incorporated fatty acid, pH, and ionic strength [26,27]. In general, pasteurization for plasma HSA is performed at 60 °C for 10 h to eliminate the contaminations, e.g., hepatitis, HIV, and herpes virus [1]. Sodium caprylate and sodium *N*-acetyl-L-tryptophanate are commonly added to stabilize the albumin structure during the heat treatment [26]. Since the thermogram of the rHSA monomer under our sample conditions (PBS, pH 7.4) showed the T_d at 63 °C, we concluded that the rHSA dimer has the same thermodynamic stability with the monomer. Only the enthalpy change during the denaturation (ΔH) was slightly lower than the twice the monomer's value.

HSA acts as a carrier for many endogenous and exogenous substances in the blood circulation, and has two major specific drug binding sites, namely the warfarin site (Site I) and the indole and benzodiazepine site (Site II) [1,28]. We then determined the binding constants of typical ligands, warfarin (Site I-ligand) and diazepam (Site II-ligand), for the rHSA dimer using the ultracentrifugation method. In contrast to the results of the control experiments with rHSA, the amount of unbound ligand decreased to nearly half. The equilibria are expressed by following equations:

$$D + L \overset{K_1}{\rightleftharpoons} D\text{-}L \quad (3)$$

$$D\text{-}L + L \overset{K_2}{\rightleftharpoons} D\text{-}L_2 \quad (4)$$

$$D + 2L \overset{K_1K_2}{\rightleftharpoons} D\text{-}L_2 \quad (5)$$

where D is the rHSA dimer and L represents the ligand. The apparent binding constants (K_1K_2) of the warfarin and diazepam to the dimer were calculated to be 9.2×10^{10} and 3.0×10^{10} M^{-2}, respectively. If the each albumin unit independently accommodates one ligand, we estimated K_1 ($=K_2$) of each ligand as the square root of these values; 3.0×10^5 and 1.7×10^5 M^{-1}, respectively. They are almost in the same range as the binding constants for the monomeric rHSA (K_1), 3.8×10^5 and 1.4×10^5 M^{-1}, which means that neither the prevention nor the cooperation of the second ligand binding occurred in the dimer.

The attempt to prepare single crystals of the rHSA dimer for X-ray structural analysis failed, probably because it is likely to be very flexible at the BMH moiety. Transmission electron microscopy of the negatively stained samples showed homogeneous round particles with a diameter of 15–20 nm (not shown), however, the image is too small to obtain precise morphological information about the molecule.

The primary physiological function of HSA is the maintenance of COP within the blood vessels. Although HSA accounts for only 60% of the mass of the plasma protein, it contributes 80% of the COP. Two-thirds of this COP is simply the van't Hoff pressure and the other third arises from the Donnan effect of the negative charges of the plasma proteins, which is essentially due to albumin [1]. The relationship between the protein concentration and COP was observed for the rHSA and rHSA dimer solutions (Fig. 5). Both lines deviated upward from the linear correlation, because of the relatively larger value of the second virial coefficient, which is an index of the COP capacity, of the albumin molecule compared to those of the other plasma proteins. The measured rHSA monomer's curve coincided well to the previously reported result of Scatchard and co-workers (dotted line) [29]. The physiological concentration (5 g dL^{-1}) of rHSA represented the COP of 19 Torr. The careful inspections of their COP curves revealed that the 8.5 g dL^{-1} dimer solution has the same COP as the 5 g dL^{-1} rHSA. The plots of [C] versus COP/[C] gave a straight line, and the extrapolations to the y intercept afford the molecular weights of the monomer and dimer of 66×10^3 and 136×10^3 Da, respectively.

3.3. Viscosity and compatibility with blood components

Viscosity is a characteristic of proteins related to their size, shape, and conformation. The PBS solution of 8.5 g dL^{-1} rHSA dimer exhibited a Newtonian flow similar to the 5.0 g dL^{-1} rHSA, and showed a viscosity of 1.2 cP at a shear rate of 230 s^{-1} (Fig. 6). The dimer solution was then mixed with freshly drawn whole blood (1:1, v/v). The obtained suspension did not show any coagulation or precipitation for 6 h at 37 °C (after 6 h, hemolysis gradually took place even in the control experiment with saline or rHSA), and its viscosity profile was again Newtonian (1.8 cP at 230 s^{-1}). This result demonstrated good compatibility of the rHSA dimer with blood.

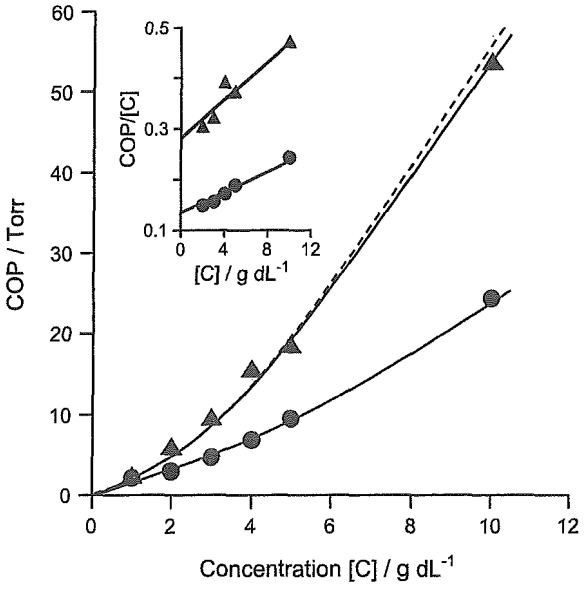


Fig. 5. Concentration [C] dependence of COP of rHSA monomer (▲) and rHSA dimer (●) in PBS (pH 7.4) at 22 °C. The dotted line represents the plasma HSA results taken from Ref. [29]. Inset shows relationship between [C] and COP/[C] for rHSA monomer (▲) and rHSA dimer (●).

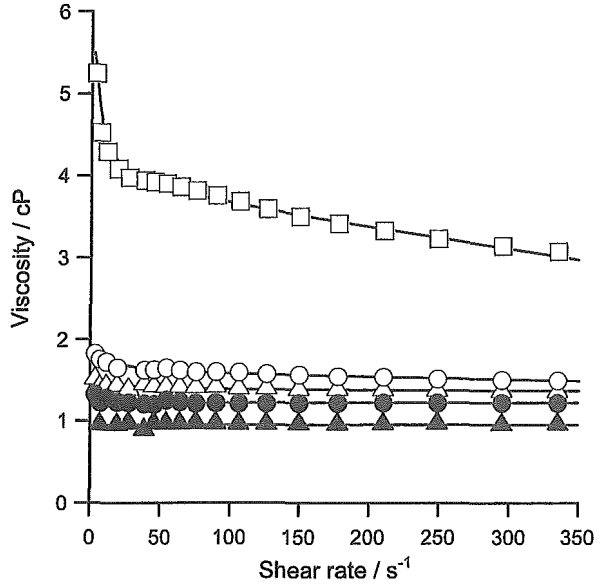


Fig. 6. Changes in the viscosity of rHSA monomer and rHSA dimer at various shear rates at 37 °C [□: whole blood, ●: 8.5 g dL^{-1} rHSA dimer, ▲: 5.0 g dL^{-1} rHSA, ○: 8.5 g dL^{-1} rHSA dimer plus whole blood (1:1, v/v), △: 5.0 g dL^{-1} rHSA plus whole blood (1:1, v/v)].

In order to evaluate the blood compatibility of the 8.5 g dL^{-1} rHSA dimer solution in detail, the changes in the number of blood cell components [RBC, white blood cells (WBC), and platelets (PLT)] have been counted after the mixture (1:1, v/v). The numbers just after the addition of the rHSA dimer to the whole blood were reasonably reduced to half the basal values; the same behavior was observed in the control experiments with the saline or 5 g dL^{-1} rHSA [Fig. 7(A)]. Optical microscopic observations revealed that the homogeneous round shape of the RBCs was completely retained [Fig. 7(B)(C)]. Therefore, it can be considered that no specific interaction occurred between the rHSA dimer and the blood cell components in vitro.

3.4. Immunogenicity

We then analyzed the immunological reactivity of the rHSA dimer against the anti-HSA polyclonal antibody. The absorption intensity of the reactant plate with the dimer showed clear concentration dependence in the same manner as those of the rHSA and plasma HSA groups (Fig. 8). It is known that HSA has five major antigenic sites by analysis using synthetic peptides [30,31]. The sites are nearly α-helical regions in the HSA molecule and include charged and/or aromatic residues which are important for the presentation of antigenic determinations. We previously reported that the cross-reactivity of the anti-HSA polyclonal antibody to BSA was extremely low, despite their homologies of the sequences over 70% and its antigenic sites in the same regions [32]. The antigenic epitopes of rHSA are preserved after bridging the Cys-34.

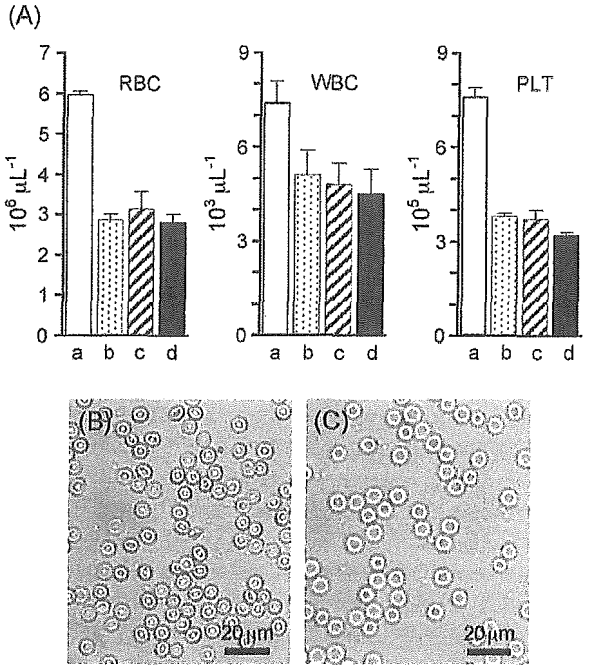


Fig. 7. (A) The blood cell (RBC, WRC, PLT) numbers in the blood suspension with the rHSA samples (1:1, v/v) at 25 °C [a: whole blood (basal value), b: with saline, c: with 5.0 g dL^{-1} rHSA, d: with 8.5 g dL^{-1} rHSA dimer]. Optical microscopic observations of (B) whole blood and (C) the blood suspension with the 8.5 g dL^{-1} rHSA dimer (1:1, v/v) (bar: 20 μm). The shape of the RBC with a diameter of ca. 8 μm did not change.

