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Oxygen-Carrying Plasma Hemoprotein Including Synthetic Heme

Teruyuki Komatsu and Eishun Tsuchida

Summary. Recombinant human serum albumin (rHSA) incorporating tetraphenylporphyrinatoiron(II) derivative with four pivaloylamino substituents (FepivP), albumin-heme, is an entirely synthetic hemoprotein that can reversibly bind and release O_2 under physiological conditions. We have recently found that replacing the substituent groups of FepivP with more hydrophobic 1-methylcyclohexanoylamino groups, affording FecycP, substantially stabilizes the formed O_2–adduct complex. The O_2- and CO-binding abilities and blood compatibility of this new rHSA-heme hybrid (rHSA-FecycP) have been investigated by spectroscopy. The maximum number of FecycP binding to one albumin was determined to be eight. Because the isoelectric point and circular dichroism (CD) spectral pattern were identical to those of rHSA itself, the two-dimensional structure of the host albumin could be unchanged after the incorporation of FecycP. Laser-flash photolysis experiments gave the association and dissociation rate constants for O_2 and CO (k_{on}, k_{off}). The rebinding kinetics of these gaseous ligands consists of multiple exponentials. We conjectured that the O_2- and CO-binding reactions are affected by the molecular environment around each of the active heme sites. rHSA-FecycP showed almost the same O_2-binding affinity($P_{1/2}{}^{O_2}$ 34 torr at 37°C) and thermodynamic parameters (ΔH, ΔS) for the oxygenation as rHSA-FepivP. In contrast, the half-life of the O_2-adduct complex (9 h, 37°C) became significantly longer than that of rHSA-FepivP (by a factor of 4.5), which is close to that of myoglobin. The obtained red solution was stable and demonstrated a long shelf life (>2 years) at room temperature. The equivalent mixture of rHSA-FecycP and whole blood exhibited no coagulation or precipitation, indicating its high blood compatibility.

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Key words. Human serum albumin, Albumin-heme, Synthetic hemoprotein, Oxygen-binding ability, Red blood cell substitute

Introduction

Human serum albumin (HSA) used for clinical treatment in Japan amounted to 1.9 million l (in terms of a blood source) in 2002 [1]. Most was administered to hemorrhagic shocked patients as a resuscitation fluid. If HSA can transport oxygen (O_2) like red blood cells, it could be of extreme medical importance not only as a blood replacement but also as an O_2 therapeutic agent.

In our circulatory system, free hemin, an iron(III) complex of protoporphyrin IX dissociated from methemoglobin, is potentially toxic because it may (1) intercalate phospholipid membranes, (2) be a major source of iron for bacterial pathogens, and (3) catalyze the formation of free radicals. Hemopexin has high affinity for binding protein with hemin, having the highest binding constant of any known protein ($K > 10^{12}\,M^{-1}$), but it releases it into liver cells via specific surface receptors [2]. Crystal structure analysis of the hemopexin-hemin complex revealed that the hemin is tightly bound by double histidine coordinations to the central ferric ion and multiple hydrogen bondings with the amino acid residues [3]. Nevertheless, the concentration of hemopexin in the plasma is rather low (<17 μM). HSA may also provide reserve binding capacity of hemin in various conditions (e.g., trauma, inflammation, hemolysis). In fact, HSA binds hemin with a relatively high affinity ($K = 10^8\,M^{-1}$) [4]. We have determined the single crystal structure of the HSA-hemin-myristate complex with a resolution of 3.2 Å [5]. Hemin is accommodated into the narrow D-shaped pocket in subdomain IB; and proximal coordination with Tyr-161 and three hydrogen bondings with basic amino acids contribute to maintaining the assembly. Addition of a sodium dithionate into this solution under an N_2 atmosphere reduced the central ferric ion to the ferrous state, although exposure to O_2 gas immediately oxidized the iron(II) center (T. Komatsu, N. Ohmichi, E. Tsuchida, unpublished data, 2004).

We have found that tetraphenylporphyrinatoiron(II) derivative with four pivaloylamino substituents (FepivP) (Fig. 1) was also incorporated into HSA, and the obtained albumin-heme (HSA-FepivP) can reversibly bind and release O_2 under physiological conditions in the same manner as hemoglobin (Hb) and myoglobin (Mb) [6–12]. Because recombinant HSA (rHSA) was manufactured on a large scale by expression in *Pichia pastoris* [13], rHSA-heme hybrid has become entirely synthetic and absolutely free of infectious pathogens. Our animal experiments have also demonstrated that rHSA-heme works as an "oxygen-carrying plasma hemoprotein" in the bloodstream [14; T. Komatsu et al., unpublished data, 2004].

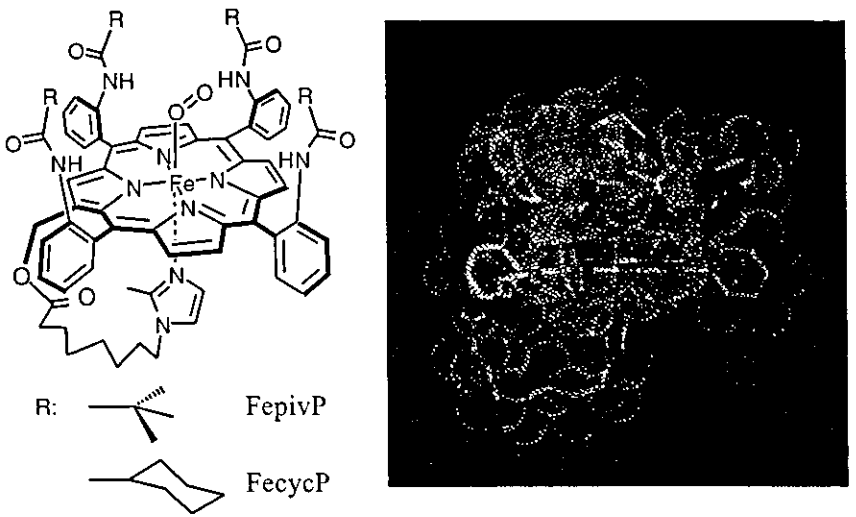

FIG. 1. Structures of the new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) and pivaloylamino substituents (FepivP), and the simulated structure of oxygenated FecycP. The extensible systematic forcefield (ESFF) simulation was performed using an Insight II system (Molecular Simulations, San Diego, CA, USA). The structure was generated by alternative minimization and annealing dynamic calculations from 1000 K to 100 K. The dielectric constant was fixed at 2.38 D, corresponding to the toluene solution. The *dotted surface* represents the van der Waals radius

Half of the Hb-based O_2 carrier in advanced clinical trials still exhibited vasoconstriction, which increased blood pressure and decreased cardiac output [15–19]. The precise mechanism of this hypertension is controversial, but many investigators suspect that the Hb molecules penetrate the vascular endothelium and bind the endothelial-derived relaxing factor (EDRF), namely nitric oxide [20–27]. Others believe that excessive delivery of O_2 to arteriolar vascular walls induces autoregulatory vasoconstriction [28–33]. Interestingly, rHSA-heme does not induce such a vasopressor effect [34]. The electrostatic repulsion between the albumin surface and glomerular basement membrane around the endothelial cell retards rapid leakage of the rHSA-heme molecule and quick scavenging of NO. Albumin-heme is now recognized to be one of the most promising materials as a new class of red blood cell substitute.

To improve the O_2-binding ability of rHSA-FepivP, we have synthesized new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) (Fig. 1) [35]. rHSA-FecycP forms a significantly stable O_2-adduct complex with

a long half-life compared to that of FepivP (by a factor of 4.5). We herein report the O_2- and CO-binding abilities of this entirely synthetic albumin-based O_2 carrier.

Incorporation of Heme into rHSA

Based on quantitative analysis of the absorption intensity for the Soret band of aqueous rHSA-FecycP, the maximum number of FecycP binding to an rHSA was determined to be eight using a molar extinction coefficient [35]. FecycP is accommodated into certain domains of rHSA with binding constants of 10^6–$10^4\,M^{-1}$.

The isoelectric points (*p*I) of the obtained rHSA-FecycP hybrid (FecycP/rHSA = 1–8 mol/mol) were 4.8, exactly the same as those of rHSA. Fatty acid binding, for example, induced a reduction in the *p*I value due to partial neutralization of the surface charge. The FecycP molecule without any ionic side chain interacts nonspecifically with a hydrophobic subdomain of rHSA, so its surface charge distributions are unaltered. Consequently, the essential biological roles as serum albumin [i.e., control of colloid osmotic pressure (COP) and plasma expansion] are essentially sustained after the incorporation of FecycP.

