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.

Acknowledgments. This work was partially supported by Health Science Research Grants from MHLW, Grant-in-Aid for Scientific Research (No. 16350093) from JSPS, and Grant-in-Aid for Exploratory Research (No. 16655049) from MEXT. The authors are grateful to NIPRO Corp. for their supporting the oxygen-infusion project. We also thank Mr. Seiji Ishihara (Waseda University) for his skilful physicochemical experiment.

References

1. Homepage of Ministry of Health, Labor, and Welfare, Japan (2003) (http://www.mhlw.go.jp/shingi/2003/06/s0627-12.html)
2. Tolosano E, Altruda F (2002) Hemopexin: structure, function, and regulation. DNA Cell Biol 21:297–306
3. Paoli M, Anderson BF, Baler HM, et al (1999) Crystal structure of hemopexin reveals a novel high-affinity heme site formed between two β-propeller domains. Nat Struct Biol 6:926–931
4. Adams PA, Berman MC (1980) Kinetics and mechanism of the interaction between human serum albumin and monomeric heamin. Biochem J 191:95–102
5. Zunszain PA, Ghuman J, Komatsu T, et al (2003) Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. BMC Struct Biol 3:6
6. Komatsu T, Hamamatsu K, Wu J, et al (1999) Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*-pivamido)phenylporphyrinatoiron(II) derivatives. Bioconjug Chem 10:82–86
7. Komatsu T, Hamamatsu K, Tsuchida E (1999) Cross-linked human serum albumin dimers incorporating sixteen (tetraphenylporphinato)iron(II) derivatives: synthesis, characterization, and O_2-binding property. Macromolecules 32:8388–8391
8. Tsuchida E, Komatsu T, Mastukawa, et al (1999) Human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinato-iron(II) derivative as a totally synthetic O_2-carrying hemoprotein. Bioconjug Chem 10:797–802
9. Komatsu T, Matsukawa Y, Tsuchida E (2000) Kinetics of CO and O_2 binding to human serum albumin-heme hybrid. Bioconjug Chem 11:772–776
10. Komatsu T, Matsukawa Y, Tsuchida E (2001) Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphyrinatoiron(II) derivatives. Bioconjug Chem 12:71–75
11. Komatsu T, Okada T, Moritake M, et al (2001) O_2-binding properties of double-sided porphyrinatoiron(II)s with polar substituents and their human serum albumin hybrids. Bull Chem Soc Jpn 74:1695–1702

12. Huang Y, Komatsu T, Nakagawa A, et al (2003) Compatibility in vitro of albumin-heme (O_2 carrier) with blood cell components. J Biomed Mater Res 66A:292–297
13. Sumi A, Ohtani W, Kobayashi K, et al (1993) Purification and physicochemical properties of recombinant human serum albumin. Biotechnol Blood Proteins 227:293–298
14. Tsuchida E, Komatsu T, Hamamatsu K, et al (2000) Exchange transfusion of albumin-heme as an artificial O_2-infusion into anesthetized rats: physiological responses, O_2-delivery and reduction of the oxidized hemin sites by red blood cells. Bioconjug Chem 11:46–50
15. Chang TMS (1997) Recent and future developments in modified hemoglobin and microencapsulated hemoglobin as red blood cell substitutes. Artif Cells Blood Substit Immobil Biotechnol 25:1–24
16. Tsuchida E (1998) Perspectives of blood substitutes. In: Tsuchida E (ed) Blood substitutes: present and future perspectives. Elsevier Science, Lausanne, pp 1–14
17. Winslow RM (1998) The role of blood substitutes in emerging healthcare systems. In: Tsuchida E (ed) Blood substitutes: present and future perspectives. Elsevier Science, Lausanne, pp 15–32
18. Winslow RM (1999) New transfusion strategies: red cell substitutes. Annu Rev Med 50:337–353
19. Squires JE (2002) Artificial blood. Science 295:1002–1005
20. Keipert P, Chang T (1998) Pyridoxylated-polyhemoglobin solution: a low viscosity oxygen-delivery blood replacement fluid with normal oncotic pressure and long term storage feasibility. Biomater Artif Cells 16:185–196
21. Keipert PE, Gonzales A, Gomez CL, et al (1993) Acute changes in systemic blood pressure and urine output of conscious rats following exchange transfusion with diaspirin-crosslinked hemoglobin solution. Transfusion 33:701–708
22. Hess JR, MacDonald VW, Brinkley WW (1993) Synthetic and pulmonary hypertension after resuscitation with cell-free hemoglobin. J Appl Physiol 74:1769–1778
23. Schultz SC, Grady B, Cole F, et al (1993) A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med 122:301–308
24. Thompson A, McGarry AE, Valeri CR, et al (1994) Stroma-free hemoglobin increases blood pressure and GFR in the hypotensive rat: role of nitric oxide. J Appl Physiol 77:2348–2354
25. Sharma AC, Singh G, Gulati A (1995) Role of NO mechanism in cardiovascular effects of diaspirin cross-linked hemoglobin in anesthetized rats. Am J Physiol 269:H1379–H1399
26. Moisan S, Drapeau G, Burhop KE, et al (1998) Mechanism of the acute pressor effect and bradycardia elicited by disapirin crosslinked hemoglobin in anesthetized rats. Can J Physiol Pharmacol 76:434–442
27. Abassi Z, Kotob S, Pieruzzi F, et al (1997) Effects of polymerization on the hypertensive action of diaspirin cross-linked hemoglobin in rats. J Lab Clin Med 129:603–610
28. Guyton AC, Ross JM, Carrier O, et al (1964) Evidence for tissue oxygen demand as the major factor causing autoregulation. Circ Res 14:1–60
29. Johnson PC (1986) Autoregulation of blood flow. Circ Res 59:483–495
30. Vandegriff KD, Winslow RM (1995) A theoretical analysis of oxygen transport: a new strategy for the design of hemoglobin-based red cell substitutes. In: Winslow RM, Vandegriff KD, Intaglietta M (eds). Blood substitutes: physiological basis of efficiency. Birkhäuser, Boston, pp 143–154
31. Tsai AG, Kerger H, Intaglietta M (1995) Microcirculatory consequences of blood substitution with αα-hemoglobin. In: Winslow RM, Vandegriff KD, Intaglietta M (eds) Blood substitutes: physiological basis of efficiency. Birkhäuser, Boston, pp 155–174

32. Rohlfs RJ, Bruner E, Chiu A, et al (1998) Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem 273:12128–12134
33. Winslow RM (2000) αα-Crosslinked hemoglobin: was failure predicted by preclinical testing? Vox Sang 79:1–20
34. Tsuchida E, Komatsu T, Matsukawa Y, et al (2003) Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging. J Biomed Mater Res 64A:257–261
35. Komatsu T, Matsukawa Y, Tsuchida E (2002) Effect of heme structure on O_2-binding properties of human serum albumin-heme hybrids: intramolecular histidine coordination provides a stable O_2-adduct complex. Bioconjug Chem 13:397–402
36. Beaven H, Chen CH, D'Albis A, et al (1974) A spectroscopic studies of the haemin-human-serum-albumin system. Eur J Biochem 41:539–546
37. Casella L, Gullotti M, Ploi S, et al (1993) Haem-protein interactions: the binding of haem complexes to serum albumin. Gazz Chim Itali 123:149–154
38. Traylor TG, Tsuchiya S, Campbell D, et al (1985) Anthracene heme cyclophanes: steric effects in CO, O_2, and RNC binding. J Am Chem Soc 107:604–614
39. Collman JP, Brauman JI, Iverson BL, et al (1983) O_2 and CO binding to iron(II) porphyrins: a comparison of the "picket fence" and "pocket" porphyrins. J Am Chem Soc 105:3052–3064
40. Sawicki CA, Gibson QH (1977) Properties of the T state of human oxyhemoglobin stideid by laser flash photolysis. J Biol Chem 252:7538–7547
41. Geibel J, Cannon J, Campbell D, et al (1978) Model compounds for R-state and T-state hemoglobins. J Am Chem Soc 100:3575–3585
42. Severinghaus JW (1966) Blood gas calculator. J Appl Physiol 21:1108–1116
43. Imai K, Yonetani T (1975) Thermodynamical studies of oxygen equilibrium of hemoglobin. J Biol Chem 250:7093–7098
44. Sugawara Y, Shikama K (1980) Autoxidation of native oxymyoglobin. Eur J Biochem 110:241–246
45. Mansouri A, Winterhalter H (1973) Nonequivalence of chains in hemoglobin oxidation. Biochemistry 12:4946–4949
46. Sugawara Y, Shikama K (1980) Autoxidation of native oxymyoglobin. Eur J Biochem 110:241–246
47. Steinmeier RC, Parkhurst LJ (1975) Kinetic studies on the five principle components of normal adult human hemoglobin. Biochemistry 14:1564–1573
48. Sharma VS, Schmidt MR, Ranney HM (1976) Dissociation of CO from carboxyhemoglobin. J Biol Chem 251:4267–4272
49. Springer BA, Sligar SG, Olson JS, et al (1994) Mechanism of ligand recognition in myoglobin. Chem Rev 94:699–714
50. Matsuura M, Tani F, Naruta Y (2002) Formation and characterization of carbon monoxide adducts of iron "twin coronet" porphyrins: extremely low CO affinity and a strong negative polar effect on bound CO. J Am Chem Soc 124:1941–1950