3.5. Circulation lifetime of ^{125}I-labeled rHSA dimer in rats

The rHSA and rHSA dimer labeled with ^{125}I were injected into rats to determine their blood circulation

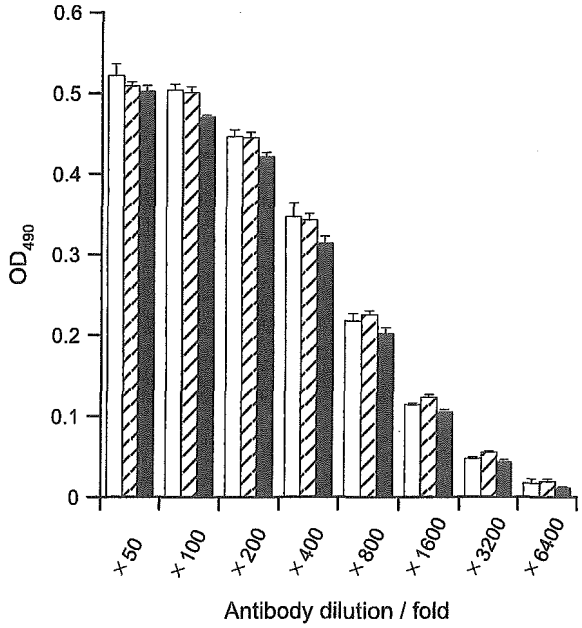


Fig. 8. Cross-reactivity of the anti-HSA polyclonal antibody with rHSA monomer and rHSA dimer [white bar: plasma HSA, diagonal bar: rHSA monomer, black bar: rHSA dimer]. All values are mean±S.D. (n=3).

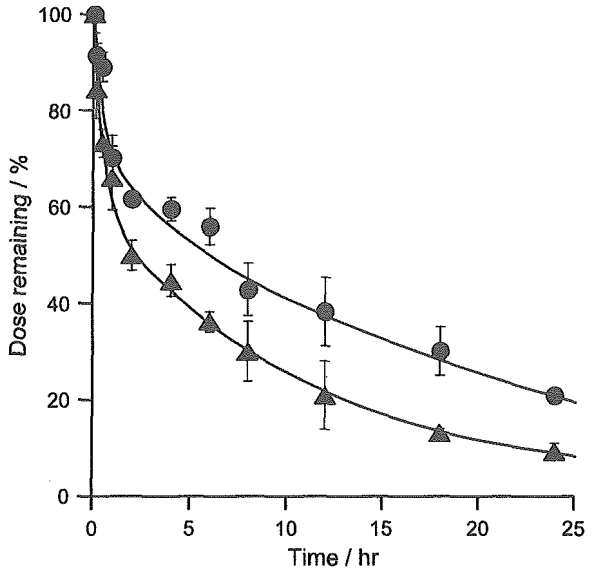


Fig. 9. Plasma levels of ^{125}I-rHSA monomer and dimer (1.0×10^7 cpm, 1.0 mg kg^{-1}) after intracardial administration into Wistar rats. All values are mean±S.D. (n=3).

lifetimes. The time courses of the radioactivity clearly showed two-phase kinetics, and significant differences between these two samples (Fig. 9). The half lifetimes ($\tau_{1/2}$) determined from the disappearance phase (β-phase) were 8.9 and 16.2 h for the monomer and dimer, respectively. The enlargement of the molecular size definitely led to retardation of extravasation through the vascular endothelium and produced a longer lifetime in the bloodstream. Radioactivity of the trichloroacetic acid precipitates recovered up to 95% the intensity, which means ^{125}I did not dissociate from the proteins. The tissue distributions of the rHSA dimer were in the skin, liver, kidney, and spleen (not shown), which took up most of the radioactivity, and we could not find any differences to that of the rHSA monomer.

More recently, McCurdy et al. [33] reported that a reiterated form of recombinant rabbit serum albumin (rRSA), in which two copies of the C34A rRSA mutant were joined with the C-terminus and N-terminus by a hexaglycine spacer, was more rapidly cleared ($\tau_{1/2}$: 3.0 days) in vivo (rabbit) than the corresponding monomer, C34A rRSA ($\tau_{1/2}$: 4.9 days). They postulated that the mechanism of this quick clearance of the dimer appears to involve the reticuloendothelial system (two binding sites on a dimer molecule to phagocytic cells may underlie the increased rate); and also suggested that the albumin dimerization through Cys-34 probably does not substantially contribute to the albumin metabolism. However, our results clearly showed that the Cys-34 BMH-bridged rHSA dimer has the longer circulation lifetime relative to that of the rHSA monomer. This opposite conclusion presumably arises from the differences in the chemical structure of the cross-linking agents, highly-ordered structure of the albumin dimer itself, and mammal species in the experimental protocol.

3.6. Albumin–heme dimer and its O_2-binding

Since a maximum of eight FecycPs (Fig. 1) was incorporated into certain domains of rHSA [12,d,], we postulated that the rHSA dimer can capture a twofold molar excess of FecycP molecules relative to the monomer. From the quantitative analyses of the amount of rHSA and FecycP in the hemoprotein, the ratio of the FecycP/rHSA (mol/mol) was determined to be 15.7 for the mixing ratio (m) of 16 and 16.4 for the m of 20. Thus, we concluded that the maximum binding numbers of FecycP to an rHSA dimer were 16, the same as FepivP [11]. The obtained red-solution of the rHSA–FecycP dimer was stored for more than 1 year at 4 °C without any aggregation and precipitation.

The UV–Vis absorption spectrum of the aqueous rHSA–FecycP dimer solution in an N_2 atmosphere showed the formation of a typical five-N-coordinate high-spin complex of porphyrinatoiron(II) (λ_{max}: 444, 539, 565 nm) [12,14,17,19]. Upon exposure of this solution to O_2, the absorption spectral pattern immediately changed to that of a dioxygenated species (λ_{max}: 426, 549 nm). This O_2-binding reaction was reversible and kinetically stable under physiological conditions (pH 7.3, 37 °C). After admitting the CO gas, the O_2–addcut complex moved to the very stable carbonyl low-spin complex (λ_{max}: 427, 539 nm) (Fig. 10).

We then employed MCD spectroscopy to characterize the coordination structure of the FecycP in the rHSA dimer. Under an N_2 atmosphere, the MCD showed a well-characterized spectrum of a mono-imidazole ligated five-

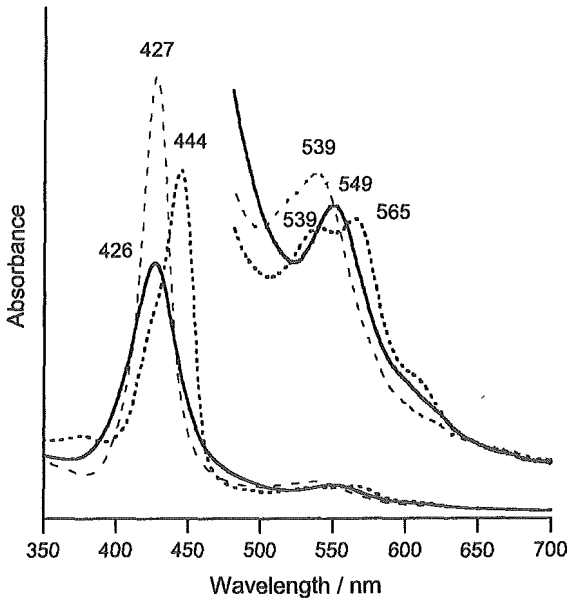


Fig. 10. UV–Vis absorption spectral changes of the rHSA–FecycP dimer in phosphate buffer solution (pH 7.3) at various conditions (25 °C) (dotted line: under N_2; solid line: under O_2; broken line: under CO).

coordinate high-spin porphyrinatoiron(II), which is in contrast to that of the six-coordinate low-spin state with the bis-imidazole ligated complex (Fig. 11) [34]. This observation indicates that no amino acid residue binds to the sixth position of FecycP in the rHSA structure under an N_2 atmosphere. The admission of O_2 gas yields an S-shaped A-term MCD in the Soret-band region, which shows the formation of an O_2–adduct complex as observed in the spectra of the other dioxygenated porphyrinatoiron(II) [34]. The CO adduct is also low spin and showed a similar A-term MCD band with a strong intensity. In all cases, the pattern in the Q-band regions coincided well with those reported previously [34].

The autooxidation reaction of the oxy state (λ_{max}: 549 nm) slowly occurred and the absorption intensity of 549 nm almost disappeared after 36 h, leading to the formation of the inactive Fe(III)cycP. The half-life of the dioxygenated species ($\tau_{1/2}$) was 6 h at 37 °C, which is significantly longer than that of our previous results for the rHSA–FepivP dimer ($\tau_{1/2}$: 2 h) [11]. The hydrophobic cyclohexanoyl fences on the porphyrin ring plane could effectively retard the autooxidation through the proton-driven process.

3.7. O_2-Binding kinetics and equilibrium of albumin–heme dimer

The association and dissociation rate constants for O_2 (k_{on}, k_{off}) were explored by laser flash photolysis. The absorption decays accompanying the O_2 recombination were composed of three-component kinetics, and the curves were fit by a triple-exponential equation [12,a,b,14]. The minor (less than 12%) component k_1, which is the fastest rate constant, is presumably correlated with a base

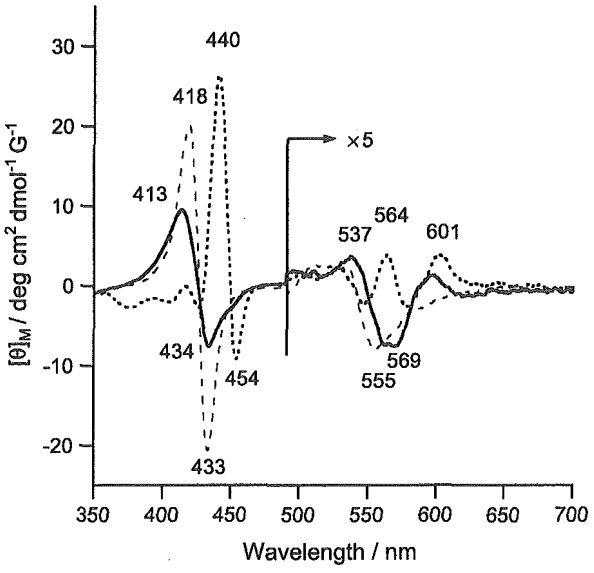


Fig. 11. MCD spectral changes of the rHSA–FecycP dimer in phosphate buffer solution (pH 7.3) at various conditions (25 °C) (dotted line: under N_2; solid line: under O_2; broken line: under CO).