The secondary and tertiary structures of rHSA and the deformation upon FecycP binding were measured by circular dichroism (CD) spectroscopy. The spectral pattern showed typical double-minimum negative peaks in the ultraviolet (UV) region independent of the number of FecycP molecular bound (Fig. 2). The estimated α-helix content was approximately 67%, suggesting that the FecycP association did not cause any high-ordered structural change in the host albumin. Moreover, rHSA-FecycP showed no induced CD in the Soret region (400–500 nm). The hemin binding to the serum albumin is accompanied by a rise in the extrinsic negative Cotton effect in the Soret region because it binds to albumin through axial coordination, allowing a large degree of immobilization [36,37]. We concluded that hydrophobic interaction is the major molecular force of FecycP binding, and its incorporation does not induce any changes in the highly ordered structure or in the surface net charges of rHSA.

O_2-Binding Property of rHSA-Heme

The UV-visible absorption spectrum of the aqueous rHSA hybrid that included carbonyl FecycP showed the formation of the typical CO-coordinated low-spin tetraphenylporphyrinatoiron(II) derivative (λ_{max}: 429, 545 nm). Light irradiation of this solution under an O_2 atmosphere led to

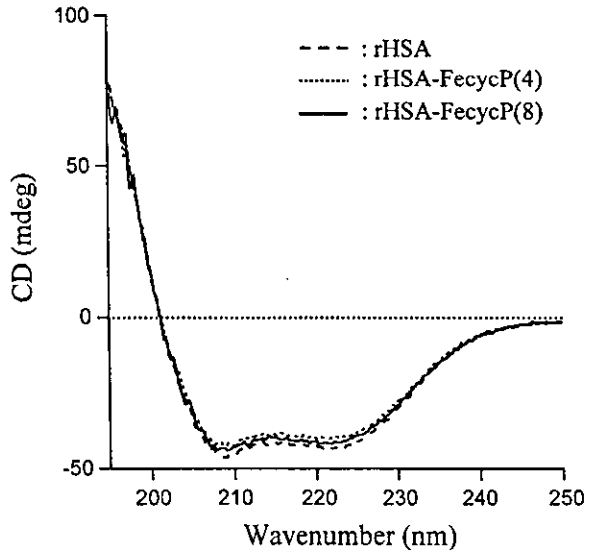

FIG. 2. Circular dichroism (*CD*) spectra of recombinant human serum albumin (*rHSA*) and rHSA-FecycP in water at 25°C

CO dissociation, giving the O_2-adduct complex (λ_{max}: 428, 555 nm). Upon exposure of the oxygenated rHSA-FecycP to N_2, the UV-visible absorption pattern changed to that of the five-*N*-coordinated high-spin iron(II) complex with an intramolecularly coordinated proximal imidazole (λ_{max}: 445, 543, 567 nm). This oxygenation was reversibly dependent on the O_2 partial pressure and sufficiently stable under physiological conditions (37°C, pH 7.4) (Fig. 3). The rate of irreversible oxidation is satisfactorily slow (vide infra).

The O_2 coordination to FecycP in human serum albumin is expressed by Eq. 1.

$$\mathrm{FeP} + \mathrm{O_2} \underset{k_{off}^{O_2}}{\overset{k_{on}^{O_2}}{\rightleftarrows}} \mathrm{FeP\text{-}O_2} \qquad (1)$$

$$\left[P_{1/2}^{O_2} = (K^{O_2})^{-1} = k_{on}^{O_2}/k_{off}^{O_2}\right]$$

The O_2 association and O_2-dissociation rate constants ($k_{on}^{O_2}$, $k_{off}^{O_2}$) were explored by laser flash photolysis (Table 1) [9,35,38–40]. The detailed kinetic evaluation of rHSA-FecycP gave the following results.

1. The absorption decays accompanying O_2 recombination were composed of three phases of first-order kinetics; the curves were fit by a triple-exponential equation [9]. The minor (<10%) and fastest component was

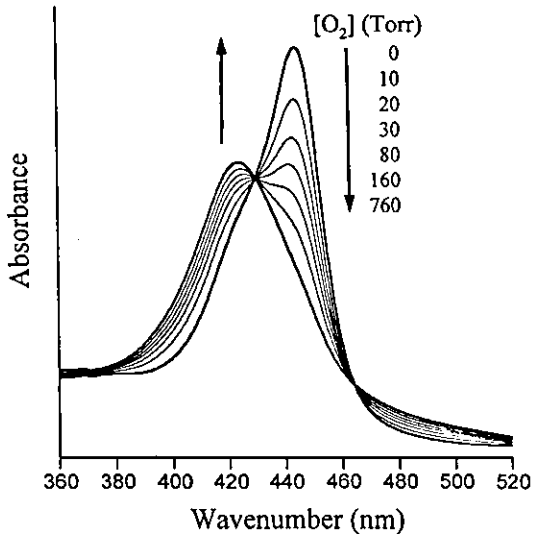

FIG. 3. Ultraviolet-visible. Absorption spectral changes of rHSA-FecycP(4) dependent on the O_2 partial pressure in phosphate-buffered solution (pH 7.3) at 37°C. The number in parenthesis is molar ratio of FecycP and rHSA

TABLE 1. O_2 association and dissociation rate constants for rHSA-FecycP in phosphate-buffered solution (pH 7.3) at 25°C

Substance	$k_{on}[M^{-1}s^{-1}]$		$k_{off}[s^{-1}]$	
	Fast	Slow	Fast	Slow
rHSA-FecycP(8)	4.6×10^7	7.3×10^6	9.8×10^2	1.6×10^2
rHSA-FepivP(8)[a]	3.4×10^7	9.5×10^6	7.5×10^2	2.0×10^2
Hb (T-state)α[b]	2.9×10^6		1.8×10^2	

rHSA, recombinant human serum albumin; FecycP, tetraphenylporphyrinatoiron(II) derivative with 1-methyl cyclohexanoylamino groups; FepivP, tetraphenyl porphyrinatoiron (II) with pivaloyamino substitute; Hb, hemoglobin.
[a]Ref. [9].
[b]pH 7, 20°C; Ref. [40].
The numbers in parenthesis is molar ratio of porphyrin and rHSA.

independent of the O_2 concentrations. It should be correlated with a base elimination [41].

2. Based on careful inspection of the two slower phases, the association rate constants for the fast and slow rebinding [k_{on}(fast) and k_{on}(slow)] of O_2 were calculated. The k_{on}(fast) values are four- to fivefold higher than the k_{on}(slow) volues.
3. The concentration ratios of the fast and slow reactions were 2:1 to 3:1.

Based on these findings, we can conclude that the O_2 association with FecycP in the hydrophobic domains of rHSA is influenced by the molecular

TABLE 2. O_2-binding equilibrium parameters and half-lifetime of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

Substance	$P_{1/2}$(torr)[a]	ΔH[kJ mol^{-1}]	ΔS[J K^{-1}mol^{-1}]	$\tau_{1/2}$[h][a]
rHSA-FecycP(4)	34	−59	−108	9
rHSA-FecycP(8)	35	−59	−107	9
rHSA-FepivP(4)[b]	36	−60	−114	2
rHSA-FepivP(8)[b]	33	−60	−112	2
Red cells[c]	27			
Hbα	40[d]	−57 to −65[e]	−116 to −133[e]	35[f]
Mb[d]	40[d]	−57 to −65[e]	−116 to −133[e]	12[g]

[a]At 37°C.
[b]Ref. [8].
[c]pH 7.4; ref. [42].
[d]T-state, pH 7, 20°C; ref. [40].
[e]pH 7.4; ref. [43].
[f]At 37°C, pH 7.2; ref. [44].
[g]At 35°C, pH 7.0; ref. [45].
The number in parenthesis is molar ratio of porphyrin and rHSA.

microenvironment around each O_2 coordination site (e.g., steric hindrance of the amino acid residue and difference in polarity).

The O_2-binding affinity for such oxygenation could be directly determined. Adequate isosbestic behavior was maintained during the course of a spectrophotometric titration of O_2 (Fig. 3). According to the kinetic experiments, the $P_{1/2}$ values were divided into two components using our previously reported equation [9]. The calculated $P_{1/2}$ for the fast and slow phases were identical in each case (Table 2). The thermodynamic parameters (ΔH, ΔS) of oxygenation were also measured by the van't Hoff plots of the K^{O_2} values (Fig. 4) [8]. The $P_{1/2}{}^{O_2}$, ΔH, and ΔS values for oxygenation of rHSA-FecycP resembled those of Hb and Mb [8,40,42–45]. Moreover, we could not find significant differences in these parameters for rHSA-FepivP and rHSA-FecycP. This result indicates that the substituent structure on the porphyrin plane does not cause any substantial change in the O_2 equilibria and kinetics of rHSA-heme.