Studies on Red Cell Substitutes in Japan and Future Perspectives

Masuhiko Takaori

Key words. Artificial blood, Chemical property, Preclinical assessment, Clinical trial, Clinical efficacy

In 1966 Toyoda [1] at the Department of Surgery, Tokyo University School of Medicine, synthesized polystyrene encapsulated hemoglobin vesicles as artificial red cells and infused them in rabbits intravenously. However, they could not confirm whether or not those vesicles actually transported oxygen to the tissues. In 1979, Naito and Yokoyama [2] of Green Cross, Japan, produced Fluosol DA (Green Cross, Osaka, Japan), mixture of perfluorodecalin and perfluorotripropylamine, which looked like milk and was called "white blood". They performed some clinical trails in Japan. We used this product for a patient who suffered from unexpected massive bleeding and could not get proper blood for transfusion. The patient recovered uneventfully. However, it could be certain whether the Fluosol DA (Green Cross) was absolutely effective for oxygen transport, as it failed to transport sufficient amounts of oxygen to the tissues under normal atmospheric pressure without a high concentration of Fluosol DA [3].

Pharmaceutical companies such as Baxter, Biopure, Hemosol, and Alliance developed various artificial blood products in the past [4]. Data from some of the clinical trials which were performed in Europe [5] and the USA [6] have been published; however, none of the products progressed to clinical use. Nevertheless, the development of artificial blood for clinical use remains an urgent problem, particularly in the face of an expected shortage of blood for transfusion in the near future. In Japan we already occasionally experience

East Takarazuka Sato Hospital, 2-1 Nagao-cho, Takarazuka, Hyogo 665-0873, Japan

shortfalls in blood supply for transfusion, and anticipate a very severe shortfall in the future. Watanabe et al. [7] demonstrated that demand for blood will exceed supply by 2005. We are collecting outdated blood and extracting hemoglobin (Hb) for storage now as a possible solution. We hope to convert the collected blood products into artificial blood for use as a supplement for blood for transfusion in the future.

Recent Progress in Artificial Blood

Since 1990 the research group at Waseda University has developed a liposome encapsulated hemoglobin vesicle (HbV) as a substitute for red blood cells. In 1995 the Terumo company, which was associated with Waseda's scientific research, produced a similar HbV called the Neo Red Cells (NRC) (Terumo, Tokyo, Japan). We tested this product in animal experiments and found that it could transport oxygen to the tissues [8]. Under hemodilution, in which the animal's hematocrit value decreased to about 12%, the mixed venous oxygen saturation could be maintained at mostly normal with NRC's (Terumo) infusion but not with a plasma substitute such as hydroxyethyl starch (HES) solution. Oxygen consumption was maintained sufficiently with NRC (Terumo) but not with HES solution (Table 1).

Similarly Motoki et al. [9] noted that NRC (Terumo) released a greater amount of oxygen in the peripheral tissues compared with autologous blood transfusion (Fig. 1). Cardiac output in our study [6] was maintained satisfactorily with NRC (Terumo). Furthermore, it was noted that the circulating blood volume in the same situation could be maintained at a normal level without adverse effects, particulary life-threating complications. Therefore we believe that NRC can be used in practice.

Yoshizu et al. [10] at Keio University hemodiluted rats in which the hematocrit value was decreased to around 20% with either albumin solution or NRC. They measured tissue oxygen tension in the kidney polarographically and noted that NRC could maintain oxygen tension higher than the albumin solution or autologous blood transfusion did. A similar trend was also noted in skeletal muscle.

Subsequently the Waseda and Keio groups improved the efficiency of HbV for oxygen transport, and recently presented the physicochemical properties [11] (Table 2).

In the meantime, Nishi and Kida [12] at the University of Kumamoto, School of Medicine, Department of Pharmacology, formed pyridoxal phosphate polyethylene conjugated hemoglobin dimer (Fig. 2) as artificial red cells and used them for organ perfusion. This product obtained 24 h half life in the circulation of rats. It was noted, however, that this product scavenged nitric

TABLE 1. Changes in oxygen partial pressure (PvO_2), oxygen saturation (SvO_2), oxygen content (CvO_2) in mixed venous blood and oxygen consumption (VO_2) following hemodilution with Neo Red Cell and HES (hydroxyethyl starch) solution

		Initial	4× hemodilutions	8× hemodilutions	1 h after hemodilution	2 h after hemodilution
PvO_2	H	62 ± 5	52 ± 4	46 ± 6	41 ± 7	41 ± 7
mmHg	N	64 ± 3	50 ± 4	45 ± 4	42 ± 6	42 ± 5
SvO_2	H	73 ± 4	66 ± 5	41 ± 4	38 ± 6	37 ± 9
%	N	73 ± 5	67 ± 6	51 ± 6**	55 ± 6**	52 ± 5**
CvO_2	H	14.7 ± 0.7	8.2 ± 0.4	2.2 ± 0.4	2.4 ± 0.5	2.4 ± 0.4
ml/dl	N	15.1 ± 0.5	8.0 ± 0.5	3.2 ± 0.4**	3.2 ± 0.6**	2.9 ± 0.5**
VO_2	H	91.4 ± 7.4	68.4 ± 5.3	56.2 ± 5.9	46.3 ± 7.5	42.4 ± 6.2
ml/min	N	94.9 ± 7.3	74.3 ± 8.6	74.1 ± 8.4**	77.2 ± 6.1**	80.5 ± 6.9**

mean ± SD.
H, hydroxyethyl starch group; N, Neo Red Cells group.
Source: Takaori M, Fukui A (1996) Treatment of massive hemorrhage with liposome encapsulated hemoglobin (NRC) and hydroxyethyl starch (HES) in beagles.
Artif Cells Blood Substs Immunob Biotechnol 24; 643–653.
Comparison between groups **: $p < 0.01$.

TABLE 2. Physicochemical Properties of HbV

Hb	10.0 g/dl
Hb/Lipid	1.7
PEG-DSPE	0.3 mol %
diameter	247 μm
allosteric effector (Pyridoxal Phosphate/Hb)	2.5 mol/mol
P50	33 mmHg
MetHb	<1.0%
HbCO	<2.0%
Suspension	Saline

Hb, hemoglobin; PEG-DSPE, polyethylene glycole; MetHb, methemoglobin; HbCO, carboxylhemoglobin.

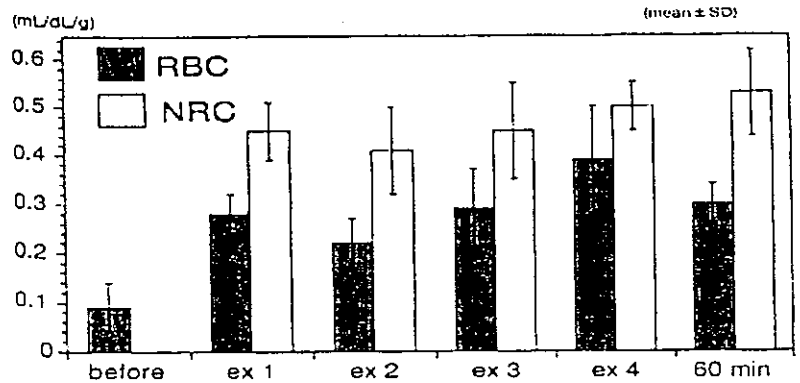

FIG. 1. AV (arteriovenous) oxygen content difference/Hb. Source: Usuba et al. [7]

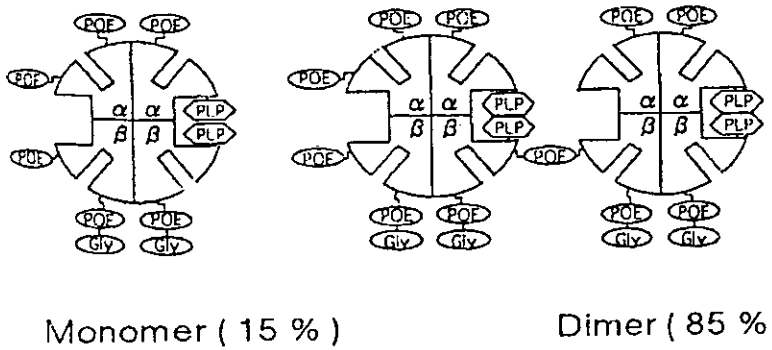

FIG. 2. Molecular structure of PHP. Source: Nishi and Kida [9]. *POE*, polyoxyethylene; *Gly*, glycine; *PLP*, phosphoenolpyruvate

oxide from the endothelium. Therefore, this product was excluded from clinical application.

Design of Artificial Red Cells

Although a search has been attempted for a substance which transports oxygen and releases it at the peripheral tissues more efficiently than hemoglobin, no such substance has yet been found. Therefore we concluded that natural Hb is best for this purpose at the present time.