Table 2
O_2-Binding parameters of the rHSA–FecycP dimer in phosphate buffer solution (pH 7.3) at 25 °C

	k_{on} (M^{-1} s^{-1})	k'_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	k'_{off} (s^{-1})	$P_{1/2}$ (Torr)[a]
rHSA–FecycP dimer	2.8×10^7	4.8×10^6	6.7×10^2	1.2×10^2	38
rHSA–FecycP	4.6×10^7	7.3×10^6	9.8×10^2	1.6×10^2	35
Hb (T-state)[b]	2.9×10^6		1.8×10^2		40

[a] At 37 °C. [b]pH 7.0, 20 °C, from Ref. [36].

elimination reaction [35]. From the slope of the linear plots of k_2 and k_3 versus the O_2 concentration, two association rate constants for the fast O_2 rebinding (k_{on}) and the slow O_2 rebinding were obtained (Table 2). The k_{on} values are 5.8-fold grater than k'_{on}, and the amplitude ratio of the fast and slow reactions was approximately 5/3. The O_2 association to FecycPs incorporated in the certain domains of the rHSA dimer is largely influenced by the protein environments surrounding each iron center of FecycP, for example, steric hindrance by the amino acid residues and/or difference in polarity. Six of the 16 sites of FecycP in the rHSA dimer are estimated to be the slow sites for the O_2 association.

The O_2-binding affinity ($P_{1/2}$) of the rHSA–FecycP dimer was determined to be 38 Torr at 37 °C on the basis of the UV–Vis spectral changes by O_2 titration [12,14,17–19]. The obtained $P_{1/2}$ is very close to that of rHSA–FecycP in the monomeric form and T-state Hb (Table 2) [12,d,35]. The O_2-dissociation rate constants were also calculated by k_{on}/K.

The rHSA–FecycP dimer did not show a cooperative O_2-binding profile like that seen in RBC; the Hill coefficient was 1.0 (Fig. 12). Although $P_{1/2}$ is slightly lower than that of RBC, the O_2-transporting efficiency (OTE) of the rHSA–FecycP dimer between the lungs (P_{O_2}: 110 Torr) and muscle

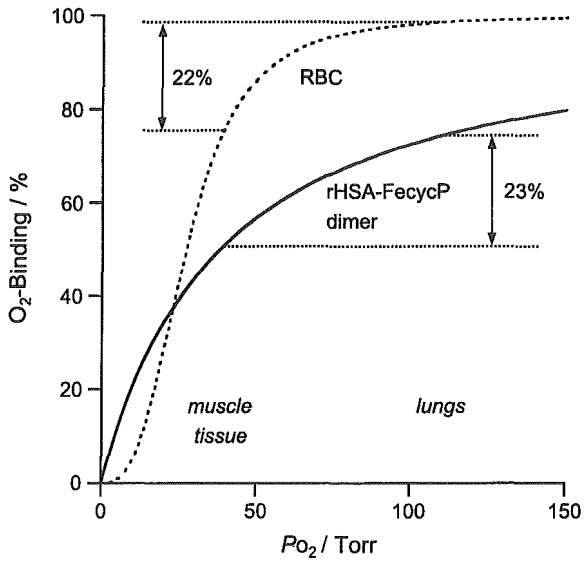


Fig. 12. OEC curve of the rHSA–FecycP dimer at 37 °C.

tissue (P_{O_2}: 40 Torr) (23%) becomes slightly higher than that of the human RBC (22%).

4. Conclusions

The obvious characteristics of the rHSA dimer cross-linked with the bola-shaped bismaleimide are as follows: (i) unaltered essential properties of the albumin units (the secondary/tertiary structure, surface net charges, thermostability), (ii) excess ligand-binding capacity relative to the monomer while maintaining its COP at the physiological value, (iii) good blood compatibility and identical antigenic epitopes with the monomer, and (iv) longer half-life in the bloodstream and similar tissue distributions with rHSA. Furthermore, (v) one molecule of the rHSA dimer incorporates 16 FecycPs, which is exactly twice the amount compared to that of the monomeric rHSA, and the obtained hemoprotein can reversibly bind and release O_2 under physiological conditions. (vi) The 8.5 g dL^{-1} rHSA–FecycP dimer solution satisfies the initial clinical requirements for the O_2-carrier as an RBC substitute, which transports 10 mM O_2 (compared to 9.2 mM in the human blood) while maintaining the COP at a constant 19 Torr.

Acknowledgements

This work was partially supported by Grant-in-Aid for Scientific Research (no. 16350093) from JSPS, and Grant-in-Aid for Exploratory Research (no. 16655049) from MEXT Japan, and Health Science Research Grants (Regulatory Science) from MHLW Japan.

References

[1] T. Peters Jr., All about Albumin. Biochemistry, Genetics, and Medical Applications, Academic Press, San Diego, 1996, and reference therein.
[2] D.C. Carter, J.X. Ho, Structure of serum albumin, Adv. Protein Chem. 45 (1994) 153–203.
[3] S. Curry, P. Brick, N.P. Franks, Fatty acid binding to human serum albumin: new insights from crystallographic studies, Biochim. Biophys. Acta 1441 (1999) 131–140.
[4] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 Å resolution, Protein Eng. 12 (1999) 439–446.
[5] DJ. Meyer, H. Kramer, N. Özer, B. Coles, B. Ketterer, Kinetics and equiliburia of *S*-nitrosothiol–thiol exchange between glutathione, cysteine, penicillamines and serum-albumin, FEBS Lett. 345 (1994) 177–180.
[6] T. Peters Jr., Serum albumin, Adv. Protein Chem. 37 (1985) 161–245.
[7] a W.L. Hughes, The proteins of blood plasma, in: H. Neurath, K. Bailey (Eds.), The Proteins, vol. IIB, Academic Press, New York, 1954, pp. 663–734;
b W.L. Hughes, H.M. Dintzis, Crystallization of the mercury dimers of human and bovine mercaptalbumin, J. Biol. Chem. 239 (1964) 845–849.
[8] R. Straessle, A disulfide dimer of human mercaptalbumin, J. Am. Chem. Soc. 76 (1954) 3138–3142.
[9] L.-O. Anderson, Hydrolysis of disulfide bonds in weakly alkaline media II. Bovine serum albumin dimer, Biochim. Biophys. Acta 117 (1966) 115–133.
[10] C.N. Cornell, R. Chang, L.J. Kaplan, The environment of the sulfhydryl group in human plasma albumin as determined by spin labeling, Arch. Biochem. Biophys. 209 (1981) 1–6.
[11] T. Komatsu, K. Hamamatsu, E. Tsuchida, Cross-linked human serum albumin dimer incorporating sixteen (tetraphenylporphinato)iron(II) derivatives: synthesis, characterization, and O_2-binding property, Macromolecules 32 (1999) 8388–8391.
[12] (a) E. Tsuchida, T. Komatsu, Y. Matsukawa, K. Hamamatsu, J. Wu, Human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) derivative as a totally synthetic O_2-carrying hemoprotein, Bioconj. Chem. 10 (1999) 797–802;
(b) T. Komatsu, Y. Matsukawa, E. Tsuchida, Kinetics of CO and O_2 binding to human serum albumin–heme hybrid, Bioconj. Chem. 11 (2000) 772–776;
(c) T. Komatsu, Y. Matsukawa, E. Tsuchida, Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(II) derivatives, Bioconj. Chem. 12 (2001) 71–75;
(d) T. Komatsu, Y. Matsukawa, E. Tsuchida, 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, Bioconj. Chem. 13 (2002) 397–402.
[13] (a) E. Tsuchida, T. Komatsu, K. Hamamatsu, Y. Matsukawa, A. Tajima, A. Yoshizu, Y. Izumi, K. Kobayashi, 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, Bioconj. Chem. 11 (2000) 46–50;
(b) Y. Huang, T. Komatsu, A. Nakagawa, E. Tsuchida, S. Kobayashi, Compatibility in vitro of albumin–heme (O_2-carrier) with blood cell components, J. Biomed. Mater. Res. 66A (2003) 292–297;
(c) E. Tsuchida, T. Komatsu, Y. Matsukawa, A. Nakagawa, H. Sakai, K. Kobayashi, M. Suematsu, Human serum albumin incorporating synthetic heme: red blood cell substitute without hypertension by nitric oxide scavenging, J. Biomed. Mater. Res. 64A (2003) 257–261.
[14] E. Tsuchida, T. Komatsu, K. Arai, H. Nishide, Synthesis and O_2-binding properties of tetraphenylporphyrinatoiron(II) derivatives bearing a proximal imidazole covalently bound at the β-pyrrolic position, J. Chem. Soc., Perkin Trans. 2 1995 (1995) 747–753.
[15] B.T. Doumas, W.A. Watson, H.G. Biggs, Albumin standards and measurement of serum albumin with bromcresol green, Clin. Chim. Acta 31 (1971) 87–96.
[16] H. Watanabe, K. Yamasaki, U. Kragh-Hansen, S. Tanase, K. Harada, A. Suenaga, M. Otagiri, In vivo and in vitro properties of recombinant human serum albumin from *Pichia pastoris* purified by a method of short processing time, Pharm. Res. 18 (2001) 1775–1781.
[17] J.P. Collman, J.I. Brauman, B.L. Iverson, J.L. Sessler, R.M. Morris, Q.H. Gibson, O_2 and CO Binding to iron(II) porphyrins: a comparison of the "picket fence" and "pocket" porphyrins, J. Am. Chem. Soc. 105 (1983) 3052–3064.
[18] T.G. Traylor, S. Tsuchiya, D. Campbell, M. Mitchell, D. Stynes, N. Koga, Anthracene heme cyclophanes: steric effects in CO, O_2, and RNC binding, J. Am. Chem. Soc. 107 (1985) 604–614.
[19] E. Tsuchida, T. Komatsu, K. Arai, H. Nishide, Synthesis and dioxygen-binding properties of double-sided porphyrinatoiron(II) complexes bearing covalently bound axial imidazole, J. Chem. Soc., Dalton Trans. 1993 (1993) 2465–2469.
[20] E. Katchalski, G.S. Benjamin, V. Gross, The availability of the disulfide bonds of human and bovine serum albumin and of bovine g-globulin to reduction by thioglycolic acid, J. Am. Chem. Soc. 79 (1957) 4096–4099.