Stability of O_2-Adduct Complex of Albumin-Heme

Accompanying the autooxidation of the central iron(II), the absorption band (λ_{max} 555 nm) slowly disappeared at 37°C, leading to formation of the inactive ferric porphyrin. The effect of the heme structure on the half-life of the O_2-adduct complex against the ferric state ($\tau_{1/2}$) was marked. The rHSA-FecycP had a $\tau_{1/2}$ of 9 h, which is 4.5-fold longer than that of rHSA-FepivP and close to that of the Mb (12 h at 37°C) [46].

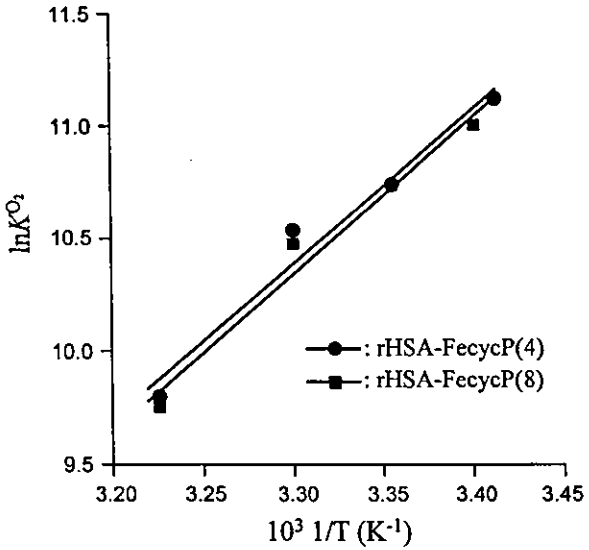

FIG. 4. Van't Hoff plots of O_2-binding affinity of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

CO-Binding Property of rHSA-Heme

Upon addition of CO gas through the deoxy or oxy state of the rHSA-FecycP solution, the spectrum immediately exhibited formation of the carbonyl complex. The CO-binding affinity ($P_{1/2}^{CO}$) of rHSA-FecycP became 2.5-fold higher than that of rHSA-FepivP (Table 3) [9,47,48]. Kinetically, this is due to the low CO dissociation rate constant, k_{off}^{CO}. More recently, CO/O_2 discrimination of Hb and Mb has not been based mainly on distal steric constraints in the heme pocket; the emphasis has shifted to polar interactions in the binding pocket [49,50]. That is, a polar environment could favor the highly polarized coordinated Fe-O_2 unit over the apolar Fe-CO moiety. In FecycP, the hydrophobic cavity around the central ferrous ion probably contributes to the rise in CO-binding affinity. This interpretation is in good agreement with assumptions by other investigators.

Blood Compatibility

The red rHSA-FecycP solution showed a long shelf life (>2 years) at temperatures of 4°–37°C without any aggregation or precipitation. The solution properties also satisfied physiological requirements. The specific gravity was 1.013 (FecycP/rHSA = 1–8 mol/mol). The viscosity of 1.2 cP (at a high shear

TABLE 3. CO-binding parameters of rHSA-FecycP in phosphate-buffered solution (pH 7.3) at 25°C

Substance	$P_{1/2}^{CO}$(torr)	k_{on}($M^{-1}s^{-1}$) Fast	Slow
rHSA-FecycP(8)	0.04	5.9×10^6	8.9×10^5
rHSA-FepivP(8)	0.10	4.9×10^6	6.7×10^5
Hb (T-state)α*	0.30	2.2×10^5	

*Aqueous, pH 7.0–7.4, 20°C; refs. [47, 48].
The number in parenthesis is molar ratio of porphyrin and rHSA.

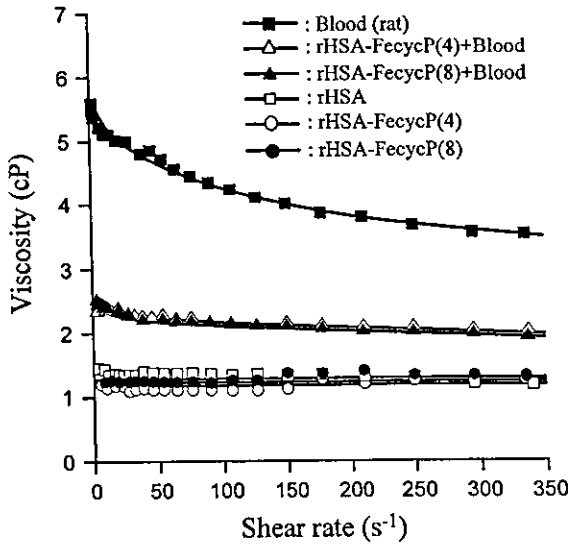

FIG. 5. Viscosity of rHSA-FecycP solution with whole blood at 37°C

rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cP) and exhibited Newtonian-type shear rate dependence similar to that of rHSA itself (Fig. 5). Furthermore, the viscosity of the mixed dispersion with freshly drawn blood (1:1, v/v) showed 2.0 cP (at 230 s^{-1}), indicating that rHSA-FecycP had good compatibility with blood. Optical microscopic observations also revealed that the homogeneous morphology of the red blood cells was not affected by mixing with whole blood (not shown).

Conclusions

Human serum albumin incorporating synthetic heme formed an O_2-adduct complex under physiological conditions. In particular, oxygenated rHSA-FecycP showed high stability compared to the previous rHSA-FepivP, and its half-life reached a value similar to that of the native Mb. It has been also found

that another rHSA-heme complex incorporating an FecycP analogue with a histidyl base at the porphyrin periphery had an extremely long half-life of the oxygenated complex (25 h) under the same conditions (in this case the O_2-binding affinity is quite high) [35]. rHSA–FecycP with a $P_{1/2}$ value (34 torr at 37°C) similar to that of red blood cells is now the most promising material to be used as an artificial O_2 carrier. Exchange transfusion with rHSA-FecycP into anesthetized beagles to evaluate its clinical safety and efficacy is now under investigation.

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PAT

Oxygen infusions (hemoglobin-vesicles and albumin-hemes) based on nano-molecular sciences†

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Since the discovery of a red-colored saline solution of a heme derivative that reversibly binds and releases oxygen (1983), significant efforts have been made to realize an oxygen infusion as a red cell substitute based on the sciences of both molecular assembling phenomena and macromolecular metal complexes. The authors have specified that hemoglobin (Hb)-vesicles (HbV) and recombinant human serum albumin-hemes (rHSA-heme) would be the best systems that meet the clinical requirements. (A) Hb is rigorously purified from outdated, donated red cells via pasteurization and ultrafiltration, to completely remove blood type antigen and pathogen. The HbV encapsulates thus purified concentrated Hb solution with a phospholipid bimolecular membrane (diameter, 250 nmø), and its solution properties can be adjusted comparable with blood. Surface modification of HbV with a water-soluble polymer ensures stable dispersion state and storage over a year at 20°C. *In vivo* tests have clarified the efficacy for extreme hemodilution and resuscitation from hemorrhagic shock, and safety in terms of biodistribution, metabolism in reticuloendothelial system (RES), clinical chemistry, blood coagulation, etc. The HbV does not induce vasoconstriction thus maintains blood flow and tissue oxygenation. (B) rHSA is now manufactured in Japan as a plasma-expander. The rHSA can incorporate eight heme derivatives (axial base substituted hemes) as oxygen binding sites, and the resulting rHSA-heme is a totally synthetic O_2-carrier. Hb binds endothelium-derived relaxation factor, NO, and induces vasoconstriction. The rHSA-heme binds NO as Hb does, however, it does not induce vasoconstriction due to its low pI (4.8) and the resulting low permeability across the vascular wall (1/100 of Hb). A 5%-albumin solution possesses a physiologic oncotic pressure. Therefore, to increase the O_2-transporting capacity, albumin dimer is effective. Albumin dimer can incorporate totally 16 hemes with a regulated oncotic pressure. The rHSA-heme is effective not only as a red cell substitute but also for oxygen therapeutics (e.g. oxygenation for tumor). Significant efforts have been made to produce HbV and rHSA-heme with a facility of Good Manufacturing Practice (GMP) standard, and to start preclinical and finally clinical trials.