We were concerned which type of artificial red cell is better, the cellular or acellular type. We concluded that the cellular type is superior to the acellular type for the following reasons:

1. Some substances, for example allostetic substances, such as pyridoxal phosphate as a substitute for 2.3 DPG (diphosphoglycerol), and a reductive substance such as homocysteine as substitute a for methemoglobin reductase, can be encapsulated in a vesicle accompanied with hemoglobin.
2. Greater blood flow may be maintained in the coronary and peripheral minute vessels, since the fluid contained in cellular particles can maintain a more similar blood viscosity, even under high-graded hemodilution.
3. Longer persistence of the artificial red cell in the circulating blood can be obtained with a cellular type. Rapid excretion in urine and in exhaled air, as seen with conjugated hemoglobin and with perfluorocarbon emulsion, respectively, will not occur with the cellular type.
4. Encapsulated Hb can be kept from direct contact with surrounding tissues, such as the endothelium and circulating blood cells, meaning the oxygen-carrying substance is protected and does not affect the surrounding tissues.

The Waseda research group enclosed pyridoxal phosphate in HbV and controlled P50 of hemoglobin at 33 mmHg (Table 2).

It has been found that extremely severe hemodilution with less than 10% of hematocrit value causes an increase in pulse pressure even when systolic pressure decreases slightly. Diastolic pressure is decreased markedly at 40 mmHg, which approaches the critical pressure for corony perfusion. However, when hemodilution can be done, with artificial blood which contains cellular type red cells and viscosity that can be maintained similarly to physiological blood, the perfusion pressure that will be created from peripheral vessel back pressure will be normal.

Retention time of artificial red cells in circulation after infusion needs to be longer than 24 h. If not, oxygen transport to the tissues will be reduced sharply. Since some physiological adaptation is needed, such as hematopoiesis

(2 ml/kg per day for red cell mass), an increase in 2,3 DPG in the red cells would need to be induced in accordance with gradual extravasation of the artificial red cells. Thus we can not safely use artificial blood with a short half life in clinical practice. The half-life of HbV is estimated as 16–18 h by the Waseda study in the rat [11] (Fig. 3). In addition, Tsutsui et al. [13] (Fig. 4), reported that the half-life is about 16–18 h in rats. However, we were recently informed that the half-life of HbV was 36–40 h in a primate (personal/communication, Ogata, Terumo).

Both the above studies present the perplexing problem of the rapid conversion from hemoglobin to metHb in vivo. To address this problem Waseda's investigators tried to encapsulate catalase, as reductase, in the vesicle. They encapsulated various doses of catalase in the vesicle, and found that encapsulation of catalase of 6000 unit/ml reduced the conversion of Hb to MetHb to $^1/_3$ of that of homocysteine alone. They observed that the suppresion of conversion seemed to be dose dependent and its optimum dose has not yet been decided [11].

With regard to direct contact of Hb with the surrounding tissues, Waseda and Keio's investigators bled 50% of circulating blood from rats and left the

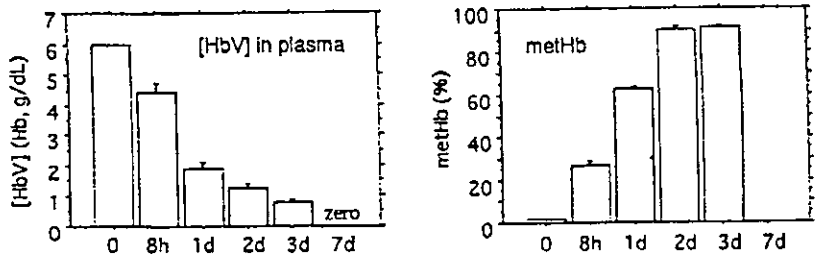

FIG. 3. Retention volume of HbV in plasma and metHb content. Source: Sakai et al. [12]

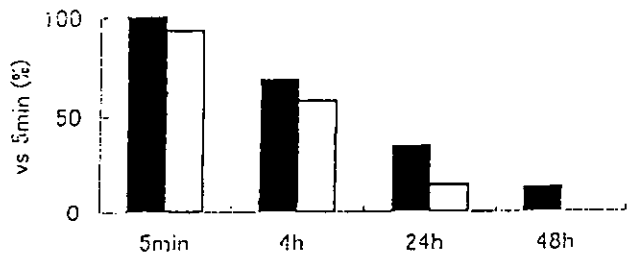

FIG. 4. Blood concentration of NRC on topload model. *Shaded*, total NRC-crit (%. vs 5 min); *unshaded*, NRC-crit with function estimated by NRC-crit and methemoglobin ratio (%. vs 5 min of total NRC-crit). Source: Tsutsui et al. [13]

blood in place for 15 min. They then replaced the blood loss with the withdrawn blood, human albumin solution, and the HbV suspended in 5% human albumin. They noted that no increase in blood pressure occurred immediately after the infusion of HbV (Fig. 5). It is suggested that encapsulation of Hb with liposome membrane blocked nitric oxide scavenging and prevented vasoconstriction [12]. Incidentally, rats treated with the HbV or autologous blood all survived but rats treated with albumin solution did not. In addition, blood lactate levels elevated transiently in a shock state and recovered rapidly after the infusion of HbV and autologous blood. However, lactate levels with the infusion of human albumin recovered in surviving animals, but may remain or elevate in nonsurvival.

On the other hand, some objections could be made against our recommendation for cellular types such as:

1. The complexity of production process. Many processes for the encapsulation of hemoglobin, allostetic substances and metHb reductase, PEGylation, such as coating the surface of HbV with polyethylene glycol, and the extraction of carbon monoxide are required.
2. The cost for cellular artificial red cells would be higher than that of acellular types.
3. Apprehension about phagocytotic tissue retention of the HbV and subsequent immunosuppression is not excluded.

Waseda's investigators observed microscopically that HbVs were captured in microphages and bone marrow cells. Therefore the weight of the liver and spleen was increased respectively. They noted a paradoxical phenomenon that phagocytotic activity for carbon particles infused intravenously increased temporarily after infusion of HbV but recovered completely 7 days later.

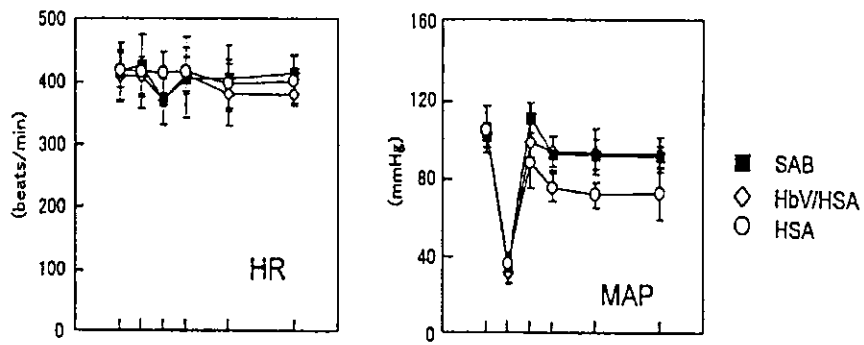

FIG. 5. *HR*, heart rate; *MAP*, mean arterial pressure; *SAB*, autologous blood; *HbV/HSA*, HbV/human albumin; *HSA*, human albumin. Source: Sakai et al. [12]

Abe et al. [15] at the Hokkaido Blood Supply Center tested the effect of HbV on complement hemolytic activity and the complement killing activity of bacteria. They noted that infusion of HbV reduced the hemolytic activity in a dose-dependent manner, and that HbV infusion suppressed the bacteria-killing activity with complements. However, they concluded that the human defense system against bacterial infection would be little influenced [15].

Yanagida et al. [16] noted a severe decrease in immunoglobulins after hemodilution with NRC, in which animal hematocrit was decreased to 7.1%. However, circulating neutrophils and lymphocytes increased after the hemodilution. These changes are similar to those we observed in severe hemodilution with dextran and HES [17]. Therefore, changes observed seem unlikely specific responses to NRC. Incidentally, changes in immunoglobulins and leukocytes recovered within a few days.

Hoka et al. [18] of the Kitazato University School of Medicine, Department of Anesthesiology, studied the effect of HbV on interaction between the neutrophil and the endothelium. Under vital microscopic observation they found in the golden hamster that the migration of the neutrophil through the endothelium layer of the buccal mucous pouch was markedly attenuated with infusion of HbV.

Safety and Efficacy of Artificial Blood

In accordance with the accumulation of data about the safety and efficacy of HbV in pre-clinical studies, a project team supported by the Japanese Ministry of Health and Welfare, sought to make safety and efficacy criteria for the clinical use of HbV.

Several studies referring to chemical and biological safety have been reported [19–22]. However, particularly with HbV, we proposed several measures recently. In the process of encapsulation of Hb in liposome vesicles, it is necessary convert Hb to carbon monoxide Hb. Therefore, it must be confirmed whether or not there will be residual carbon monoxide Hb and carbon monoxide in the solution. Likewise there must be checks to see if excess phospholipid of lipsosome or polyethylene glycol remain in the solution. Obviously endotoxin contamination cannot be allowed. It must be also kept absolutely sterile during storage. The physicochemical properties of HbV as shown in Table 2 have been tested and guaranteed to be absolutely safe.