[21] D.R. Grassetti, J.F. Murray Jr., Determination of sulfhydryl groups with 2,2′ - or 4,4′ -dithiopyridine, Arch. Biochem. Biophys. 119 (1967) 41–49.
[22] A.O. Pedersen, J. Jacobsen, Reactivity of the thiol group in human and bovine albumin at pH 3–9, as measured by exchange with 2,2′ - dithiodipyridine, Eur. J. Biochem. 106 (1980) 291–295.
[23] E.J. Cohn, The properties and functions of the plasma proteins, with a consideration of the methods for their separation and purification, Chem. Rev. 28 (1941) 395–417.
[24] I. Sjöholm, I. Ljungstedt, Studies on the tryptophan and drug-binding properties of human serum albumin fragments by affinity chromatography and circular dichroism measurements, J. Biol. Chem. 248 (1973) 8434–8441.
[25] A. Sumi, W. Ohtani, K. Kobayashi, T. Ohmura, K. Yokoyama, N. Nishida, T. Suyama, Purification and physicochemical properties of recombinant human serum albumin, in: C. Rivat, J.-F. Stoltz (Eds.), Biotechnology of Blood Proteins, vol. 227, John Libbey Eurotext, Montrouge, 1993, pp. 293–298.
[26] S. Gumpen, P.O. Hegg, H. Martens, Thermal stability of fatty acid–serum albumin complexes studies by differential scanning calorimetry, Biochim. Biophys. Acta 574 (1979) 189–196.
[27] T. Kosa, M. Maruyama, M. Otagiri, Species differences of serum albumins: II. Chemical and thermal stability, Pharm. Res. 15 (1998) 449–454.
[28] a G. Sudlow, D.J. Birkett, D.N. Wade, The Characterization of two specific drug binding sites on human serum albumin, Mol. Pharmacol. 11 (1975) 824–832;
b G. Sudlow, D.J. Birkett, D.N. Wade, Further characterization of specific drug binding sites on human serum albumin, Mol. Pharmacol. 12 (1976) 1052–1061.
[29] G. Scatchard, A.C. Batchelder, A. Brown, Chemical, clinical, and immunological studies on the products of human plasma fractionation: VI. The osmotic pressure of plasma and of serum albumin, J. Clin. Invest. 23 (1944) 165–170.
[30] S. Sakata, M.Z. Atassi, Immunochemistry of serum albumin. X. Five major antigenic sites of human serum albumin are extrapolated from bovine albumin and confirmed by synthetic peptides, Mol. Immunol. 17 (1980) 139–142.
[31] C. Lapresle, N. Doyen, Studies of an antigenic site of human-serum albumin with monoclonal-antibodies, Mol. Immunol. 20 (1983) 549–555.
[32] W. Ohtani, Y. Nawa, K. Takeshima, H. Kamuro, K. Kobayashi, T. Ohmura, Physicochemical and immunochemical properties of recombinant human serum albumin from *Pichia pastoris*, Anal. Biochem. 256 (1998) 56–62.
[33] T.R. McCurdy, S. Gataiance, L.J. Eltringham-Smith, WP. Sheffield, A covalently linked recombinant albumin dimer is more rapidly cleared in vivo than are wild-type and mutant C34A albumin, J. Lab. Clin. Med. 143 (2004) 115–124.
[34] a J.P. Collman, J.I. Brauman, K.M. Doxsee, T.R. Halbert, E. Bunnenberg, R.E. Linder, G.N. LaMar, J.D. Guadio, G. Lang, K. Spartalian, Synthesis and characterization of "tailed picket fence" porphyrins, J. Am. Chem. Soc. 102 (1980) 4182–4192;
b J.P. Collman, F. Basolo, E. Bunnenberg, T.C. Collins, J.H. Dawson, P.E. Ellis Jr., M.L. Marrocco, A. Moscowitz, J.L. Sessler, T. Szymanski, Use of magnetic circular dichroism to determine axial ligation for some sterically encumbered iron(II) porphyrin complexes, J. Am. Chem. Soc. 103 (1981) 5636–5648;
c J.P. Collman, J.I. Brauman, T.J. Collins, B.L. Iverson, G. Lang, R.B. Pettman, J.L. Sessler, M.A. Walters, Synthesis and characterization of the "pocket" porphyrins, J. Am. Chem. Soc. 105 (1983) 3038–3052.
[35] J. Geibel, J. Cannon, D. Campbell, T.G. Traylor, Model compounds for R-state and T-state hemoglobins, J. Am. Chem. Soc. 100 (1978) 3575–3585.
[36] C.A. Sawicki, Q.H. Gibson, Properties of the T state of human oxyhemoglobin studied by laser photolysis, J. Biol. Chem. Soc. 252 (1977) 7538–7547.

Biomaterials

Safety Evaluation of an Artificial O_2 Carrier as a Red Blood Cell Substitute by Blood Biochemical Tests and Histopathology Observations

YUBIN HUANG,* TERUYUKI KOMATSU,* HISASHI YAMAMOTO,† HIROHISA HORINOUCHI,‡ KOICHI KOBAYASHI,‡ AND EISHUN TSUCHIDA*

Recombinant human serum albumin (rHSA) incorporating synthetic heme with a covalently linked proximal base (albumin-heme [rHSA-heme]) is an artificial O_2 carrier that can transport O_2 like hemoglobin does in the blood stream. To evaluate the clinical safety of this compound, 20% and 40% exchange transfusions with rHSA-heme into anesthetized rats were followed by blood biochemical tests and histopathologic observations for 7 days. In the 20% rHSA-heme group, a total of 30 analytes by blood biochemical tests showed almost the same values as those observed in the reference 20% rHSA group. Although some abnormal values for liver parameters were found in the 40% rHSA-heme group, they returned to normal after 7 days. Histopathologic observations indicated that the administration of rHSA-heme in a volume of 20% total blood volume did not produce any negative side effects on the vital organs. *ASAIO Journal* 2004; 50:525–529.

Hemoglobin (Hb)-based O_2 carriers have been studied as red blood cell substitutes or as an O_2 therapeutic.[1–3] The advantages of these O_2 carriers are 1) the absence of a blood type antigen and infectious virus, 2) a small particle size for penetration through constricted vessels where red blood cells cannot penetrate, and 3) stability for long-term storage. The first generation products (Hemolink, Polyheme, and Hemopure),[4] which are currently in clinical testing, have been followed by second generation materials (Hemospan™ [MP4],[5] adenosine-GSH-Hb,[6] and SOD-catalase-Hb[7] under development. We have developed an entirely synthetic O_2 carrier without using Hb. Recombinant human serum albumin (rHSA) incorporates a synthetic heme (2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis {α,α,α,α-*o*-(1-methylcyclohexanamido)}phenylporphinatoiron(II) (**Figure 1**), providing an artificial hemoprotein (albumin-heme [rHSA-heme]), which has the potential to bind and release O_2 under physiologic conditions (pH 7.3, 37°C) in the same manner as Hb.[8–12] Because the rHSA-heme molecule is totally synthetic, there is absolutely no concern for infection with pathogens and virus. The *in vitro* experiments have indicated that the rHSA-heme solutions have a high compatibility with blood cell components.[12] Furthermore, we found that vasoconstriction was not observed after administration of rHSA-heme into the circulatory system because of its low permeability through the vascular endothelium; rHSA-heme does not deplete nitric oxide (endothelium-derived relaxing factor).[13] Our recent study of a 30% exchange transfusion with rHSA-heme after 70% hemodilution with 5% (by weight) rHSA in anesthetized rats showed that injection of this material improved the circulatory blood volume and resuscitated the animals from shock.[14] To evaluate the clinical safety of this material as a red blood cell substitute, 20% and 40% exchange transfusions in anesthetized rats were studied using blood biochemical testing and histopathologic observations for 7 days.

Materials and Methods

Preparation of rHSA-Heme

The rHSA (Albrec, 25% by weight) was provided by the NIPRO Corp. (Osaka, Japan). The rHSA-heme solution (rHSA: 4.9% rHSA by weight, pH 7.45; heme: 2.8 mmol/L heme, colloid osmotic pressure (COP) 18 torr, osmolarity 300 mOsm, viscosity 1.1 cP, and endotoxin <0.1 EU/ml) was prepared according to our previously reported procedure.[10,14,15] The other physicochemical properties of rHSA-heme (molecular weight 72.3 kDa, O_2 binding affinity [$p_{1/2}O_2$] 37 torr, and isoelectric point 4.8) have been reported elsewhere.[10] The half-life of the oxygenated rHSA-heme against the ferric state was 9 hours at 37°C *in vitro*.[10]

Exchange Transfusion with rHSA-Heme in Anesthetized Rats

The investigations were carried out in 60 male Wister rats (312 ± 3.0 gm). The details of the experimental setup (anesthesia and catheterization) were the same as our former protocol reported elswhere.[14,15] The total blood volume of a rat was estimated to be 64 ml/kg body weight. The 20% exchange transfusion was achieved by four cycles of repeated blood withdrawal *via* the common carotid artery (1 ml, 1 ml/min) and the rHSA-heme infusion into the femoral vein (1 ml, 1 ml/min; 20% rHSA-heme group, n = 12). The 40% exchange transfu-

From the *Advanced Research Institute for Science and Engineering, Waseda University, Shinjuku-ku, Tokyo; the †Pharmaceutical Research Center, NIPRO Corp., Kusatsu-shi, Shiga; and the ‡Department of General Thoracic Surgery, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan.

Submitted for consideration June 2004; accepted for publication in revised form August 2004.

This work was partially supported by a grant-in-aid for scientific research (No. 16350093) from JSPS, a grant-in-aid for exploratory research (No. 16655049) from MEXT Japan, and health science research grants (regulatory science) from MHLW Japan.

Address correspondence to: Eishun Tsuchida, Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan.