KEYWORDS: oxygen infusion; blood substitutes; surface modification; water-soluble polymers; biomaterials

INTRODUCTION

For human beings to survive, it is necessary to continuously deliver O_2 that is needed for the respiration of all tissue cells. Blood, a so-called moving internal-organ, reversibly binds and releases O_2 under physiological conditions. From this point of view, realization of red blood cell (RBC) substitutes, or O_2-infusions, would contribute significantly to human health and welfare. In this research field, the basic sciences for macromolecular complexes, molecular assemblies, and nano-molecular sciences play fundamental roles. The authors have systematically studied the metal complexes (synthetic heme derivatives) embedded into a hydrophobic cluster in aqueous medium, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. As a result, the reaction activity is observed as cooperative phenomena with the properties of the molecular atmosphere. In other words, the development of our O_2-infusion has been based on "the regulation of the electronic process on macromolecular metal complexes".[1,2]

To reproduce the O_2-binding ability of RBCs, that is, the development of a synthetic O_2-carrier that does not need hemoglobin (Hb), was the starting point of the idea for this study. In general, central ferric iron of a heme is immediately oxidized by O_2 in water, preventing the O_2 coordination process from being observed. Therefore, the electron transfer

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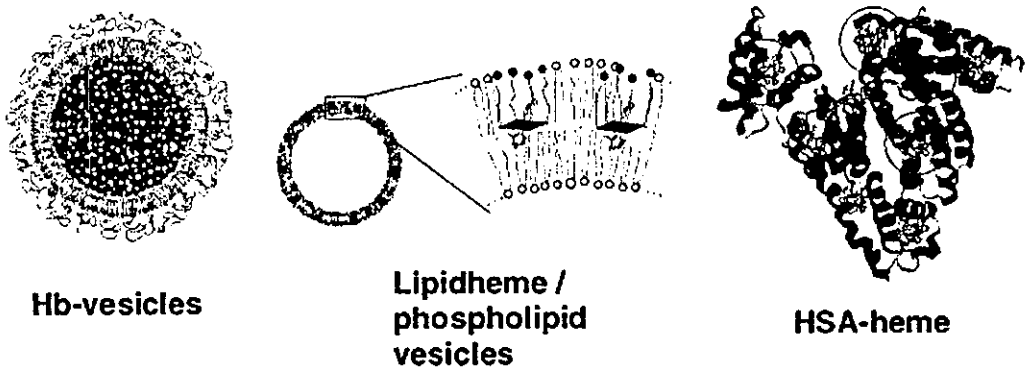

Figure 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme.

must be prevented. Fortunately, the formation of the O_2-adduct complex could be detected but for only several nano-seconds by utilizing the molecular atmosphere and controlling the electron density in the iron center. Based on this finding, the authors succeeded in reversible and stable O_2-coordination in 1983 and preparing phospholipid vesicles embedded amphiphilic-heme, known as lipidheme/phospholipids vesicles (Fig. 1).[3–5] This was the first example of reversible O_2-binding taking place under physiological conditions. For example, human blood can dissolve about 27 ml of O_2 per dl, however a 10 mM lipidheme-phospholipid vesicle solution can dissolve 29 ml of O_2 per dl. This material is suitable for "O_2-infusion". Thus over hundred types of heme derivatives have been synthesized, and recently new lipidheme bearing phospholipid groups have been synthesized, which completes self-organization in water to form stable vesicles.[6]

In 1985, Dr Sekiguchi at Hokkaido Red Cross Blood Center proposed Waseda group to consider the utilization of Hb in outdated RBCs. Thus the research of Hb-vesicles (HbV) based on molecular assembly technologies was started. In the latter 1990s, a mass-production system for recombinant human serum albumin (rHSA) was established and then albumin-heme hybrids (rHSA-heme) using its non-specific binding ability was prepared, which is now considered to be a promising synthetic material. Based on the effective integration of nano-molecular science and technologies for functional materials developed by Waseda University, and the outstanding evaluation system of safety and efficacy developed by Keio University using animal experiments, strong progress on the research of the O_2-infusion project has been made. In the near future, mass production and clinical tests of O_2-infusion will be started by the pharmaceutical industry.

DEVELOPMENT OF Hb-BASED O_2-CARRIERS AND THE CHARACTERISTICS OF HbV

Historically, the first attempt of Hb-based O_2-carrier in this area was to simply use stroma-free Hb (Fig. 2). However, several problems became apparent, including dissociation into

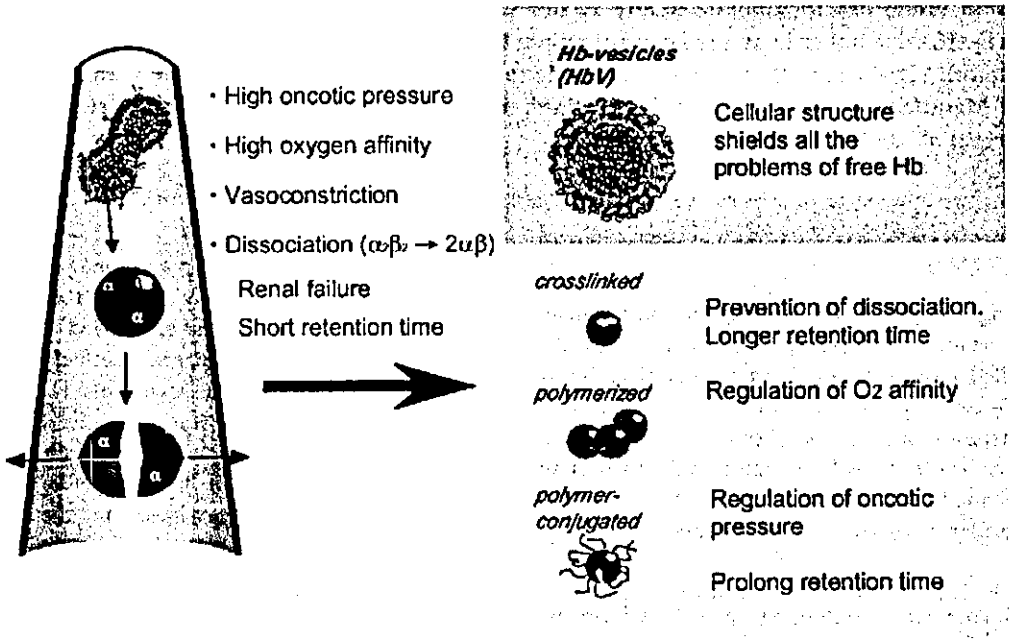

Figure 2. Approaches to solve the problems of utilization of Hb as an O_2-carrier, chemical modification or encapsulation of Hb.

dimers that have a short circulation time, renal toxicity, high oncotic pressure and high O_2-affinity. Since the 1970s, various approaches were developed to overcome these problems.[7,8] This includes intra-molecular crosslinking, polymerization and polymer-conjugation. However, in some cases the significantly different structure in comparison with RBCs resulted in side effects such as vasoconstriction.[9]

Another idea is to encapsulate Hb with a lipid bilayer membrane to solve all the problems of molecular Hb.[10] RBCs have a biconcave structure with a diameter of about 8000 nm. RBCs can deform to a parachute-like configuration to pass through narrow capillaries. The possibility of infection and blood-type mismatching, and short shelf life are the main problems. The idea of Hb encapsulation with a polymer membrane mimicking the structure of RBC is originated from Dr Chang at McGill University.[7] After that, the encapsulation of Hb within a phospholipid vesicle was studied by Dr Djordjevich at the University of Illinois in the 1970s.[11] However, it was not so easy to make HbV with a regulated diameter and adequate O_2-transport capacity, the authors made a breakthrough in routinely producing HbV by using fundamental knowledge of macromolecular and supramolecular sciences.[12–19] Several liters of HbV are routinely prepared in a completely sterile condition. Hb is purified from outdated RBCs, and concentrated to 40 g/dl. Virus removal is performed using a combination of pasteurization at 60° and filtration with a virus removal filter. The Hb encapsulation with phospholipids bilayer membrane and size regulation was performed with an extrusion method. The vesicular surface is modified with polyethylene glycol (PEG) chains. The suspension of Hb-vesicles is deoxygenated at the final stage.

The particle diameter of HbV is regulated to about 250 nm, therefore, the bottle of HbV is turbid. One vesicle contains about 30,000 Hb molecules so that it does not show oncotic pressure. There is no chemical modification of Hb. O_2-affinity is controllable with an appropriate amount of allosteric effectors, pyridoxal 5-phosphate. Hb concentration is regulated to 10 g/dl, and the weight ratio of Hb to total lipid approaches 2.0 by using an ultra pure and concentrated Hb solution of 40 g/dl, which is covered with a thin lipid bilayer membrane. The surface is modified with 0.3 mol% of PEG-lipid. Viscosity, osmolarity, and oncotic pressure are regulated according to the physiological conditions.

HbV can be stored for over 2 years in a liquid state at room temperature.[17] There is little change in turbidity, diameter, and P_{50}. Methemoglobin (MetHb) content decreases due to the presence of reductant inside the HbV, which reduces the trace amount of metHb during storage. This excellent stability is obtained by deoxygenation and PEG-modification. Deoxygenation prevents metHb formation. The surface modification of HbV, with PEG chains prevents vesicular aggregation and leakage of Hb and other reagents inside the vesicles. Liquid state storage is convenient for emergency infusion compared to freeze-dried powder or the frozen state.