Concerning the clinical safety of artificial blood, several studies have reported as above. However, a check list for finding adverse effects of HbV on vital functions is shown in Table 3.

Most of those have been tested in pre-clinical studies. However, due to present inability to mass produce HbV, a few items remain to be tested in the near future. Incidentally, the criteria of the Japanese Ministry of Health and Welfare regarding the severity of adverse effects should be applied in pre-clinical studies and clinical trials.

TABLE 3. Safety of HbV solution

Clinical Assessment	
Psychological function and behavior	Mental and nervous function
Tendon and muscle function	Heart and circulartory function
Respiratory and gas exchange function	Hepatic function
Body fluid buffering capacity	Serum electrolyte composition
Renal function	Digestive function
Hemostatic and fibrinolytic function	Hematopoietic function
Endocrine functions	Defensive and immune function
Reproductive function	Teratogenicity
Tumorigenicity	Interaction with commonly used drugs

Regarding the efficacy of artificial blood, however, few criteria have been established. As with the safety criteria, efficacy of HbV as artificial blood should be evaluated from physicochemical activity and clinical efficacy points of view. The following should be considered: (1) oxygen-deliverying capacity, which is mainly controlled by the amount of Hb and the oxygen dissociation curve of Hb inside of the vesicles; (2) conversion rate of Hb to metHb during storage and in vivo after infusion; (3) dispersibility of the vesicles in the solution and blood; (4) size of the vesicle; (5) viscosity of the solution; (6) homogeneity and stability of the vesicles in the solution; and (7) pH of the solution.

The most important property in clinical efficacy is an ability for the oxygen supply to reach tissues. It depends on good pulmonary oxygenation, oxygen extraction in the tissues, a lower conversion rate from Hb to metHb, and having a cardiac output associated with normal circulating blood volume after infusion for blood loss. Finally it depends on adequate retention of the vesicles in the circulation.

After confirmation of safety and efficacy, clinical trials must be performed before use in practice. The Society of Blood Substitutes, Japan, provided guidelines for clinical trials of artificial blood 6 years ago. We intend to follow those guidelines to clinical trial.

Design of Clinical Trials

Clinical trials should satisfy the following two points: (1) good design to obtain definite results for evidence, and (2) obtaining proper informed consent from subjects.

Table 4. Ethical considerations for clinical trials

Clinical trials should be done for the treatment or therapy for patients
Clinical trials should be equivalent or superior to conventional treatment or therapy
Clinical trials must be performed with informed consent

Table 5. Criteria for selection for clinical trial

Replacement of surgical blood loss (15–20 ml/kg)
Hemodilutional autologous blood transfusion
Blood transfusion for unexpected intraoperative bleeding
Blood transfusion for emergent surgery without proper blood
Transfusion for patients with uncommon blood types whose surgery is relatively urgent

Limit to anticipated blood loss of 15–20 ml/kg.

For achievement of definite satisfactory results, four items should be prepared, namely: (1) proper setting of controls for the procedure or treatment, (2) establishing control measures before the procedure or treatment begins, (3) application of routine laboratory tests and simple procedures, and (4) exclusion of subjects with severe illness or subjects with complicated or unstable conditions.

Regarding medical ethics, we must recognize and observe three items listed in Table 4, namely: (1) clinical trials should be performed for treatment or therapy of the patient, (2) the treatment or therapy in clinical trials should be equivalent or superior to conventional treatment or therapy, and (3) clinical trials must be done with the proper informed consent of the patient.

Considering the above restrictions, we designed a clinical trial (Table 5). It will be limited to treatment of 15–20 ml/kg blood loss, such as hemodilutional autologous blood transfusion, transfusion for unexpected surgical bleeding, transfusion in emergent surgery without proper blood preparation, and transfusion for patients with an uncommon blood type whose surgery is relatively urgent.

Some other applications might be proposed (Table 6). We would like to avoid other applications in the first clinical trial, since pathological conditions or illness influence procedures for measurement and accuracy, and so data obtained from those patients would skew the results.

Conclusions

Further assessment and confirmation remain to be done for the safety of HbV. Further improvement of the physicochemical properties of HbV and its mass production should be done in the near future. For example, a fine filter with

Table 6. No program projected first clinical trial for new artificial blood

Post-traumatic hypovolemia
Hemorrhagic shock
Myocardial and cerebral ischemia
Supplemental treatment for idiopathic anemia
Priming for extraorporeal circuit
Superoxygenation for malignant neoplasma therapy
Liquid ventilation
Perfusion and preservation for organ transplantation

Table 7. Social needs for artificial blood

Disaster use	long-term shelf storage
Emergent use	before arrival of matched or O-blood
Shortage of blood for transfusion	
Recycle use of hemoglobin from donated blood	
Application as universal blood	
Avoiding transfusion complications and human errors	
Transfusion for patients with uncommon blood type or with refusal of donated blood	

0.2 μ diameter is used for elimination of bacteria from the product at the present time; however more definite methods for asepsis such as carbon dioxide replenishment or gamma radiation, should be introduced.

Social needs for artificial blood are shown in Table 7. They are all urgent. In my view, blood transfusions as routinely performed in practice today do not fit well with sophisticated medicine. It seems that, in the future, transfusion medicine, particularly red cell transfusion, will be replaced by infusion of artificial blood. Therefore I earnestly hope for great development of artificial blood and its use in clinical practice in the near future.

References

1. Toyoda T (1966) Preclinical studies of artificial blood. J Jpn Soc Surg 1:36–57 (in Japanese)
2. Ohyanagi H, Toshima K, Sekita M, et al (1979) Clinical studies of perfluorochemical whole blood substitutes: Safety of Fluosol-DS (20%) in normal human volunteers. Clin Ther 2:306–312
3. Motoki R, Usuba A (1996) Artificial oxygen-carrier from view point of surgery. Artif Blood 4:56–64
4. Takaori M (2002) Designs for clinical trial of artificial blood (HbV) for evaluation of its efficacy and safety. Artif Blood 10:99–106

5. Spahn DR, van Brempt R, Theilmeier G, et al (1999) Perflubron, emulsion delays blood transfusions in orthopedic surgery. Anesthesiology 91:1195–1208
6. Sloan EP, Koenigsberg M, Gens D, et al (1999) Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial. JAMA 282:1857–1864
7. Watanabe Y, Takahashi K, Kakegawa H, et al (1998) Extrapolation of demand and supply of blood for the next 30 years in Japan based on published goverment demographic projection. Jpn J Transf Med 44:328–335
8. Takaori M, Fukui A (1996) Treatment of massive hemorrhage with liposome encapsulated hemoglobin (NRC) and hydroxy Ethyl starch (HES) in beagles. Artif Cells Blood Substit Immmobil Biotechnol 24:643–653
9. Usuba A, Motoki R, Fukui H, et al (1996) Effect of liposome encapsulated hemoglobin "Neo Red Cells" as a resuscitation fluid for severe hemorrhagic shock. Artif Blood 4:69–78
10. Yoshizu A, Yamahata T, Izumi Y, et al (1997) The oxygen-transporting capability of hemoglobin vesicle, as an artificial oxygen carrier, evaluated in a rat hemorrhagic shock model. Artif Blood 5:18–22
11. Sakai H, Takeoka S, Tsuchida E (2002) Retention volume of HbV in plasma and metHb content. The project study of Japanese Ministry of Health and Welfare reported on April 25th, 2002 Tokyo
12. Nishi K, Kida Y (1996) Effects of hemoglobin-based oxygen-carrying artificial blood substitutes on the circulatory system. Artif Blood 2:82–93
13. Tsutsui Y, Kimura T, Ishizuka T, et al (2002) Duration of efficacy NRC (Neo Red Cell) in a rat hemodilution model. Artif Blood 10:36–41
14. Sakai H, Takeoka S, Tsuchida E (2002) Changes in arterial blood pressure following infusion of HbV for treatment of hemorrhagic shock. The project study of Japanese Ministry of Health and Welfare reported on April 25th, 2002 Tokyo
15. Abe H, Azuma H, Ikeda H (2002) Serum complement hemolytic activity after infusion of HbV. The project study of Japanese Ministry of Health and Welfare reported on April 25th, 2002 Tokyo
16. Yanagida N, Kawamura A, Tanaka M, et al (2001) Study on immune state of high grade blood exchange (Hemodilution Hct 43.5 → 7.1) using NRC in rats. Artif Blood 9:42–45
17. Lewis J, Szeto ILF, Bayer WL, et al (1966) Severe hemodilution with hydroxyethyl starch and dextrans. Arch Surg 93:941–950
18. Hoka S, Ozawa A, Nara Y (2003) Effect of artificial oxygen carrier on neutrophil movement in the microvasculature of hamster cheek pouch. Artif Blood 11:67
19. Center for Biologic Evaluation and Research (1991) Points to consider in the safety evaluation of hemoglobin-based oxygen carriers. Transfusion 31:369–371
20. Center for Biologic Evaluation and Research (1994) Points to consider in the safety evaluation of hemoglobin-based and perfluorocarbon-based oxygen carriers. Transfusion 34:712–713
21. Przybelski RL, Daily EK, Stern KN, et al (1997) A grade scale for assessment of safety of blood substitutes. Transfusion 37:749–751
22. Fratantoni JC (1998) Red cell substitutes: evolution of approaches for demonstrating efficacy. In: Tsuchida E (ed) Blood Substitutes—Present and Future Perspectives. Elsevier, Amsterdam, pp 33–39

POLYMERS FOR ADVANCED TECHNOLOGIES
Polym. Adv. Technol. 2005; 16: 73–83
Published online 7 January 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/pat.559

PAT

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

Eishun Tsuchida[1]*, Hiromi Sakai[1], Teruyuki Komatsu[1], Shinji Takeoka[1], Yubin Huang[1], Keitaro Sou[1], Akito Nakagawa[1], Yuji Teramura[1] and Koichi Kobayashi[2]

[1]Advanced Research Institute for Science & Engineering, Waseda University, Tokyo 169-8555, Japan
[2]Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan

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

*Correspondence to: E. Tsuchida, Professor, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan.
E-mail: eishun@waseda.jp
†Selected paper presented at the 7th International Symposium on Polymers for Advanced Technologies, 21–24 September 2003, Fort Lauderdale, Florida, USA.