DOI: 10.1097/01.MAT.0000144361.60280.DA

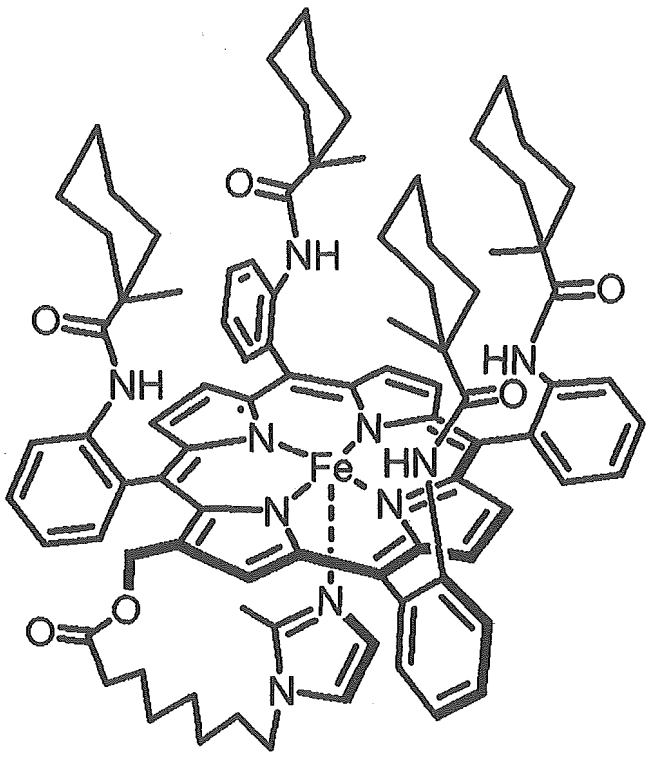


Figure 1. Chemical compound of entirely synthetic O_2 carrier without using Hb.

sion was performed by eight cycles of identical withdrawal/infusion (40% rHSA-heme group, $n = 12$). As a reference, a 5 gm/dl rHSA solution was given to other rats in the same ratios (20% rHSA group and 40% rHSA group, $n = 12$ each). Furthermore, 12 rats without blood exchange (anesthesia and surgery only) were also used as the control group. After the blood exchange transfusion, the animals were sutured and returned to their home cages. Their appearance and body weights were observed on days1, 3, and 7 after surgery.

After days 1 and 7, 4 ml of venous blood was taken from 6 rats in each group and centrifuged at 4°C (Beckman Coulter Co., Optima LE-80K for 3,500 rpm, 10 min). The plasma phase was frozen (−20°C) for the blood biochemical tests. The rats were sacrificed by venesection and the weights of the isolated liver, kidney, spleen, lung, and heart were measured. All animal handling and care were in accordance with NIH guidelines. The protocol details were approved by the Animal Care and Use Committee of Keio University.

Blood Biochemical Tests

A total of 30 analytes (total protein, albumin, albumin-globulin ratio, aspartate aminotransferase [AST], alanine aminotransferase [ALT], lactate dehydrogenase [LDH], alkaline phosphatase, γ-glutamyltransferase, leucine aminopeptidase [LAP], choline esterase, total bilirubin, direct bililubin, creatinine, blood urea nitrogen, uric acid, amylase, lipase, creatine phosphokinase, total cholesterol, free cholesterol, cholesterol ester [EChol], β-lipoprotein, high density lipoprotein [HDL] cholesterol, neutral fat [*i.e.*, triglyceride, TG], total lipid, free fatty acid, phospholipids [PhL], K^+, Ca^{2+}, and Fe^{3+}) were measured by the Kyoto Microorganism Institute (Kyoto, Japan).

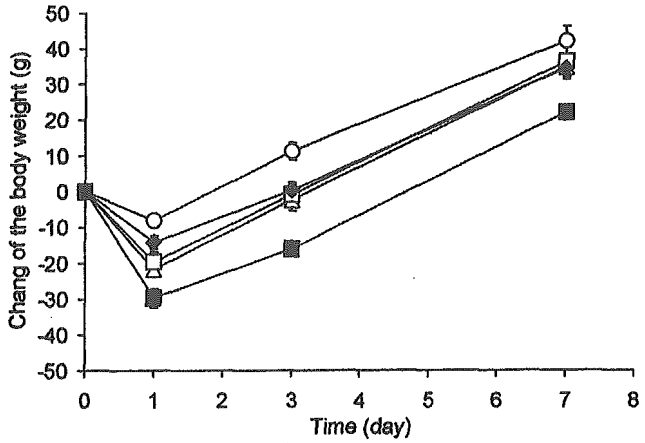


Figure 2. Body weight changes in male Wistar rats subjected to 20% and 40% blood exchange with recombinant human serum albumin (rHSA) and rHSA-heme solutions. Each value represents the mean±SEM. ○, Control group; ◆, 20% rHSA-heme group; △, 20% rHSA group; ■, 40% rHSA-heme group; and □, 40% rHSA group.

Histopathologic Observations

Paraffin sections were prepared from the 10% formalin fixed organs and stained with hematoxylin-eosin stain and Berlin blue. All histopathologic observations were carried out by Panapharm Laboratories Co., Ltd. (Kumamoto, Japan).

Data Analysis

The data for increased body weight, organ weight, and blood biochemical tests are expressed as mean ± SEM. A statistical analysis was performed using the Bartlett test followed by the Tukey-Kramer multiple comparison test. Values of $p < 0.05$ were considered significant. The statistical analytical software was StatView (SAS Institute Inc., Cary, NC).

Results

Appearance and Body Weight

In the control, 20% rHSA, and 20% and 40% rHSA-heme groups, all animals survived for 7 days without any change in their appearance and behavior. In contrast, one rat died after 3 days in the 40% rHSA group; the remaining five rats in this group did survive for 7 days.

In the control group, the change in body weight from the basal value decreased by 8.3 ± 1.5 gm after 1 day (**Figure 2**). However, it increased to 11.2 ± 2.4 gm after 3 days and to 42.1 ± 4.3 gm after 7 days.

In the 20% and 40% rHSA groups, body weights decreased by 21.8 ± 1.8 gm and 19.6 ± 2.8 gm, respectively, after 1 day, but they returned to basal levels after 3 days and increased to 34.9 ± 2.9 gm and 36.3 ± 4.0 gm, respectively, after 7 days.

In the 20% rHSA-heme group, body weight decreased by 14.3 ± 1.8 gm after 1 day. It recovered to the starting level after 3 days and increased to 34.9 ± 2.9 gm after 7 days. This change was almost the same as those observed in the 20% and 40% rHSA groups. In the 40% rHSA-heme group, the weight declined by 29.8 ± 2.5 gm after 1 day. Although it increased to 22.1 ± 2.1 gm after 7 days, the differences from the other groups were constant during the experimental period.

Weight of Vital Organs

We could not find any remarkable difference in the weights of the vital organs of the 20% rHSA and 20% rHSA-heme groups compared with the control group, except for spleen weight (**Figure 3**). Spleen weight was significantly increased in the 20% rHSA group after 1 and 7 days and in the 20% rHSA-heme group after 7 days.

In the 40% rHSA group, liver weight was significantly decreased after 1 day, and spleen weight increased after 7 days compared with that in the control group. In the 40% rHSA-heme group, spleen weight was significantly increased after 1 and 7 days *versus* the control group. There was no remarkable change in the weights of lungs, heart, liver, and kidney.

Blood Biochemical Tests

The 30 analytes from the blood biochemical tests of rat plasma are summarized in **Figure 4**. In the 20% and 40% rHSA groups, most of the parameters did not show any significant difference *versus* the control group, except for the iron decrease in the 20% rHSA group after 1 day and the choline esterase decrease after 7 days.

In the 20% rHSA-heme group, the decreases in LAP, TG, total lipid, PhL and iron after 1 day were significant compared with values in the control group. After 7 days, all analytes returned to the same levels as in the control group. With respect to the 20% rHSA group, the decrease in PhL after 1 day and the decrease in iron after 7 days were significant. In the 40% rHSA-heme group, the increase in total protein and AST and the decrease in total cholesterol, EChol, HDL cholesterol, and total lipid after 1 day were significant relative to the control group. Moreover, large increases in ALT and LDH were observed in three of the six samples. In comparison with the 40% rHSA group, the increase in LDH after 1 day, the decrease in EChol and HDL cholesterol after 1 day, the increase in HDL cholesterol after 7 days, and the decrease in TG after 7 days were significant.

Histopathologic Observations

A mild or modulate extramedullary hematopoiesis in the spleen was often found in all groups after the surgical operation at 7 days. In the 20% rHSA group, a slight erythrophagocytosis in the Kupffer cells of the liver (three samples) was observed after 7 days. In the 40% rHSA group, slight mineralization in the tubule of the kidney (three samples) after 1 day and a mild hyaline droplet in the tubule epithelial cells of the

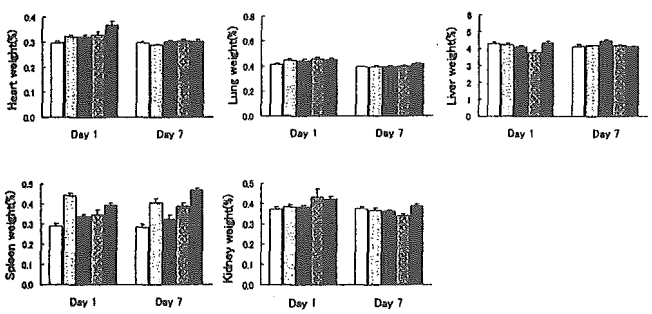


Figure 3. Relative organ weights (as a percentage of body weight) in male Wistar rats subjected to 20% and 40% blood exchange with recombinant human serum albumin (rHSA) and rHSA-heme solutions. Each value represents the mean±SEM. □, Control group; ▨, 20% rHSA group; ▩, 20% rHSA-heme group; ▨, 40% rHSA group; and ■, 40% rHSA-heme group.