IN VIVO EFFICACY OF HbV

The efficacy of HbV has been confirmed mainly with isovolemic hemodilution and resuscitation from hemorrhagic shock.[20–28] In this review two important cases are described. One is isovolemic hemodilution with 90% blood exchange in a rat model. The other is resuscitation from hemorrhagic shock in a hamster model.

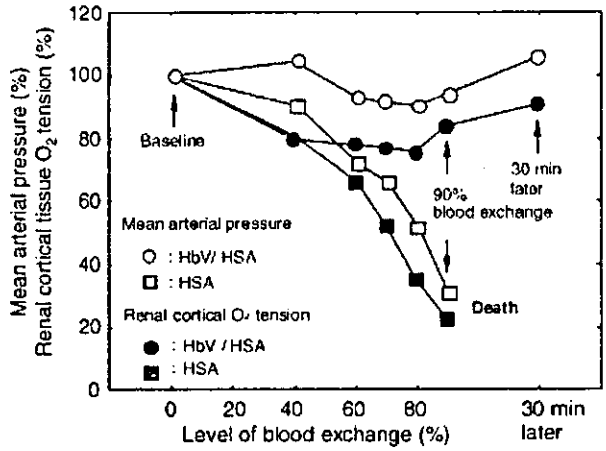

Figure 3. Ninety per cent exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored.

To confirm the O_2-transporting ability of HbV, extreme hemodilution was performed with HbV suspended in human serum albumin (HSA)[21,23] (Fig. 3). The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex, and the blood flow rate in the abdominal aorta was measured with the pulsed Doppler method. Hemodilution with albumin alone resulted in significant reductions in mean arterial pressure and renal cortical O_2 tension, and finally all the rats died of anemia. However, hemodilution with HbV, suspended in HSA sustained both blood pressure and renal cortical O_2 tension, and all the rats survived. These results clearly demonstrate that HbV has sufficient O_2 transporting capability.

To observe the microcirculatory response to the infusion of Hb products, intravital microscopy was used equipped with all the units to measure blood flow rates, vascular diameter, O_2 tension, and so on, in collaboration with Dr Intaglietta at the University of California, San Diego. The hamster dorsal-skin fold preparation allows observation of blood vessels from small arteries down to capillaries. The HbV suspension, as a resuscitative fluid for hemorrhagic-shocked hamsters was evaluated.[26] About 50% of the blood was withdrawn, and the blood pressure was maintained at around 40 mmHg for 1 hr, and the hamsters either received HbV suspended in HSA (HbV/HSA), HSA alone, or shed blood (Fig. 4). Immediately after infusion, all the groups showed increases in mean arterial pressure. However, only the albumin infusion resulted in incomplete recovery. However, the HbV/HSA group showed the same recovery with the shed autologous blood infusion. During the shock period, all the groups showed significant hyperventilation that was evident from the significant increase in arterial O_2 tension. Simultaneously, base excess and pH decreased significantly. Immediately after resuscitation, all the groups tended to recover. However, only the HSA group showed sustained hyperventilation. Base excess for the HSA group remained at a

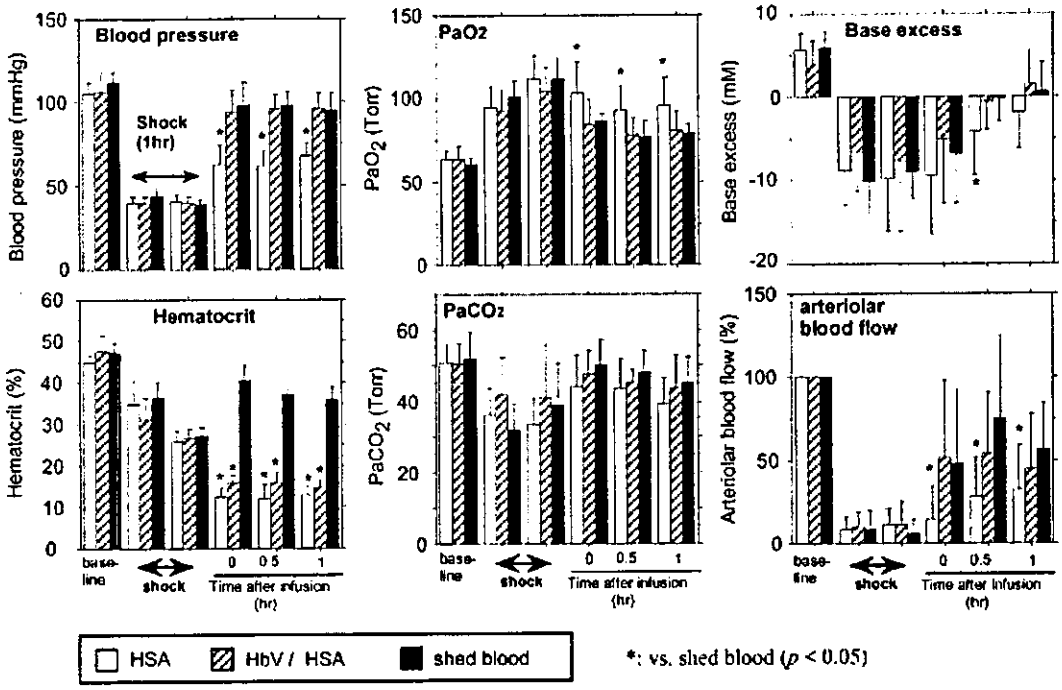

Figure 4. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD.

significantly lower value 1 hr after resuscitation. Blood flow decreased significantly in arterioles to 11% of basal value during shock. The HbV/HSA and shed autologous blood groups immediately showed significant increases in blood flow rate after resuscitation, while the albumin group showed the lowest recovery.

SAFETY EVALUATION OF HbV

The safety profile of HbV such as cardiovascular responses, pharmacokinetics, influence on RES, influence on clinical measurements and daily repeated infusions were further examined.[29–37]

The microvascular responses to the infusion of intramolecularly crosslinked Hb (XLHb) and HbV were studied using conscious hamsters. XLHb (7 nm in diameter) showed a significant increase in hypertension equal to 35 mmHg, and simultaneous vasoconstriction of the resistance artery equal to 75% of the baseline levels[30] (Fig. 5). However, HbV with diameter of 250 nm showed minimal changes. The small acellular XLHb is homogeneously dispersed in the plasma, and it diffuses through the endothelium layer of the vascular wall and reaches the smooth muscle. XLHb traps nitric oxide (NO) as an endothelium-derived relaxation factor, and induces vasoconstriction, and hypertension. However, the large HbV stay in the lumen and does not induce vasoconstriction. Several mechanisms are proposed for Hb-induced vasoconstriction. These include NO-binding, excess O_2 supply, reduced shear stress, or the presence of Hb recognition site on the endothelium. But it is clear that Hb-encapsulation shields against the side effects of acellular Hbs.

Professor Suematsu at Keio University has revealed the effects of Hb-based O_2 carriers in hepatic microcirculation[29,32] (Fig. 6). On the vascular wall of the sinusoid in hepatic microcirculation, there are many pores, called fenestration, with a diameter of about 100 nm. The small Hb

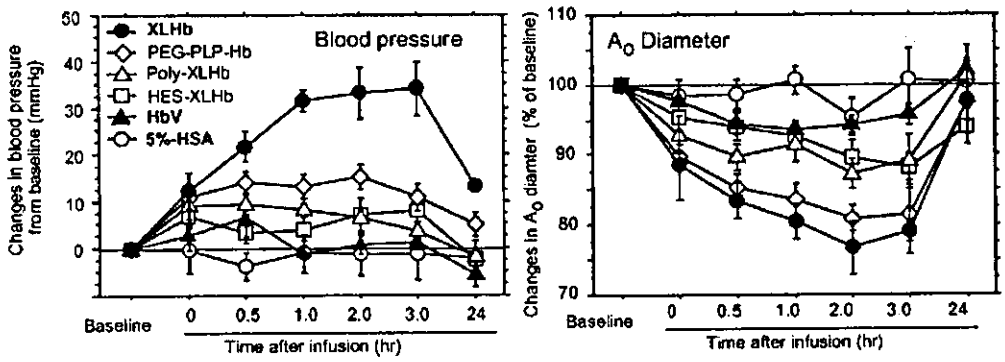

Figure 5. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2-carriers. Mean ± SD.