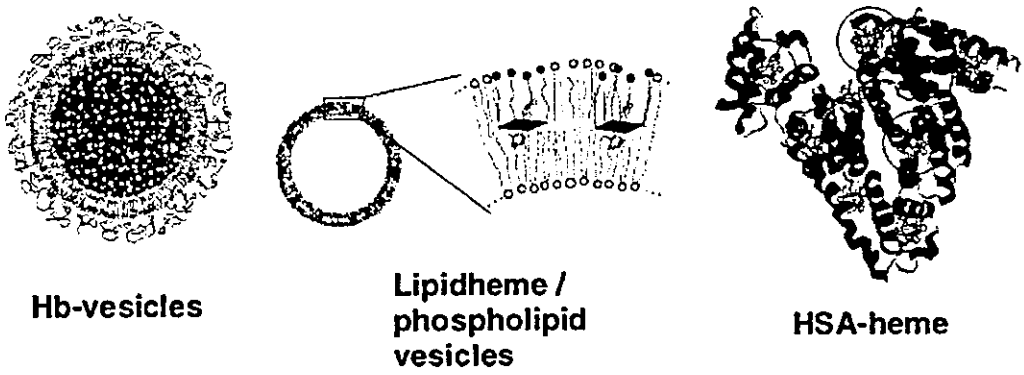

Figure 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme.

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

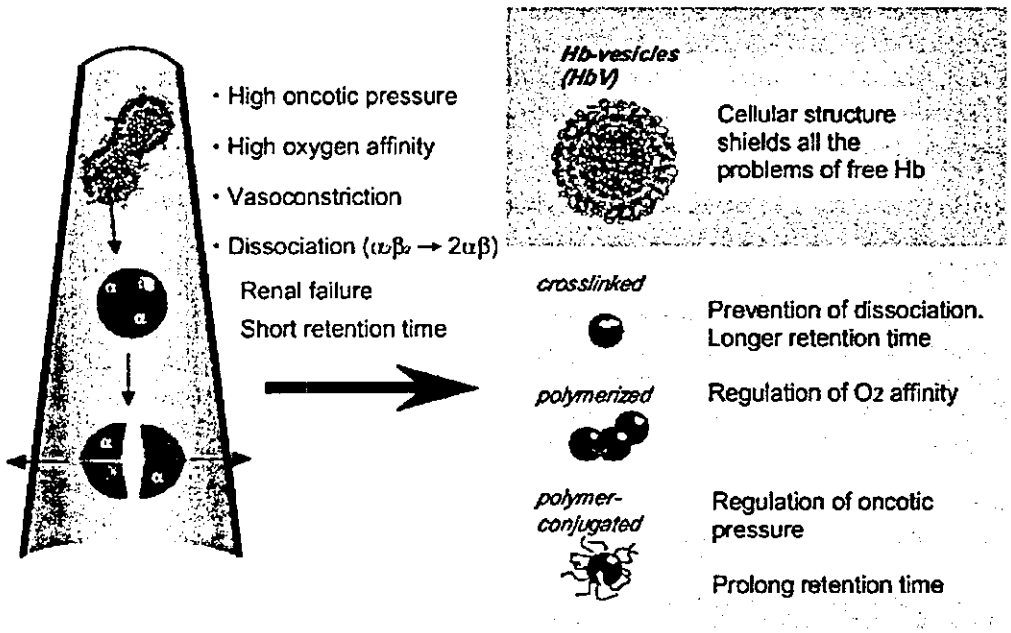

Figure 2. Approaches to solve the problems of utilization of Hb as an O_2-carrier, chemical modification or encapsulation of Hb.

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.

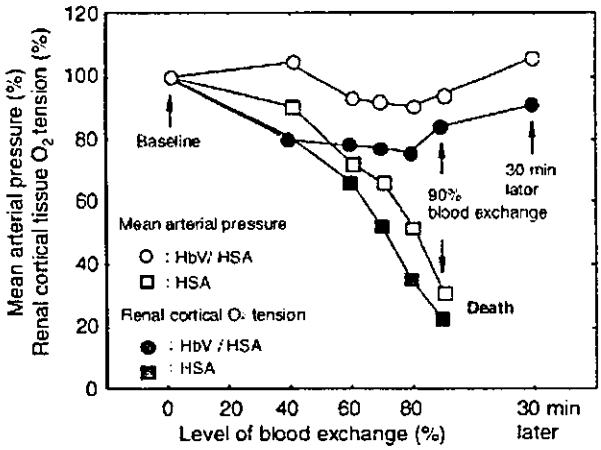

Figure 3. Ninety per cent exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored.

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

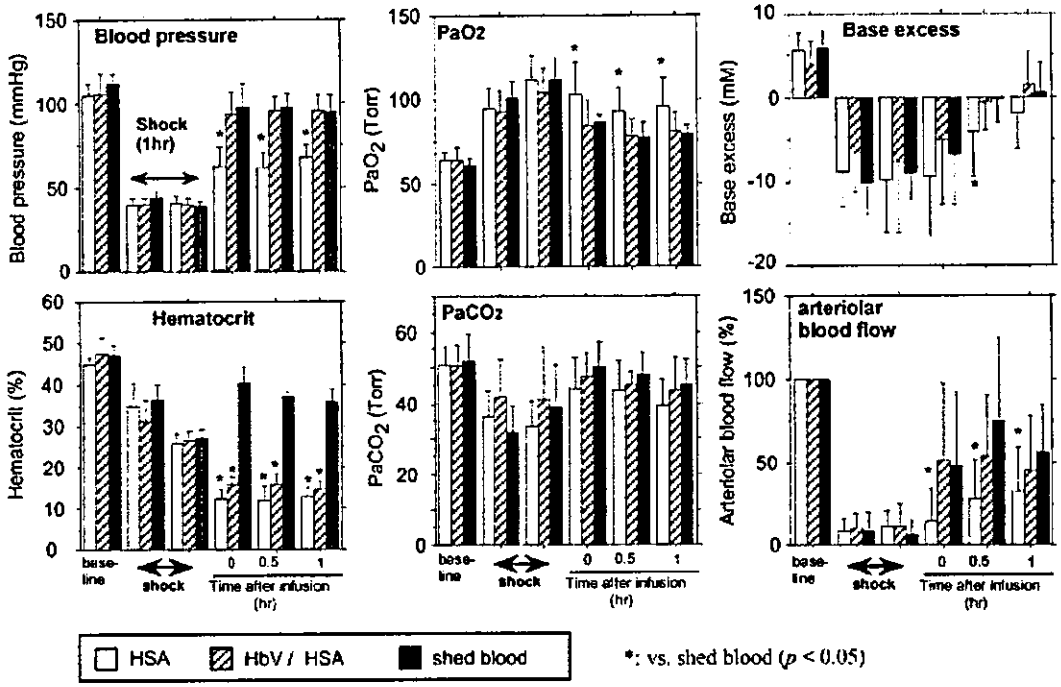

Figure 4. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD.

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

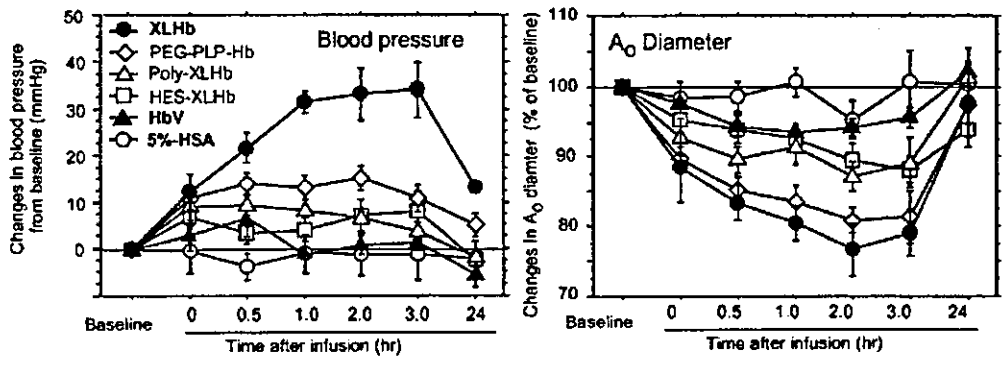

Figure 5. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2-carriers. Mean ± SD.