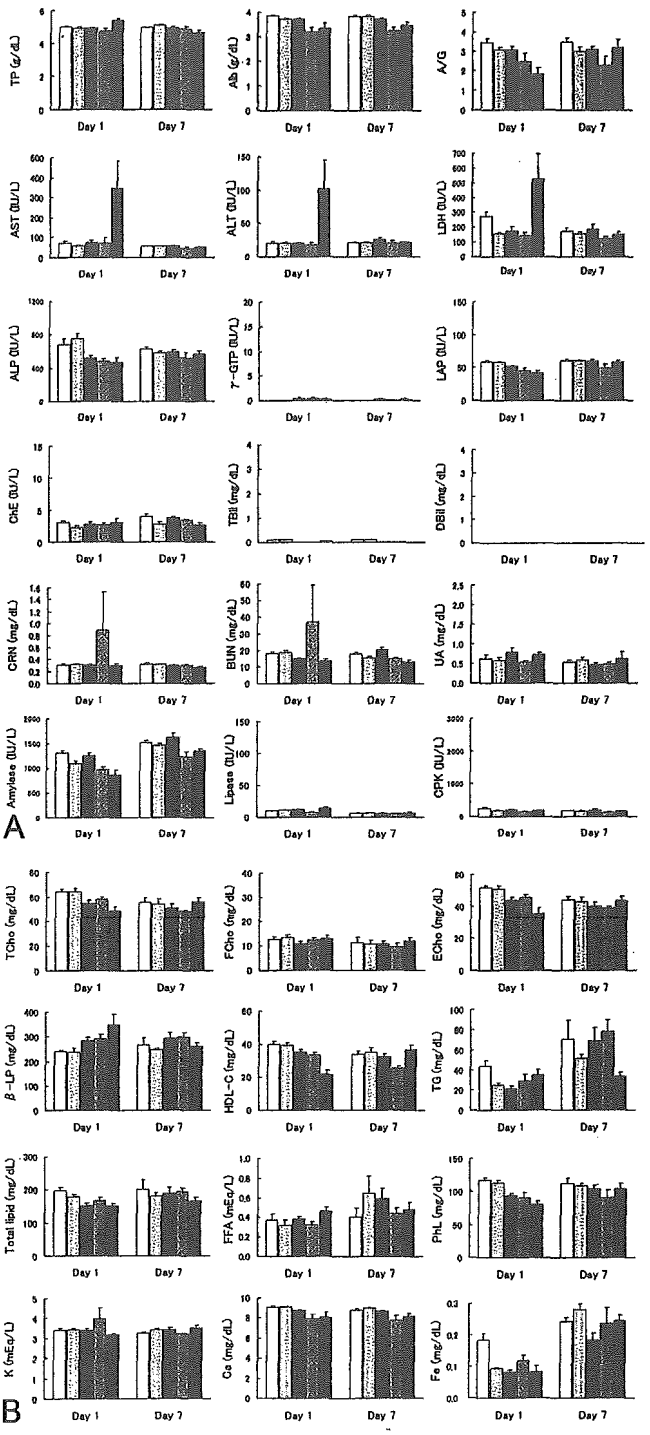


Figure 4. Blood biochemical tests of rat plasma after 20% and 40% blood exchange with recombinant human serum albumin (rHSA) and rHSA-heme solutions after 1 and 7 days. Each value represents the mean±SEM. □, Control group; ▨, 20% rHSA group; ▩, 20% rHSA-heme group; ▨, 40% rHSA group; and ■, 40% rHSA-heme group.

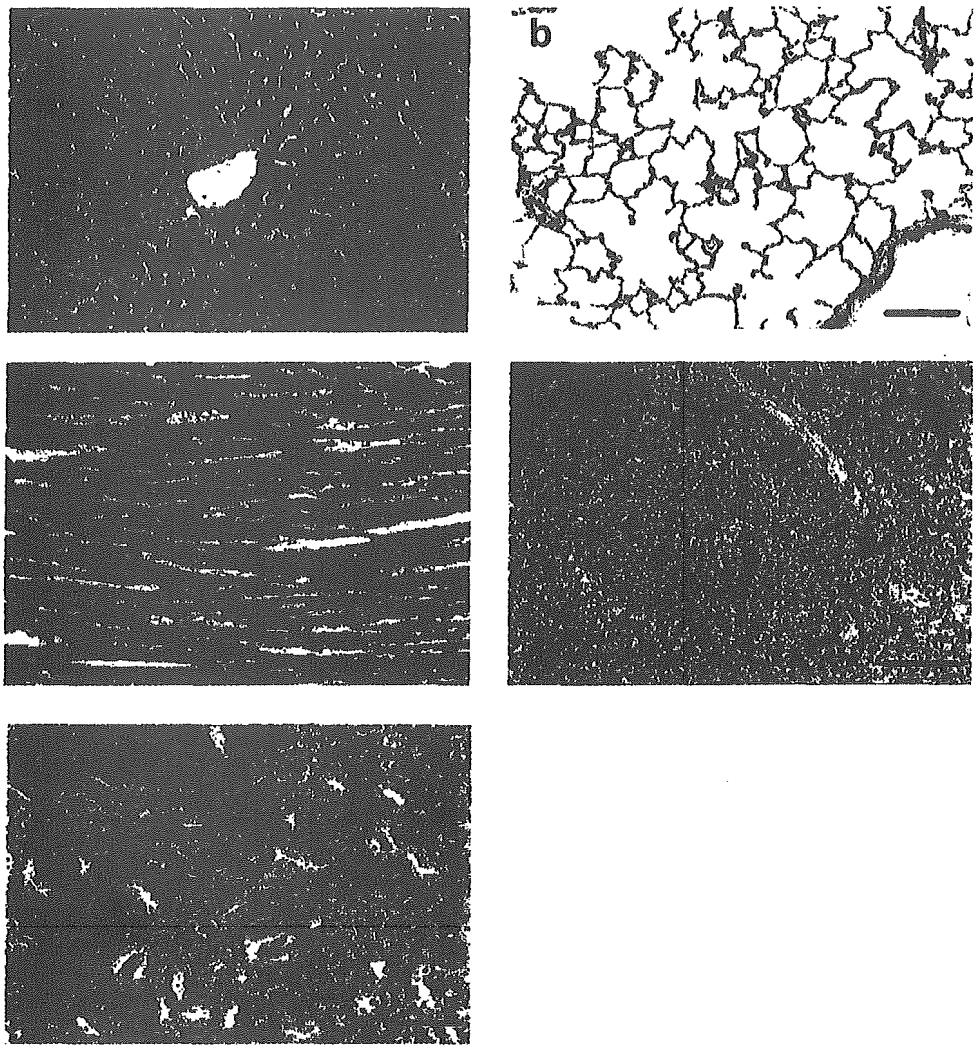


Figure 5. Microscopic observations of stained speciments of organs recovered from a rat 1 day after a 20% exchange with recombinant human serum albumin-heme. Bars = 100 mm, hematoxylin-eosin stain: a, liver; b, lung; c, heart; d, spleen; and e, kidney.

kidney (three samples) after 7 days were observed. There was no remarkable change in the lung, heart, and spleen.

In the 20% rHSA-heme group, a mild cellular infiltration in macrophages and a mild vacuolization in the Kupffer cells/macrophages of the liver were found in all six samples after 1 day (**Figure 5**). A mild brown pigment deposition appeared in the Kupffer cells/macrophages of the liver (five samples) after 7 days. There was no change in the heart, lung, and kidney. In the 40% rHSA-heme group, mild focal necrosis in the hepatocytes was visible in six samples after 1 day. The mild brown pigment deposition in the Kupffer cells/macrophages of the liver was also observed after 1 and 7 days. The mild hyaline droplet in the tubule epithelial cells in the kidney (three samples) was sometimes seen. There was no unusual change in the heart and lung.

Discussion

In the control group, although anesthesia and the surgical operation temporarily decreased the body weight of the animals after 1 day, it exceeded basal values after 3 days. The somewhat larger declines in body weight after the exchange transfusion with rHSA or rHSA-heme solution compared with the control group are considered to be the effect of hemodilution. To produce Hb, the iron concentration was reduced by consumption after 1 day. The decreased body weight increased at almost the same rate in all groups, and iron concentrations returned to control levels after 7 days.

The decrease in LAP, TG, total lipid, PhL, and iron in the 20% rHSA-heme group could not be related to acute toxicity. In addition, the mild cellular infiltration in macrophages and the mild vacuolization of Kupffer cells/macrophages in the liver observed after 1 day are considered to be a nonspecific biologic reaction to removal of the rHSA-heme, which was recognized to be an exogenous compound. More recently, we have found that >90% of the heme from the rHSA-heme is captured by the liver with high selectivity (Y. Huang *et al.*, unpublished data) After 7 days, a brown pigment deposit appeared in the Kupffer cells/macrophages of the liver. It gradually became fainter and finally disappeared after 2 months. If the heme is decomposed by hemeoxygenase in the same manner as protoheme IX from Hb, hemosiderin including free iron can be observed by Berlin blue staining. Contrary to our expectation, there was a very slight signal with the Berlin blue stain in the macrophages. We can conclude that the 20% blood exchange with the rHSA-heme solution did not produce any negative side effects in the rats.

We made further evaluations of the rHSA-heme solution with 40% blood replacement. In the 40% rHSA group, one rat died after 3 days. Although the reason is not clear, histopathologic data suggest occurrence of an unexpected disorder in the urinary system. No significant difference in the blood biochemical tests and organ weights of the 40% rHSA group in comparison with the control group (except for the decrease in liver weight after 1 day and an increase in spleen weight after 7 days) could be found. The increase in spleen weight was a specific biologic reaction to the administration of rHSA to the rats.

After a 40% blood exchange with the rHSA-heme solution, some liver function markers (*e.g.*, AST, ALT, and LDH) became elevated in three samples, and mild focal necrosis was observed in the liver after 1 day. Because these parameters in the 20% rHSA-heme group were similar or lower than those of the control group, this implies that some disorder in liver function might occur with a 40% blood exchange with the rHSA-heme solution. However, other liver function markers (γ-glutamyltransferase, total bilirubin, and direct bilirubin) remained unaltered after sample infusion, so the "disorder" seems to have been temporary and not serious. Similar to the 20% rHSA-heme group after 7 days, the brown pigment deposit in the Kupffer cells/macrophages, which is presumably heme itself or a decomposition thereof, was also found. The results of the blood biochemical tests and the histopathologic observations returned to normal after 7 days.

In conclusion, the 20 % blood replacement with rHSA-heme in rats did not yield any toxic side effects for 7 days. A mild liver disorder occurred in the 40% rHSA-heme group; however, liver function returned normal within 7 days. Thus, administration of the rHSA-heme solution in a volume of 20% total blood volume did not produce any dysfunction of the vital organs, which allows us to undertake further advanced preclinical testing of this synthetic O_2 carrier as a red blood cell substitute.