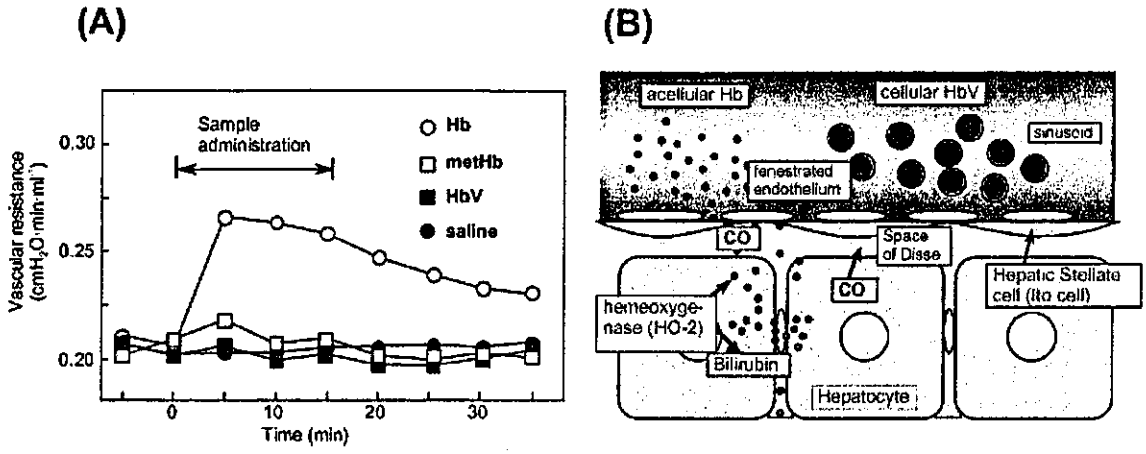

Figure 6. (A) Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. (B) Schematic representation of hepatic microcirculation: the small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolized by hemeoxygenase-2 (HO-2) and CO is released as a vasorelaxation factor. However, the excess amount of the extravasated Hb traps CO and induces vasoconstriction and the resulting higher vascular resistance. However, the larger HbV retains in the sinusoid and there is no extravasation and vasoconstriction.

molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. However, HbV particles, which are larger than the pores, do not extravasate. Heme of extravasated Hb is excessively metabolized by hemeoxygenase-2 in hepatocyte to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb rapidly binds CO, resulting in the vasoconstriction and an increase in vascular resistance. Furthermore, HbV (250 nm in diameter) is large enough to remain in the sinusoid, and the vascular resistance is maintained.

From these results, the optimal molecular dimension of Hb-based O_2 carriers can be proposed. The upper limitation is below the capillary diameter to prevent capillary plugging, and for sterilization by membrane filters (Fig. 7). However, smaller sizes exhibit a higher rate of vascular wall permeability with side effects such as hypertension and neurological disturbances. HbV exhibits a very low level of vascular wall permeability. Therefore, the HbV appears to be appropriate from the viewpoint of hemodynamics. However, the influence of HbV on the RES has to be clarified, because the fate of HbV is RES trapping.

Circulation persistence was measured by monitoring the concentration of radioisotope-labeled HbV in collaboration with Dr Phillips at the University of Texas at San Antonio. The circulation half-life is dose dependent, and when the dose rate was 14 ml/kg, the circulation half-life was 35 hr in rats. The circulation time in the case of the human body can be estimated to be twice as long; or about 3 days at the same dose rate. Gamma camera images of radioisotope-labeled HbV

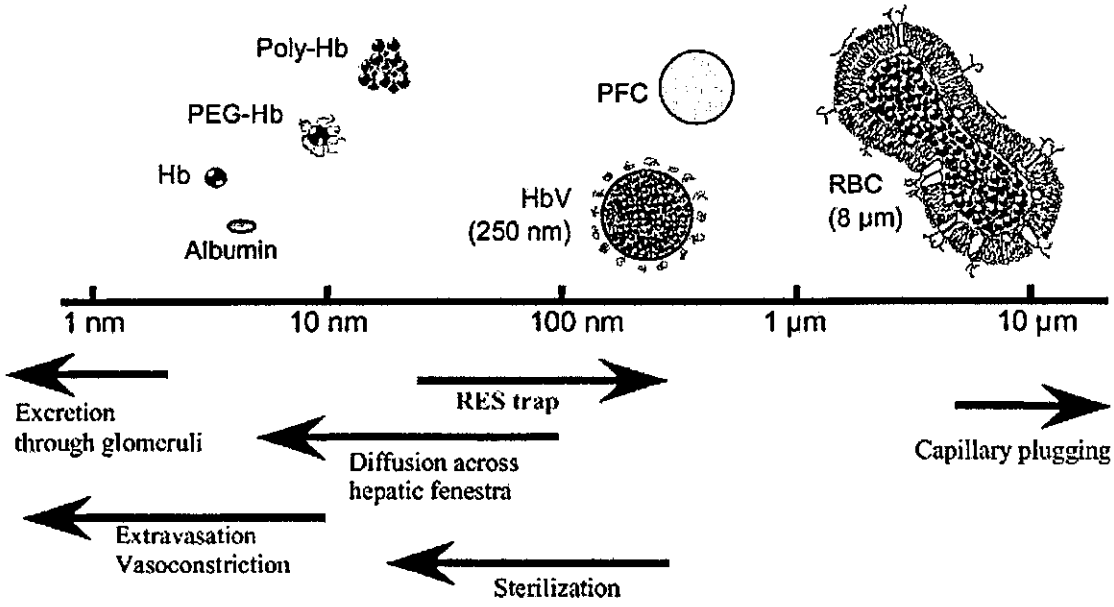

Figure 7. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process.

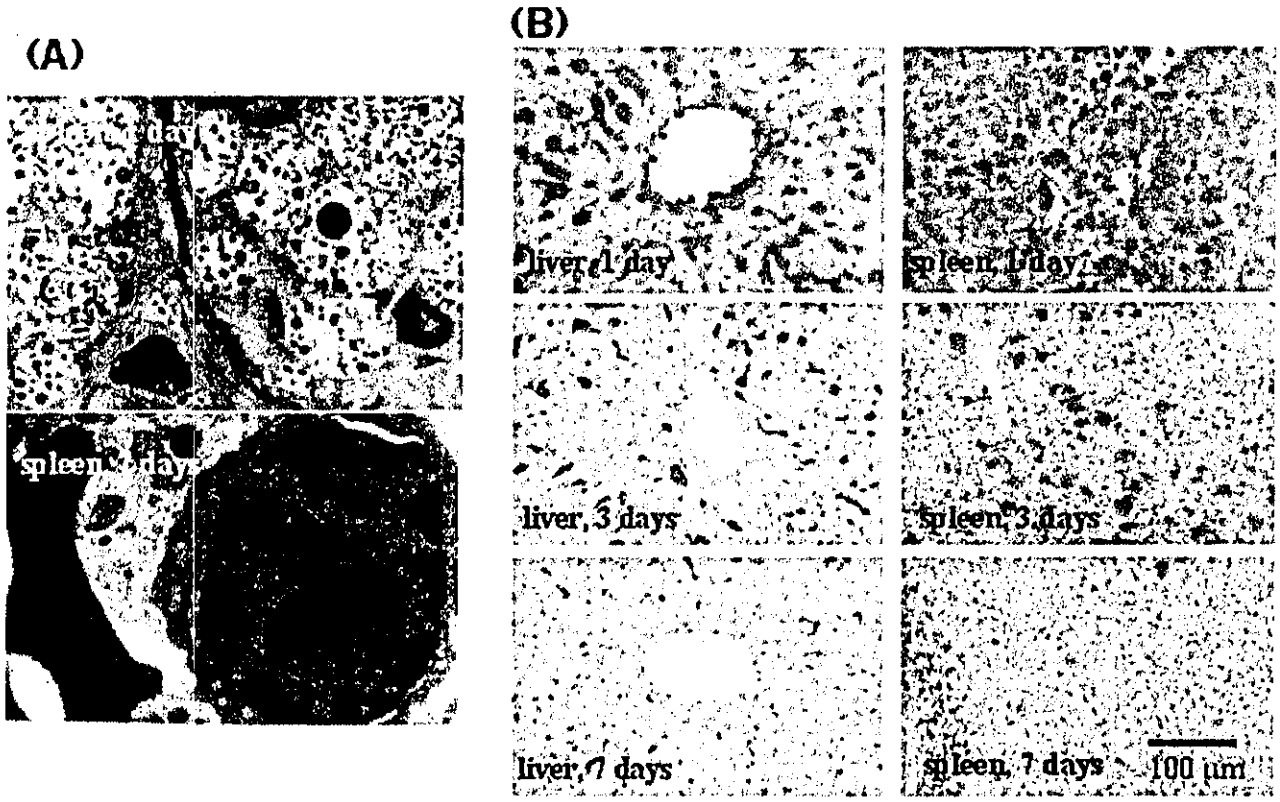

Figure 8. (A) TEM of rat spleen 1 day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. (B) Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. HbV particles disappeared within 7 days.

showed the time course of biodistribution. After HbV finished playing its role in O_2-transport, a total of 35% of HbV are finally distributed mainly in the liver, spleen and bone marrow. The transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes[34] (Fig. 8). RBCs and HbV contain a lot of ferric ion with a high electron density, so that they show strong contrast in TEM. However, after 7 days, the HbV structure cannot be observed. There were no abnormalities in the tissues and no irreversible damages to the organs. A polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV. This antibody does not recognize rat Hb. The red colored parts indicate the presence of Hb in HbV, and they have almost disappeared after 7 days in both the spleen and liver. Therefore, this shows that HbV can be metabolized quite promptly.