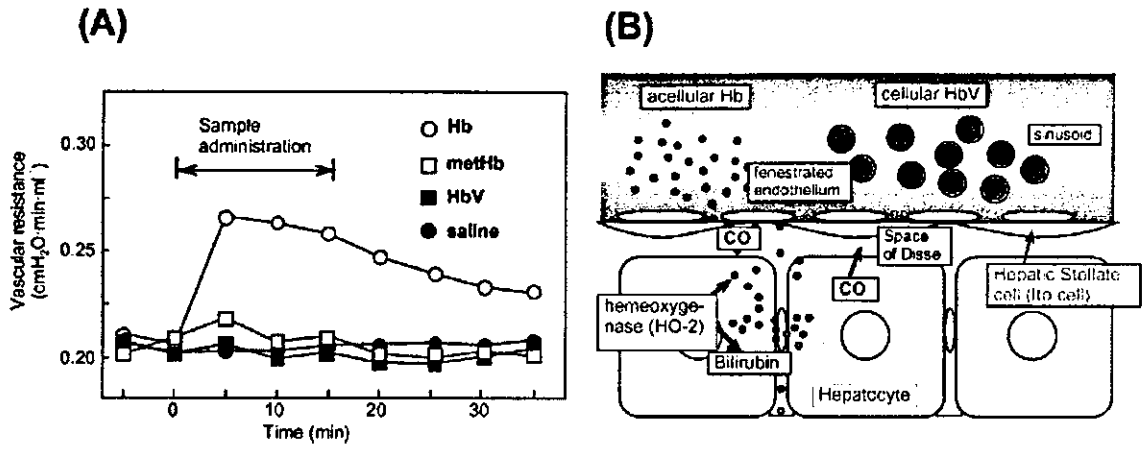

Figure 6. (A) Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. (B) Schematic representation of hepatic microcirculation: the small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolized 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

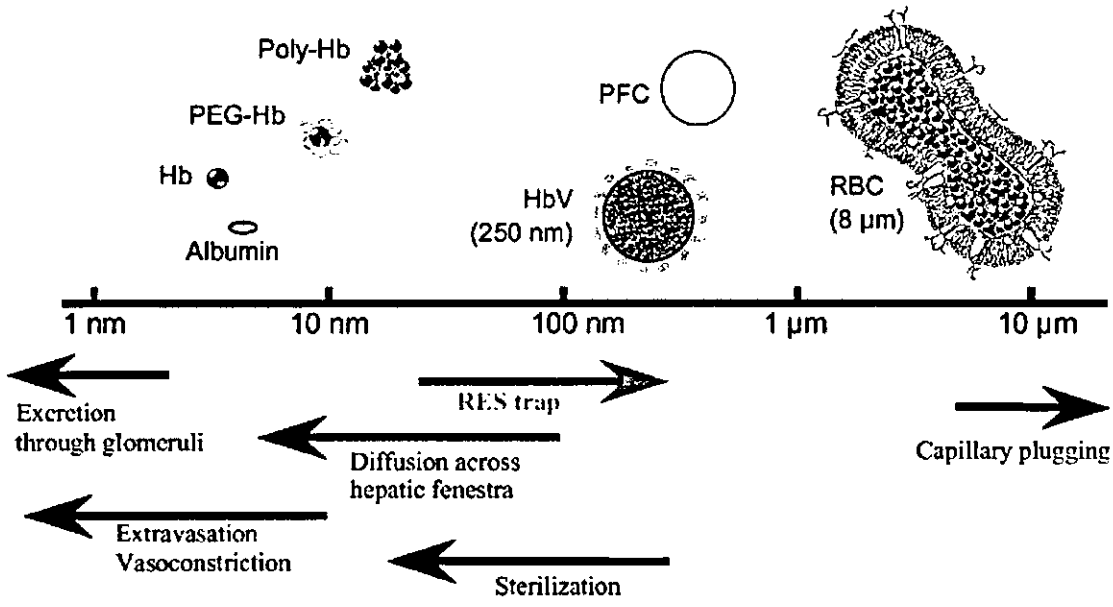

Figure 7. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process.

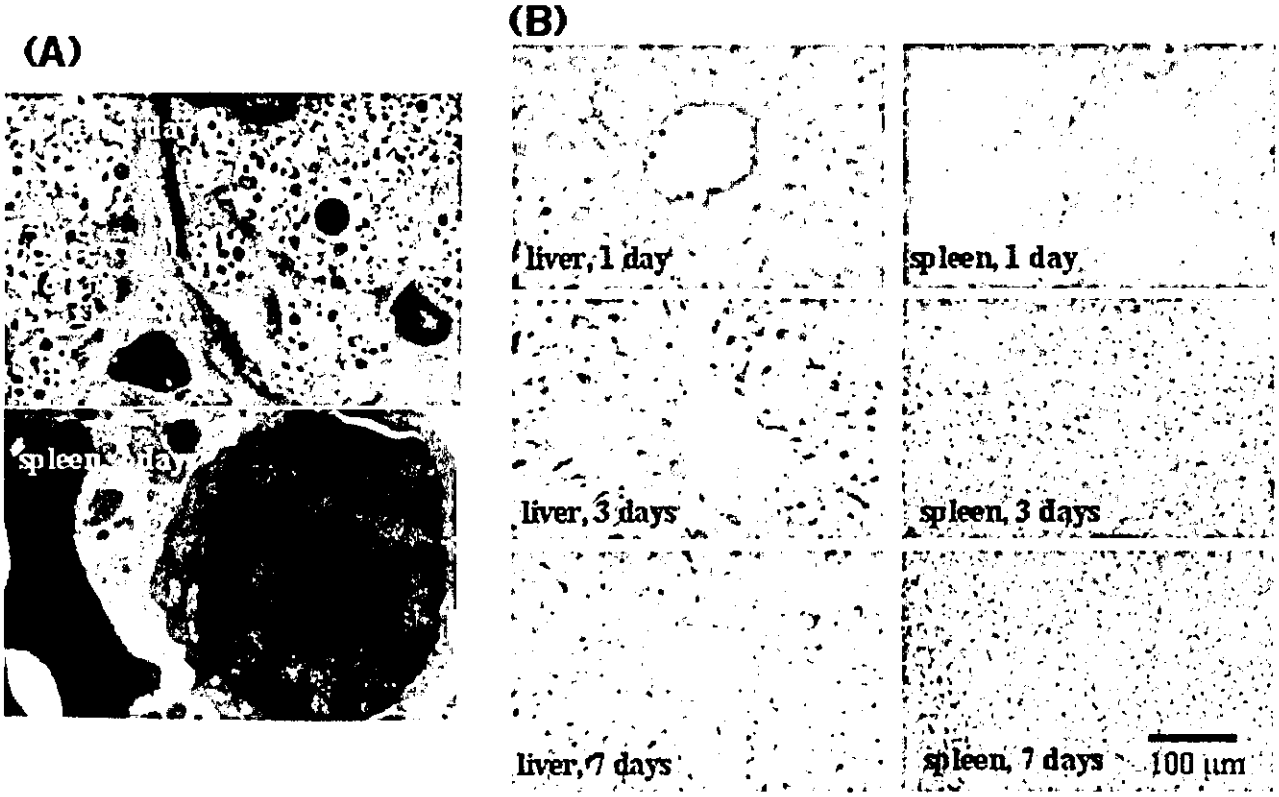

Figure 8. (A) TEM of rat spleen 1 day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. (B) Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. HbV particles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

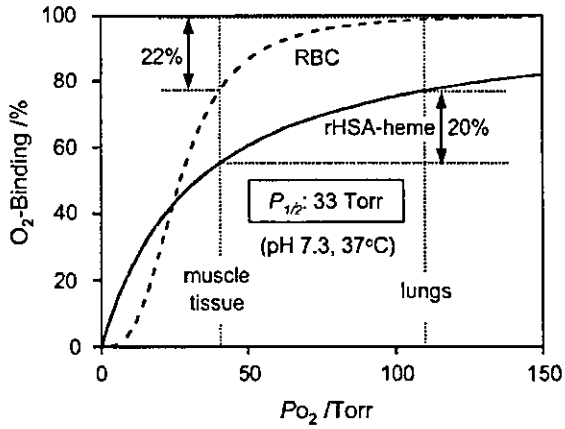

Figure 10. O_2-binding equilibrium curve of albumin-heme.

