapsulating purified and concentrated human hemoglobin (Hb-vesicle, HbV) have been developed as a transfusion alternative. They are void of blood-type antigens and infectious viruses; they are stable and suitable for long-term storage. The cellular structure of HbV (particle diameter, ca. 250 nm) prevents direct contact of Hb with the blood components and the endothelial lining shielding cells from the side effects of Hb molecules. Microcirculatory observations show that the cellular structure of HbV is important to control reactions with endothelium-derived vasorelaxation factors. Animal studies of extreme hemodilution and resuscitation from hemorrhagic shock attest to the sufficient oxygen transporting capacity of HbV. Studies of biodistribution and metabolism reveal that HbVs are captured eventually in the reticuloendothelial system, and degraded within one week. In a joint collaboration partnership of academia, a biotech venture company and a corporation, we plan to produce HbV with good manufacturing practices, and to start preclinical and, finally, clinical trials within a few years.

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Keywords: Artificial oxygen carriers; Hemoglobin-vesicles; Transfusion alternative

1. INTRODUCTION

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed for use as a transfusion alternative. Some of them are undergoing clinical trials [1,2]. Advantages of the HBOCs include the absence of blood-type antigenicity and infectious pathogens, and better stability for long-term storage than RBC transfusion [1,3]. Phospholipid vesicles or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) have been developed as O_2 carriers [1–6]. The HbV cellular structure (particle diameter, ca. 250 nm) has characteristics that are similar to those of natural RBCs because both have lipid bilayer membranes that prevent direct contact of Hb with blood components and the endothelial lining. Reasons for the Hb encapsulation in red blood cells (RBCs) include: (1) a decreased high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of Hb removal from the blood circulation; and (3) preservation of the chemical environment in RBCs such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes. Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs), many side effects of molecular Hb have become apparent. These side effects of molecular Hb imply the importance of the cellular structure.

After the unforgettable tragedies of HIV transmission because of the distribution of non-pasteurized plasma-derived products and the Great Hanshin Earthquake in 1995, the Japanese government aggressively pursued development of artificial blood. In 1997, the Ministry of Health and Welfare declared a framework of scientific research grants intended for developing a substitute for three major blood components: artificial red cells, artificial platelets, and artificial antibodies. The most promising artificial red cell in Japan should be HbV. The in vitro and in vivo studies of HbV in collaboration with Waseda-Keio and other research institutes have revealed sufficient O_2 transporting efficiency comparable to RBCs, safety in terms of blood compatibility [7,8], and prompt degradation in the reticuloendothelial system, all of which makes us confident about advancing to further development of HbV. In this review paper, we demonstrate some important results that show the safety and efficacy of HbV as a transfusion alternative.

2. EFFICACY OF HBV SUSPENDED IN AN ALBUMIN SOLUTION

One HbV contains about 30,000 Hb molecules. For that reason, the suspension of HbV does not show colloid osmotic pressure (COP). Accordingly, the

infusion of a large amount of HbV suspension requires the addition of a plasma expander such as human serum albumin (HSA). Surface modification of HbV with polyethylene glycol (PEG) is effective to prevent intervesicular aggregation in the presence of HSA by the steric hindrance of PEG chains (Fig. 1). This measure guarantees homogeneous dispersion of HbV in the microcirculation. When HbV is suspended in 5-g/dl HSA, the COP and the viscosity of the suspension are respectively regulated to 20 mmHg, and 3 cP, which are comparable with those of blood.

A clear result of O_2 transporting ability of HbV was obtained using the extreme hemodilution with HbV suspended in HSA [9–11]. The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex and skeletal muscle; then the blood flow rate in the abdominal aorta was measured using the pulsed Doppler method. Hemodilution with albumin alone engendered marked reductions in mean arterial pressure and renal cortical O_2 tension. Finally, all the rats died of anemia. On the other hand, hemodilution with HbV suspended in HSA sustained both blood pressure and renal cortical O_2 tension: all the rats survived. These results demonstrate that HbV has sufficient O_2 transporting capability. It has been confirmed in a rat model that HbV suspended in recombinant HSA (rHSA) is effective as a priming solution for cardiopulmonary bypass circuit (about 50% hemodilution). Quite recently, we

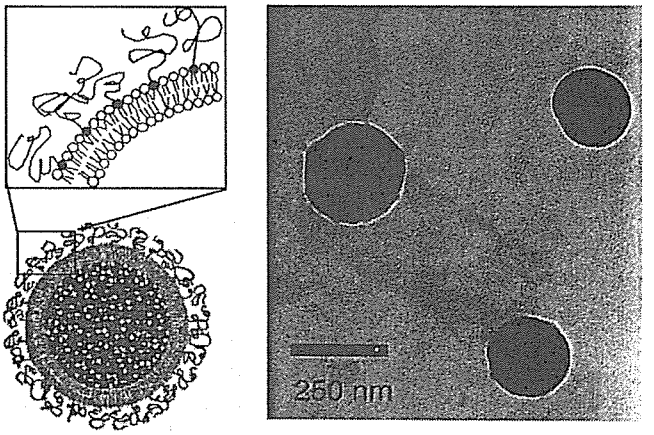

Figure 1. Highly purified and concentrated human-derived Hb solution is encapsulated in phospholipid vesicles (liposomes). One particle contains about 30,000 Hb molecules. The outer surface of one HbV is modified with about 1,500 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the blood stream. The transmission electron micrograph (TEM) clearly depicts the well-regulated particle size and high Hb content within the vesicles.