One issue of the Hb-based O_2-carriers is that they have a significant influence on clinical laboratory tests. They remain in the plasma phase in hematocrit capillaries after centrifugation of blood samples, and interfere with the colorimetric and turbidimetric measurements. However, HbV can be simply removed from blood plasma either by ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran to enhance precipitation. A very clear supernatant for accurate analyses can be obtained.[35] This is one advantage of HbV in comparison with acellular Hb solutions. Accordingly, the influence on organ functions by serum clinical laboratory tests after the bolus infusion of HbV at a dose rate of 20 ml/kg was examined. Albumin, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, which reflect the liver function, moves their values within normal range.[36] Concentrations of bilirubin and ferric ion are maintained at a low level. The concentration of lipids transiently changed. In particular, the cholesterols increased significantly. And phospholipids slightly increased, however, they returned to the original level after 7 days. These results indicate that the membrane components of HbV, once they reappear from RES, are metabolized on the physiological pathway.

A test of daily repeated infusion is required to evaluate the safety of a new drug. The daily repeated infusion of HbV in Wistar rats at a dose rate of 10 ml/kg/day for 14 days, everyday was tested.[37] The total infusion volume (140 ml/kg) was 2.5 times as much as the volume of the whole blood (56 ml/kg), however, all rats tolerated it well and survived. The body weight showed a monotonous but slightly depressed increase in comparison with the saline. However, after 2 weeks there was no significant difference with the saline control group. All the rats seemed very healthy and active. Histopathological examination 1 day after the final infusion of HbV showed significant accumulation of HbV in spleen macrophages, and liver Kupffer cells, and they mostly disappeared after 14 days. There were no irreversible other morphological abnormalities, and the serum clinical chemistry indicated transient but reversible increases in lipid components. AST and ALT were within the normal range. From these results the authors are confident with the safety of HbV.

DESIGN AND PHYSICOCHEMICAL PROPERTIES OF rHSA-HEME

In this study research on totally synthetic O_2-carriers, or so-called albumin-heme that does not require Hb has been conducted. HSA is the most abundant plasma protein in our blood stream, but its crystal structure has not been elucidated for a long time. In 1998, Dr Stephen Curry of the Imperial College London first elucidated the crystal structure of the HSA complexed with seven molecules of myristic acids.[38] He found that the dynamic conformational changes of albumin take place by the binding of fatty acid. However, in Japan, rHSA is now manufactured on a large scale by expression in the yeast *Pichia pastoris*, and it will appear on the market soon.[39] A large-scale plant, which can produce one million vials per year, has been already established. From the viewpoint of clinical application, O_2-carrying albumin is quite exciting and may be of extreme medical importance. With this background, it has been found that synthetic heme derivative is efficiently incorporated into rHSA, creating a red-colored rHSA-heme hybrid. This rHSA-heme can reversibly bind and release O_2 molecules under physiological conditions in the same manner as Hb. In other words, the rHSA-heme hybrid is a synthetic O_2-carrying hemoprotein, and it is believed that its saline solution will become a new class of RBC substitute.[40–51]

Figure 9 summarizes the structure of the rHSA-heme molecule. The maximal binding numbers of heme to one albumin are eight, and the magnitude of the binding constants ranged from 10^6 to 10^4 (M^{-1}). The isoelectric point of rHSA-heme was found to be 4.8, independent of the binding numbers of heme. This value is exactly the same as that of albumin itself. Furthermore, the viscosity and density did not change after the incorporation of heme molecules, and the obtained solution showed a long shelf life of almost 2 years at room temperature. Since the O_2-binding sites of rHSA-heme are iron-porphyrin, the color of the solution changed in a similar way to Hb. Upon addition of O_2 gas through this solution, the visible absorption pattern immediately changed to that of the O_2-adduct complex. Moreover, after bubbling carbon monoxide gas, rHSA-heme formed a very stable carbonyl complex.

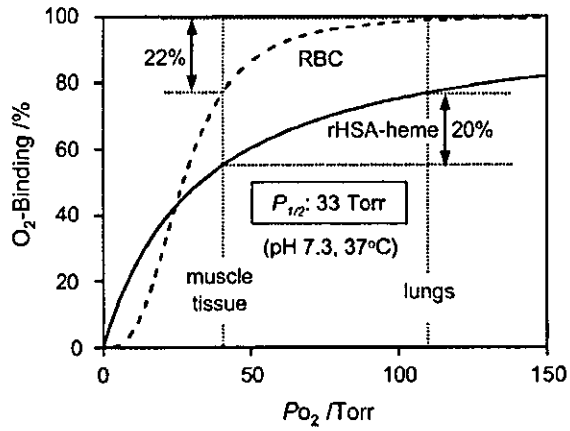

Figure 10. O_2-binding equilibrium curve of albumin-heme.

Figure 10 shows the O_2-binding equilibrium curve of rHSA-heme. The O_2-binding affinity of rHSA-heme is always constant independent of the number of heme, and the O_2-binding profile does not show cooperativity. However, the O_2-transporting efficiency of rHSA-heme between the lungs measuring 110 Torr and muscle tissue measuring 40 Torr increases to 22%, which is identical to the 22% efficiency for RBCs. The O_2-binding property of rHSA-heme can be controlled by changing the chemical structure of heme derivatives incorporated. More recently, it has been found that a protoheme derivative is also incorporated into albumin and can bind and release O_2 as well.[52]

IN VIVO SAFETY AND EFFICACY OF rHSA-HEME

Based on these findings, it can be said that rHSA-heme can become an entirely synthetic O_2-carrier, and satisfy the initial clinical requirements for a RBC substitute. However, there is another problem to solve before this material can be used as an O_2-carrier in the circulatory system. This problem is NO scavenging. Of course, rHSA-heme can bind NO, and it may be anticipated that the injection of rHSA-heme also induce hypertensive action. The authors have evaluated the

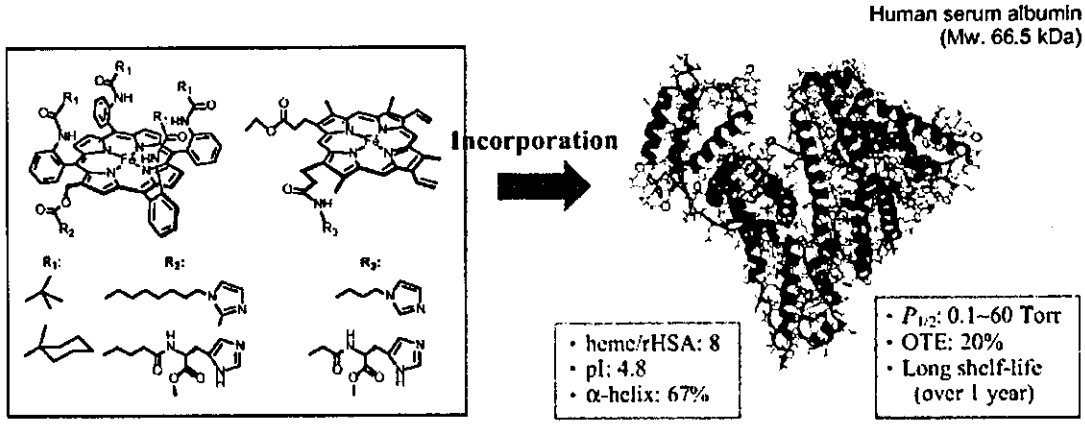

Figure 9. Structure of the albumin-heme molecule.

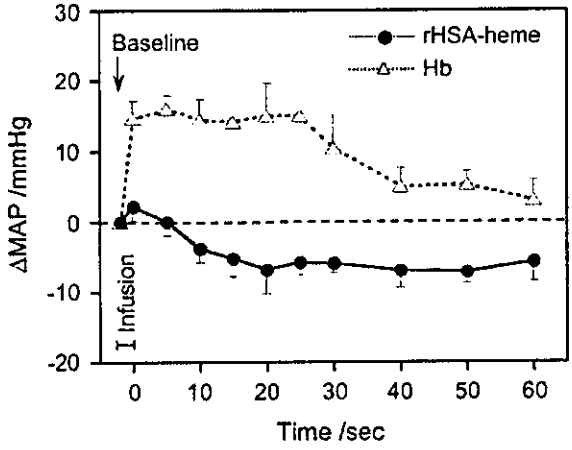

Figure 11. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats ($n=5$). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± SE. Basal value is 90.1 ± 3.0 mmHg.

efficacy and safety of this rHSA-heme solution with animal experiments.