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

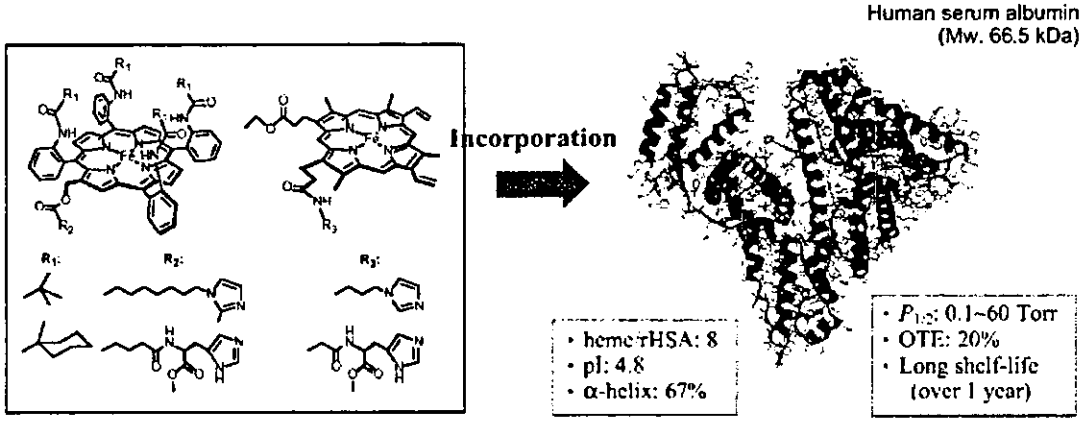

Figure 9. Structure of the albumin-heme molecule.

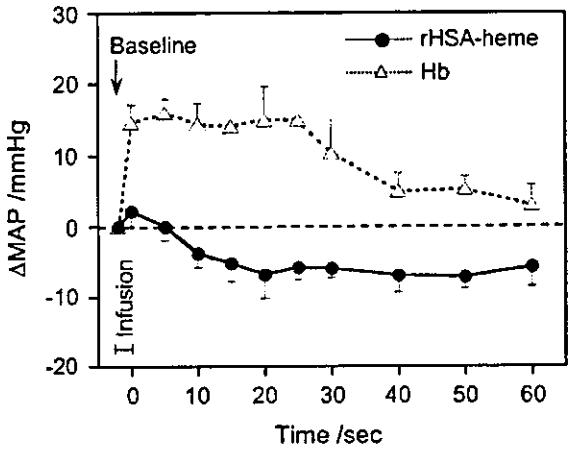

Figure 11. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats ($n=5$). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± SE. Basal value is 90.1 ± 3.0 mmHg.

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.

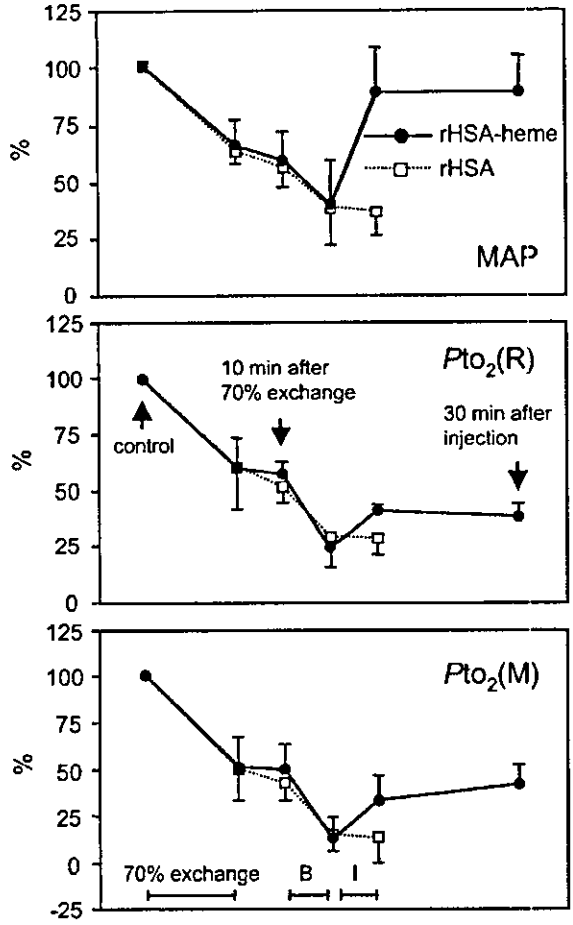

Figure 12. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats ($n=5$). All data are shown as changes from the basal values and expressed as mean ± SE.

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

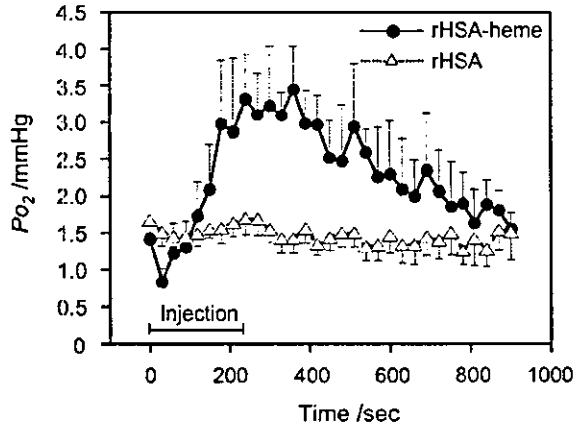

Figure 13. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats ($n=4$ each). All data are shown as changes from the basal values (P_{O_2}) just before the infusion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

Acknowledgements

This work has been supported by The Ministry of Education, Culture, Sports, Science and Technology and The Ministry of Health, Labor and Welfare.

REFERENCES

1. Tsuchida E (ed.). *Macromolecular Complexes, Dynamic Interactions and Electronic Processes*. VCH: New York, 1991.
2. Ciardelli F, Tsuchida E. Wöhrle D (eds). *Macromolecule-metal Complexes*. VCH: New York, 1996.
3. Matsushita Y, Hasegawa E, Eshima K, Tsuchida E. Synthesis of amphiphilic porphinatoiron complexes having phosphocholine groups. *Chem. Lett.* 1983; 1387–1389.
4. Tsuchida E. Liposome-embedded iron-porphyrins as an artificial oxygen carrier. *Ann. New York Acad. Sci.* 1985; **446**: 429–442.
5. Tsuchida E, Nishide H. Hemoglobin model—artificial oxygen carrier composed of porphinatoiron complexes. *Top. Curr. Chem.* 1986; **132**: 63–99.
6. Komatsu T, Moritake M, Nakagawa A, Tsuchida E. Self-organized lipid-porphyrin bilayer membranes in vesicular form: nanostructure, photophysical properties and dioxygen coordination. *Chem. Eur. J.* 2002; **8**: 5469–5480.
7. Chang TMS. *Blood Substitutes: Principles, Methods, Products, and Clinical Trials*. Karger: Basel, 1997.
8. Riess JG. Oxygen carriers ("blood substitutes")-raison d'etre, chemistry, and some physiology. *Chem. Rev.* 2001; **101**: 2797–2919.
9. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, Rodman G Jr. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock. *JAMA* 1999; **282**: 1857–1864.