Acknowledgment

The authors are grateful to NIPRO Corp. for their supporting the O_2 infusion project.

References

1. Tsuchida E: Perspectives of blood substitutes, in Tsuchida E (ed), *Blood Substitutes: Present and Future Perspectives,* Lausanne: Elsevier Science, 1998, pp. 1–14.
2. Winslow RM: The role of blood substitutes in emerging healthcare systems, in Tsuchida E (ed), *Blood Substitutes: Present and Future Perspectives,* Lausanne: Elsevier Science, 1998, pp. 15–32.
3. Squires JE: Artificial blood. *Science* 295: 1002–1005, 2002.
4. Greenburg AG, Kim HM: Hemoglobin-based oxygen carriers. *Crit Care* 8: S61–64, 2004 (and references therein).
5. Vandegriff KD, Malavalli A, Wooldridge J, Lohman J, Winslow RM: MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion* 43: 509–516, 2003.
6. Simoni J, Simoni G, Wesson DE, Griswold JA, Feola M; A novel hemoglobin-adenosine-glutathione based blood substitute: Evaluation of its effect on human blood *ex vivo. ASAIO J* 46: 679–692, 2000.
7. Powanda DD, Chang TMS: Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artif Cells Blood Subs Immobil Biotechnol* 30: 23–27, 2002.
8. Komatsu T, Hamamatsu K, Wu J, Tsuchida E: Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*- pivamido)phenylporphyrinatoiron(II) derivatives. *Bioconj Chem* 10: 82–86, 1999.
9. Tsuchida E, Komatsu T, Matsukawa Y, Hamamatsu K, Wu J: Human serum albumin incorporating tetrakis(*o*-pivalamido)-phenylporphinatoiron(II) derivative as a totally synthetic O_2-carrying hemoprotein. *Bioconj Chem* 10: 797–802, 1999.
10. 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. *Bioconj Chem* 13: 397–402, 2002.
11. Tsuchida E, Komatsu T, Hamamatsu K, *et al:* 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. *Bioconj Chem* 11: 46–50, 2000.
12. Huang Y, Komatsu T, Nakagawa A, Tsuchida E, Kobayashi S: Compatibility *in vitro* of albumin-heme (O_2 carrier) with blood cell components. *J Biomed Mater Res* 66A: 292–297, 2003.
13. Tsuchida E, Komatsu T, Matsukawa Y, *et al:* Human serum albumin incorporating synthetic heme: Red blood cell substitute without hypertension by nitric oxide scavenging. *J Biomed Mater Res* 64A: 257–261, 2003.
14. Komatsu T, Yamamoto H, Huang Y, Horinouchi H, Kobayashi K, Tsuchida E: Exchange transfusion with synthetic oxygen-carrying plasma protein 'albumin-heme' in acute anemia rat model after seventy-percent hemodilution. *J Biomed Mater Res* 71A: 2004, in press.
15. Huang Y, Komatsu T, Yamamoto H, Horinouchi H, Kobayashi K, Tsuchida E: Exchange transfusion with entirely synthetic red-cell substitute albumin-heme into rats: physiological responses and blood biochemical tests. *J Biomed Mater Res* 71A: 63–69, 2004.

Human Serum Albumin Bearing Covalently Attached Iron(II) Porphyrins as O_2-Coordination Sites

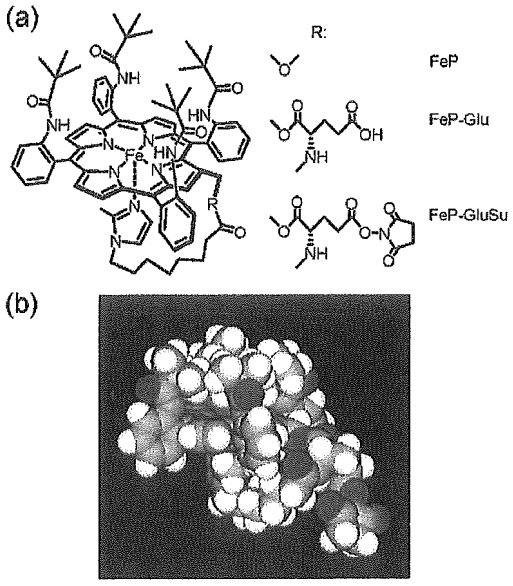
Rong-Min Wang,[†,‡] Teruyuki Komatsu,[†] Akito Nakagawa,[†] and Eishun Tsuchida*[,†]

Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan, and Institute of Polymer, Northwest Normal University, Lanzhou 730070, China . Received June 17, 2004; Revised Manuscript Received September 25, 2004

Tetrakis{(α,α,α,α-*o*-pivalamido)phenyl}porphinatoiron(II) with a bifunctional tail possessing an axially coordinated imidazolyl group and a protein attachable succinimidyl(glutamyl) group (FeP-GluSu) has been synthesized. It can efficiently react with the lysine residues of recombinant human serum albumin (rHSA), giving a new albumin–heme conjugate [rHSA(FeP-Glu)]. MALDI-TOFMS showed a distinct molecular ion peak at *m/z* 70 643, which indicates that three FeP-Glu molecules were covalently linked to the rHSA scaffold. The binding number of FeP-Glu is approximately three (mol/mol) and independent of the mixing ratio. The CD spectrum and Native PAGE revealed that the albumin structure remained unaltered after the covalent bonding of the hemes. This rHSA(FeP-Glu) conjugate can bind and release O_2 reversibly under physiological conditions (pH 7.3, 37 °C) in the same manner as hemoglobin and myoglobin. The O_2-adduct complex had a remarkably long lifetime ($\tau_{1/2}$: 5 h). The O_2-binding affinity [$P_{1/2}^{O_2}$: 27 Torr] was identical to that of human red cells. Laser flash photolysis experiments gave the O_2- and CO-association rate constants and suggested that there are two different geometries of the imidazole binding to the central ion.

Human serum albumin (HSA), which is the major plasma protein component in our bloodstream, has no prosthetic group; however it nonspecifically captures many endogenous and exogenous compounds by weak interactions, *e.g.,* H-bond, ionic attraction, and hydrophobic interaction, namely noncovalent bonds (*1–3*). Synthetic heme, 2-[{8-*N*-(2-methylimidazolyl)octanoyl}-oxy]methyl-5,10,15,20-tetrakis{(α,α,α,α-*o*-pivalamido)-phenyl}porphinatoiron(II) (FeP, Chart 1 a) is also incorporated into recombinant HSA (rHSA), and the obtained albumin–heme (rHSA-FeP) hybrid can reversibly coordinate O_2 under physiological conditions (pH 7.3, 37 °C) (*4*). This O_2-carrying plasma hemoprotein could be of medical importance as a blood replacement composition (*4e–g*). Nevertheless, the major driving force of the heme-binding to albumin is a hydrophobic interaction; therefore, its binding constants (10^4–10^6 M^{-1}) are not high enough to maintain the heme concentration in the circulatory system for a long period (*4a*). The administration of the albumin–heme hybrid solution into rats demonstrated that the lifetimes of the heme was less than 6 h (*4e, 5*). To immobilize the heme group to the albumin scaffold more tightly and retain its O_2-transport efficacy, we have combined the O_2-coordination site FeP to the rHSA structure through a covalent bond. In this communication, we report, for the first time, the synthesis of a novel FeP analogue with a bifunctional branched-tail including an axially coordinated imidazolyl group and a protein-attachable succinimidyl(glutamyl) group (FeP-GluSu, Chart 1 a), and the properties of the rHSA conjugate bearing covalently linked FeP-Glu as a new O_2-carrying hemoprotein.

Chart 1. (a) 5,10,15,20-Tetrakis{(α,α,α,α-*o*-pivalamido)-phenyl}porphinatoiron Derivatives with a Bifunctional Tail Group. (b) Space-Filling Representation of the Oxygenated FeP-GluSu by Insight II (see ref 11)



As a functional side-chain of FeP, which directly makes a covalent bond to rHSA, we selected the succinimidyl group, because it selectively reacts with the NH_2 group of lysine in the range of pH 6.3–8.6 with high yield. The branched tail that includes the imidazolyl and succnimidyl groups via a glutamate junction was introduced into the parent porphyrin **1** (*6*) as shown in Scheme 1 (*7*).

* Corresponding author. Phone: +81 3-5286-3120. Fax: +81-3-3205-4740. E-mail: eishun@waseda.jp.
† Waseda University.
‡ Northwest Normal University.

10.1021/bc049859m CCC: $30.25
Published on Web 11/12/2004

Scheme 1. Synthetic Scheme of FeP-GluSu

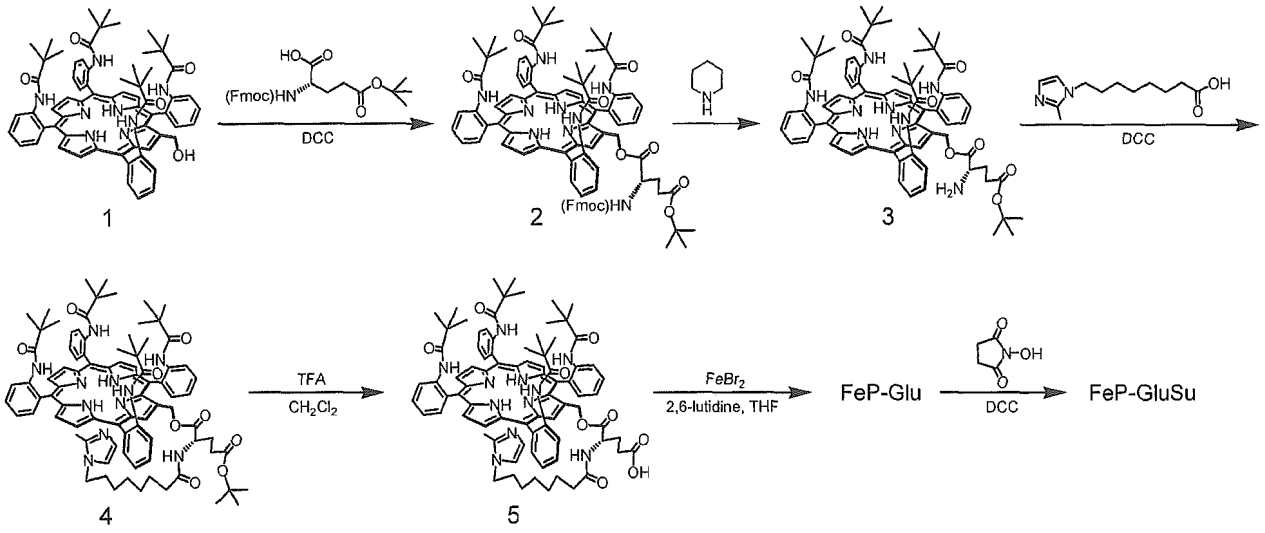


Table 1. CO-and O_2-Binding Parameters of rHSA(FeP-Glu) Conjugate in Phosphate–Buffered Solution (pH 7.3) at 25 °C

	$10^{-6}\,k_{on}^{CO}(M^{-1}s^{-1})$		$10^{-7}\,k_{on}^{O_2}(M^{-1}s^{-1})$		$10^{-2}\,k_{off}^{O_2}(s^{-1})$		$P_{1/2}^{O_2}$(Torr)[a]
system	fast	slow	fast	slow	fast	slow	
rHSA(FeP-Glu) conjugate	6.2	1.1	2.8	–	3.3	–	9 (27)
rHSA-FeP hybrid[b]	4.7	0.66	3.2	1.0	7.2	2.2	13 (35)
Hb(T-state)α[c]	0.22	–	0.29	–	1.8	–	40

[a] At 37 °C in parenthesis. [b] From ref 4c. [c] From refs 13–15.