As described earlier, small Hb molecules extravasate through the vascular endothelium and react with NO, thus inducing vasoconstriction and acute increases in systemic blood pressure. Contrary to the expectations, the observation of the intestinal microcirculation after the infusion of rHSA-heme into an anesthetized rat revealed that the diameters of the venules and arterioles were not deformed at all.[53] Indeed, only a small change in the mean arterial pressure was observed after the administration of the rHSA-heme solution (Fig. 11). In contrast, the infusion of Hb elicited an acute increase in blood pressure. Why does rHSA-heme not induce vasoconstriction or hypertension? The answer probably lies in the negatively charged molecular surface of albumin. One of the unique characteristics of serum albumin is its low permeability through the muscle capillary pore, which is less than 1/100 that for Hb due to the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells.

Thus the authors are now evaluating the O_2-transporting ability of this rHSA-heme molecule in the circulatory system with further animal experiments.[54] First, the physiological responses to exchange transfusion with rHSA-heme solution into rats after 70% hemodilution and 40% hemorrhage was determined (Fig. 12). The declined mean arterial pressure and blood flow after a 70% exchange with albumin and further 40% bleeding of blood showed a significant recovery of up to 90% of the baseline values by the infusion of the rHSA-heme solution. However, all rats in the control group only injected with albumin died within 30 min. Furthermore, muscle tissue O_2-tension significantly increased. These responses indicate the *in vivo* O_2-delivery of the rHSA-heme solution.

More recently, HSA dimer, which can incorporate 16 hemes in its hydrophobic domain has been synthesized.[55] The human serum rHSA-heme dimer solution dissolves 1.3-times more O_2 compared to that of RBC and keeps its colloid osmotic pressure at the same level as the physiological value.

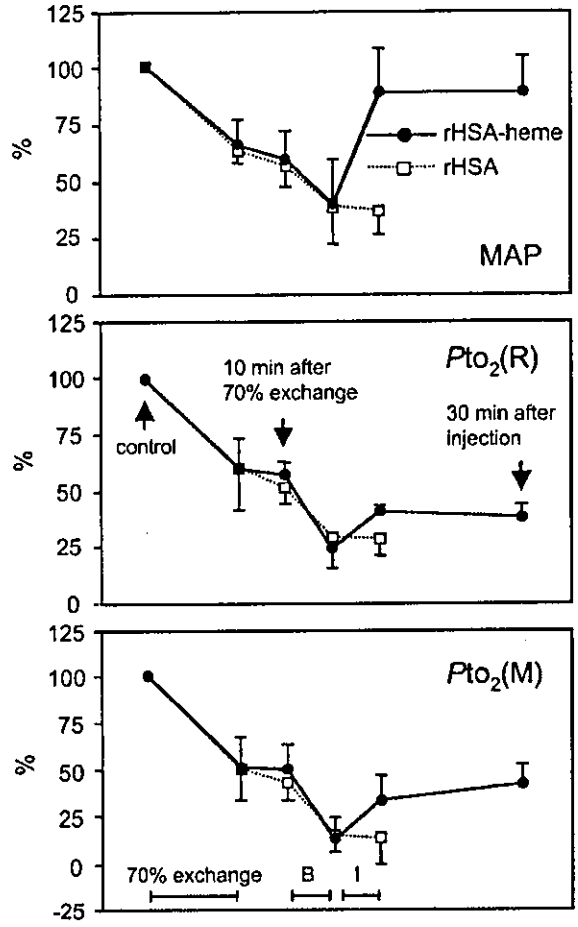

Figure 12. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats ($n=5$). All data are shown as changes from the basal values and expressed as mean ± SE.

POTENTIAL APPLICATIONS OF ARTIFICIAL O_2 CARRIERS

As described earlier the primary application of artificial O_2-carriers would be the resuscitative fluid for hemorrhage. Since some of the characteristics of artificial O_2-carriers overwhelm those of donated blood, there are many potential applications other than blood substitutes.

Tumor oxygenation

Unlike vessels in normal tissues, the development of a vasculature in a tumor lacks regulation and is hence, highly heterogeneous. Consequently, areas of hypoxia are quite common in tumors. In these hypoxic regions, it can be added that tumor cells acquire resistance to treatments such as chemotherapy and radiation. The rHSA-heme was injected into the responsible artery that supplies circulation to an implanted tumor (Fig. 13).[56] O_2-tension of the tumor rises immediately after intra-arterial infusion of albumin heme up to 2.4 times that of the baseline value. The findings in animals indicate that tumor tissue O_2-levels can be elevated by the administration of artificial O_2-carriers due to the

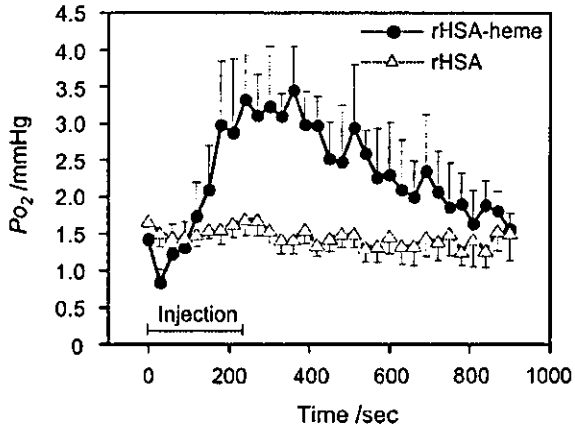

Figure 13. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats ($n=4$ each). All data are shown as changes from the basal values (P_{O_2}) just before the infusion and expressed as mean ± SE.

difference in O_2-transporting properties from RBCs. Whether this increase in tissue O_2 can potentiate cancer treatment is currently under investigation.

Oxygenation of ischemic tissue

Tissue ischemia can ensue from impairment of peripheral perfusion due to a variety of diseases such as arteriosclerosis obliterans, diabetes, and Burger's disease. The key event in the progression of ischemic diseases is the inability of red cells to flow through the capillaries, beyond which point ulceration and gangrene formation become imminent. It is believed that this critical phase can be avoided or delayed by the application of artificial O_2-carriers, which can be designed to flow even through these damaged capillaries.[27,28]

Organ preservation

One of the most important agenda in transplantation medicine is long-term organ preservation and circumvention of ischemia reperfusion injuries. It is believed that artificial O_2-carriers can be applied as a perfusate for donor tissue in order to overcome these problems. In particular, its O_2 carrying capacity has the potential to significantly extend the preservation period. This will make it easier to transport organs. Also, utilizing the extra time, it may be possible in the future to perform additional organ tests for better compatibility, or even perform genetic modifications during this period. It is believed that through these applications, the concept of organ preservation can be expanded to culture organs, and furthermore to include the preservation of cells derived from donor tissues.

Extracorporeal circulation

Extracorporeal circulation is quite common in cardiac surgery. Improvements are being made in the priming solutions but red cells are often still required to fill the device circuit, particularly in compromised cases and in children.[57] It is believed that the use of artificial O_2-carriers in the priming solution can decrease or completely eliminate the need for a transfusion in such cases, and hence reduce the incidence of infection or graft-versus-host disease (GVHD).

Liquid ventilation for acute lung injury

For patients who present acute lung injury or acute respiratory distress syndrome (ARDS), gas exchange in the lung exhibits severe deterioration and sometimes even the newest mechanical ventilation method fails to establish adequate oxygenation of the blood. In this type of critical case, liquid ventilation using an artificial O_2-carrier can establish optimal oxygenation of the blood and may reproduce the integrity of lung parenchyma.[58] Briefly explained, oxygenated liquid ventilation fluid is administered into the lung through trachea and O_2 molecules are transferred through diseased alveolus by diffusion and oxygenate the blood. Currently, this method is thought to be effective for patients with congenital diaphragmatic herniation. Efficacy for adult acute lung injuries is now under investigation. Perfluorochemicals are the main fluid used for clinical use, however, aqueous artificial O_2-carriers may have the potential to be used for liquid ventilation.

FUTURE SCOPE

The research field of the red cell substitutes is moving forward very rapidly, and the paradigm in this field is expanding from red cell substitutes to "O_2 therapeutics". Significant efforts have been made to produce HbV and albumin-heme with a facility of GMP standard, and to start preclinical and finally clinical trials. We look forward to the day that our research will play an effective role in treating patients.

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分野：基礎、臨床、その他

ランニングタイトル(簡略タイトル)：Developmental Trend of Artificial Blood

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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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