10. Tsuchida E. *Blood Substitutes: Present and Future Perspectives*. Elsevier: Amsterdam, 1998.
11. Djordjevich L, Mayoral J, Miller IF, Ivankovich AD. Cardiorespiratory effects of exchange transfusions with synthetic erythrocytes in rats. *Crit. Care Med.* 1987; **15**: 318–323.
12. Takeoka S, Ohgushi T, Yokohama H, Sakai H, Nishide H, Tsuchida E. Preparation conditions of human hemoglobin vesicles covered with lipid membrane. *Artif. Organs Today* 1993; **3**: 129–136.
13. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E. Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif.* 1993; **4**: 563–569.
14. Takeoka S, Ohgushi T, Terase K, Ohmori T, Tsuchida E. Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir* 1996; **12**: 1755–1759.
15. Naito Y, Fukutomi I, Masada Y, Sakai H, Takeoka S, Tsuchida E, Abe H, Hirayama J, Ikebuchi K, Ikeda H. Virus removal from hemoglobin solution using Planova membrane. *J. Artif. Organs* 2002; **5**: 141–145.
16. Fukutomi I, Sakai H, Takeoka S, Nishide H, Tsuchida E, Sakai K. Carbonylation of oxyhemoglobin solution using a membrane oxygenator. *J. Artif. Organs* 2002; **5**: 102–107.
17. Sou K, Endo T, Takeoka S, Tsuchida E. Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjugate Chem.* 2000; **11**: 372–379.
18. Sakai H, Tomiyama K, Sou K, Takeoka S, Tsuchida E. Poly(ethyleneglycol)-conjugation and deoxygenation enable long term preservation of hemoglobin vesicles as oxygen carriers. *Bioconjugate Chem.* 2000; **11**: 425–432.
19. Sou K, Naito Y, Endo T, Takeoka S, Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using the freeze–thawing and extrusion. *Biotechnol. Prog.* 2003; **19**: 1547–1552.
20. Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, Kobayashi K. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. *Crit. Care Med.* 1996; **24**: 1869–1873.
21. Izumi Y, Sakai H, Kose T, Hamada K, Takeoka S, Yoshizu A, Horinouchi H, Kato R, Nishide H, Tsuchida E, Kobayashi K. Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J.* 1997; **43**: 289–297.
22. Kobayashi K, Izumi Y, Yoshizu A, Horinouchi H, Park SI, Sakai H, Takeoka S, Nishide H, Tsuchida E. The oxygen carrying capability of hemoglobin vesicles evaluated in rat exchange transfusion models. *Artif. Cells Blood Substitues Immobilization Biotechnol.* 1997; **25**: 357–366.
23. Sakai H, Takeoka S, Park SI, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, Tsuchida E. Surface modification of hemoglobin vesicles with poly(ethyleneglycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjugate Chem.* 1997; **8**: 23–30.
24. Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, Tsuchida E, Intaglietta M. Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. *J. Biomed. Mater. Res.* 1998; **40**: 66–78.
25. Sakai H, Tsai AG, Rohlfs RJ, Hara H, Takeoka S, Tsuchida E, Intaglietta M. Microvascular responses to hemodilution with Hb-vesicles as red cell substitutes: influences of O_2 affinity. *Am. J. Physiol.* 1999; **276**: H553–H562.
26. Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, Tsuchida E. Systemic and microvascular responses to the hemorrhagic shock and resuscitation with Hb-vesicles. *Am. J. Physiol. Heart Circ. Physiol.* 2002; **283**: H1191–H1199.
27. Erni D, Wettstein R, Schramm S, Contaldo C, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A. Normovolemic hemodilution with hemoglobin-vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am. J. Physiol. Heart Circ. Physiol.* 2003; **284**: H1702–H1709.
28. Contaldo C, Schramm S, Wettstein R, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A, Erni D. Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol.* 2003; **285**: H1140–H1147.
29. Goda N, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M. Distribution of heme oxygenase isoform in rat liver: topographic basis for carbon monoxide-mediated micorvascular relaxation. *J. Clin. Invest.* 1998; **101**: 604–612.
30. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension in conscious hamster model. *Am. J. Physiol. Heart Circ. Physiol.* 2000; **279**: H908–H915.
31. Wakamoto S, Fujihara M, Abe H, Sakai H, Takeoka S, Tsuchida E, Ikeda H, Ikebuchi K. Effects of PEG-modified hemoglobin vesicles on agonist induced platelet aggregation and RANTES release *in vitro*. *Artif. Cells Blood Substitues Immobilization Biotechnol.* 2001; **29**: 191–201.
32. Kyokane T, Norimizu S, Taniai H, Yamaguchi T, Takeoka S, Tsuchida E, Naito M, Nimura Y, Ishimura Y, Suematsu M. Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. *Gastroenterology* 2001; **120**: 1227–1240.
33. Ito T, Fujihara M, Abe H, Yamaguchi M, Wakamoto S, Takeoka S, Sakai H, Tsuchida E, Ikeda H, Ikebuchi K. Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on *N*-formyl-methionyl-leucyl-phenylalanine induced responses of polymorphonuclear neutrophils *in vitro*. *Artif. Cells Blood Substitues Immobilization Biotechnol.* 2001; **29**: 427–438.
34. Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial systems. *Am. J. Pathol.* 2001; **159**: 1079–1088.
35. Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, Tsuchida E. Pretreatment of serum containing Hb-vesicles (oxygen carriers) to avoid their interference in laboratory tests. *Clin. Chem. Lab. Med.* 2003; **41**: 222–231.
36. Sakai H, Horinouchi H, Masada Y, Takeoka S, Kobayashi K, Tsuchida E. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials* 2004; **25**: 4317–4325.
37. Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Kobayashi K, Tsuchida E. Physiologic capacity of reticuloendothelial system for degradation of homoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J. Pharmacol. Exp. Ther.* 2004; **311**: 874–884.
38. Curry S, Mandelkow H, Brick P, Franks N. Crystal structure of human serum albumin complexes with fatty acid reveals an asymmetric distribution of binding sites. *Nature Struct. Biol.* 1998; **5**: 827–835.
39. Sumi A, Ohtani W, Kobayashi K, Ohmura T, Yokoyama K, Nishida M, Suyama T. Purification and physicochemical properties of recombinant human serum albumin. In *Biotechnology of Blood Proteins*, Rivat C, Stoltz JF (eds). John Libbey Eurotext: Montrouge, 1993; vol. 227, 293–298.
40. Komatsu T, Ando K, Kawai N, Nishide H, Tsuchida E. O_2-transport albumin: a new hybrid-haemoprotein incorporating tetraphenylporphinatoiron(II) derivative. *Chem. Lett.* 1995; 813–814.
41. Tsuchida E, Ando K, Maejima H, Kawai N, Komatsu T, Takeoka S, Nishide H. Properties of oxygen binding by albumin-tetraphenylporphyrinatoiron(II) derivative complexes. *Bioconjugate Chem.* 1997; **8**: 534–538.
42. Wu J, Komatsu T, Tsuchida E. Resonance raman studies of O_2-binding to *ortho*-substituted tetraphenyl- and tetranaphthyl-porphinatoiron(II) derivatives with a covalently linked axial imidazole. *J. Chem. Soc., Dalton Trans.* 1998; 2503–2506.
43. Komatsu T, Hamamatsu K, Wu J, Tsuchida E. Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) serivatives. *Bioconjugate Chem.* 1999; **10**: 82–86.
44. Tsuchida E, Komatsu T, Mastukawa Y, Hamamatsu K, Wu J. Human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) derivative as a totally

synthetic O_2-carrying hemoprotein. *Bioconjugate Chem.* 1999; **10**: 797–802.
45. Komatsu T, Matsukawa Y, Tsuchida E. Kinetics of CO and O_2 binding to human serum albumin-heme hybrid. *Bioconjugate Chem.* 2000; **11**: 772–776.
46. Komatsu T, Matsukawa Y, Tsuchida E. Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(II) derivatives. *Bioconjugate Chem.* 2001; **12**: 71–75.
47. Nakagawa A, Komatsu T, Tsuchida E. Photoreduction of autooxidized albumin-heme hybrid in saline solution: revival of its O_2-binding ability. *Bioconjugate Chem.* 2001; **12**: 648–652.
48. Komatsu T, Okada T, Moritake M, Tsuchida E. O_2-Binding properties of double-sided porphyrinatoiron(II)s with polar substituents and their human serum albumin hybrids. *Bull. Chem. Soc. Jpn* 2001; **74**: 1695–1702.
49. Wu Y, Komatsu T, Tsuchida E. Electrochemical studies of albumin-heme hybrid in aqueous media by modified electrode. *Inorg. Chim. Acta* 2001; **322**: 120–124.
50. Komatsu T, Matsukawa Y, Tsuchida E. Effect of heme structure on O_2-binding properties of human serum albumin-heme hybrids: intramolecular histidine coordination provides a stable O_2-adduct complex. *Bioconjugate Chem.* 2003; **13**: 397–402.
51. Tsuchida E, Komatsu T, Yanagimoto T, Sakai H. Preservation stability and *in vivo* administration of albumin-heme hybrid solution as an entirely synthetic O_2-carrier. *Polym. Adv. Technol.* 2002; **13**: 845–850.
52. Nakagawa A, Komatsu T, Ohmichi N, Tsuchida E. Synthetic dioxygen-carrying hemoprotein: human serum albumin including iron(II) complex of protoporphyrin IX with an axially coordinated histidylglycyl-propionate. *Chem. Lett.* 2003; **32**: 504–505.
53. Tsuchida E, Komatsu T, Matsukawa Y, Nakagawa A, Sakai H, Kobayashi K, Suematsu M. Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging. *J. Biomed. Mater. Res.* 2003; **64A**: 257–261.
54. Tsuchida E, Komatsu T, Hamamatsu K, Matsukawa Y, Tajima A, Yoshizu A, Izumi Y, Kobayashi K. Exchange transfusion of albumin-heme as an artificial O_2-infusion into anesthetized rats: physiological responses, O_2-delivery and reduction of the oxidized hemin sites by red blood cells. *Bioconjugate Chem.* 2000; **11**: 46–50.
55. Komatsu T, Hamamatsu K, Tsuchida E. Cross-linked human serum albumin dimers incorporating sixteen (tetraphenylporphinato)iron(II) derivatives: synthesis, characterization, and O_2-binding property. *Macromolecules* 1999; **32**: 8388–8391.
56. Kobayashi K, Komatsu T, Iwamaru A, Matsukawa Y, Horinouchi H, Watanabe M, Tsuchida E. Oxygenation of hypoxic region in solid tumor by administration of human serum albumin incorporating synthetic hemes. *J. Biomed. Mater. Res.* 2003; **64A**: 48–51.
57. Kobayashi K, Izumi Y, Yamahata T, Sakai H, Takeoka S, Nishide H, Tsuchida E. Efficacy of synthetic oxygen-carrying substances. In *Int. Congr. Ser. 1995, 1102 (Shock: from Molecular and Cellular Level to Whole Body)*. Elsevier: Amsterdam, 1996; 305–310.
58. Horinouchi H, Tajima A, Kobayashi K. Liuid ventilation using artificial oxygen carrier. *Artif. Blood* 2001; **9**: 2–5 (in Japanese).