First, Fmoc-L-Glu(g-*tert*-butyl ester) was bound to the OH group at the β-pyrrolic position of the porphyrin **1** by DCC. After removal of the Fmoc protecting group with piperidine, 8-*N*-(2-methylimidazolyl)octanoic acid was reacted with the obtained compound **3** in CH_2Cl_2, giving the imidazolyl-tailed porphyrin (**4**). The *tert*-butyl group was then removed by TFA, and the central iron insertion was carried out by the general $FeBr_2$ method to afford the iron-porphryin FeP-Glu. Finally, the reaction of *N*-hydroxysuccinimide with DCC gave the FeP-GluSu. All reactions can be performed at room temperature with high yields. The analytical data of all compounds described above were satisfactory obtained (*7*).

The FeP-Glu was converted to the ferrous complex by reduction in a heterogeneous two-phase system (toluene/aqueous $Na_2S_2O_4$) under an N_2 atmosphere (*6, 8*). The UV–vis absorption spectrum of the orange solution showed five-*N*-coordinated Fe(II) species (λ_{max}: 440, 531, 563 nm) via intramolecular imidazole binding (*6, 8, 9*). Upon exposure to CO, its UV–vis absorption immediately moved to that of the CO adduct complex. On the other hand, the dioxygenation was unstable at 25 °C, which is likely due to the presence of the neighboring glutamic acid proton.

The EtOH solution of the carbonyl FeP-GluSu (2 mL) was then injected into the phosphate-buffered solution of rHSA (8 mL, pH 7.3) (molar ratio 4/1), and the mixture was gently stirred for 1 h at room temperature. The solution was dialyzed against phosphate buffer (pH 7.3) to remove EtOH. The MALDI-TOFMS demonstrated a single molecular ion peak at *m/z* 70 643 (Figure 1). Attempts to measure the molecular weight of the rHSA-FeP hybrid, in which the FePs are noncovalently accommodated, failed using MALDI- and ESI-TOFMS; the molecular ion peak of rHSA (65 500) was only observable because the FePs are dissociated from the albumin during the ionization process (*10*). Therefore, we can conclude that the FeP-Glu is conjugated with rHSA through amide bond formation. The average number of FeP-Glu in an rHSA was estimated to be 2.9–3.5, and this number is not dependent on the mixing molar ratio of FeP-GluSu/rHSA that ranged from 4 to 10. There are a total of 59 NH_2 groups in the rHSA structure, but only three of them are presumably active for the FeP-GluSu binding.

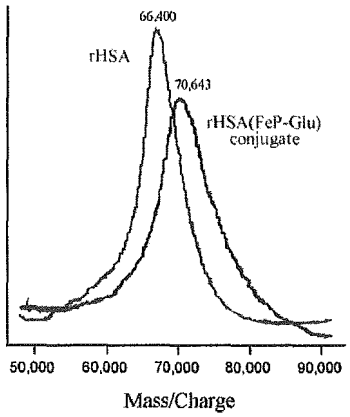


Figure 1. MALDI-TOFMS of the rHSA(FeP-Glu) conjugate. Matrix: 2,5-dihydroxybenzoic acid.

The conjugation of FeP-GluSu did not induce any change in the circular dichroism spectrum of rHSA in the 200–250 nm region. The Native PAGE of rHSA(FeP-Glu) also showed a single band with same migration distance of rHSA. Both results suggested that the secondary structure, molecular shape, and surface charge of albumin remained unaltered after the covalent binding of the hemes.

The UV–vis absorption spectrum of the rHSA(FeP-Glu) conjugate under an N_2 atmosphere showed a typical five-*N*-coordinated complex as seen in the toluene solution of FeP-Glu (Figure 2) (*4a,b, 6, 8, 9*). Upon exposure

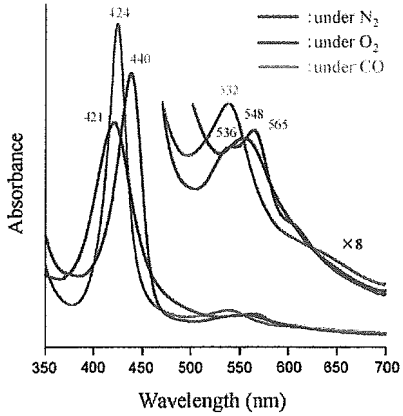


Figure 2. UV−vis absorption spectral changes of the rHSA-(FeP-Glu) conjugate in phosphate-buffered solution (pH 7.3) at 25 °C.

of this solution to O_2, the spectrum changed to that of the O_2-adduct complex under physiological conditions (pH 7.3, 37 °C) (*4a−c*). This dioxygenation was reversibly observed to be dependent on the O_2 partial pressure in the same manner as hemoglobin (Hb) and myoglobin. The half-lifetime of the O_2 adduct (ca. 5 h at 37 °C) was significantly longer than that of the noncovalent rHSA-FeP hybrid ($\tau_{1/2}$: 2 h) (*4d*). The covalent linkages of FeP-Glu to the protein scaffold obviously retarded the oxidation process of the central ferrous ion. Molecular simulation of the structure of FeP-GluSu revealed that the geometry of the imidazole ring against the porphyrin platform was perpendicular, which suggests that the spacer moiety between the imidazole and the porphyrin periphery does not produce an unfavorable distortion of the axial coordination and will not influence the O_2-binding behavior (see Chart 1b) (*11*).

The O_2-binding affinity [$P_{1/2}{}^{O_2}$] of the rHSA(FeP-Glu) conjugate was determined to be 27 Torr at 37 °C (*3b,c, 6, 7, 9, 10*), which is almost the same as that of the rHSA-FeP hybrid [$P_{1/2}{}^{O_2}$: 33 Torr] (*3b−d*) and identical to that of human red cells (*12*). The laser flash photolysis experiments provided the association rate constants of the O_2- and CO-bindings ($k_{on}{}^{O_2}$, $k_{on}{}^{CO}$) (*6, 8, 9a*). The absorption decays accompanying the O_2- and CO-recombination to the noncovalent rHSA-FeP hybrid were composed of two phases of the first-order kinetics, and the curves were fit by a double-exponential equation to determine k_{on} (fast) and k_{on} (slow) (Table 1) (*4c*). We supposed that the O_2- and CO-association to the FeP in the hydrophobic domains of the albumin was influenced by the molecular microenvironments around each O_2-coordination site, e.g., steric hindrance of the amino acid residue and difference in polarity (*4b−d*). The time dependence of the absorption change in the CO recombination to the rHSA(FeP-Glu) conjugate also showed double-exponential profile, but the rebinding process of O_2 obeyed monophasic decay. On the basis of studies on synthetic model hemes, it has been known that the proximal-side effect is the only primary factor which influences the association rate for CO but not for O_2 (*8, 9a*). We assume that there are two different geometries of the imidazole coordination and that each one shows the individual kinetics of the CO association. The covalent linkages between the axially coordinated imidazolyl side-chain and the albumin structure may provide an additional strain of the Fe−N(imidazole) bond and gives two conformations of the proximal-base binding. Since the $k_{on}{}^{O_2}$ value of rHSA(FeP-Glu) was nearly the same as the $k_{on}{}^{O_2}$ (fast) of the rHSA-FeP hybrid (Table 1), the FeP-Glu molecules are likely to locate on the surface of rHSA.

In conclusion, reaction of the newly synthesized tetrakis{(α,α,α,α-*o*-pivalamido)phenyl}porphinatoiron(II) with a proximal base and succinimidyl(glutamyl) group to rHSA produced a novel albumin conjugate bearing covalently attached heme groups as O_2-coordination sites. The molecular weight of rHSA(FeP-Glu) was directly measured by MALDI-TOF MS. In nature, one can find unique heme-linked proteins, e.g., cyctochrome *c*. The rHSA(FeP-Glu) conjugate presumably becomes a valuable model of these hemoproteins. The obtained rHSA-(FeP-Glu) can reversibly absorb O_2 under physiological conditions, and its O_2-binding affinity showed an identical value to that for human erythrocytes. These results suggest that this novel plasma protein may efficiently transport O_2 in the bloodstream as an O_2-carrier with a long circulation time.

ACKNOWLEDGMENT

This work was partially supported by Grant-in-Aid for Scientific Research (No. 16350093) from JSPS, Grant-in-Aid for Exploratory Research (No. 16655049) from MEXT Japan, and Health Science Research Grants (Regulatory Science) from MHLW Japan. R.M.W. acknowledges NNSFC (No. 20274034). The authors are grateful to NIPRO Corp. for their supporting the oxygen-infusion project.

Supporting Information Available: Experimental details of the compounds **2**, **3**, **4**, **5**, FeP-Glu, and FeP-GluSu and their spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Peters, T., Jr. (1996) All about albumin. *Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego; and reference therein.

(2) Kragh-Hansen, U. (1981) Molecular aspects of ligand binding to serum albumin. *Pharmacol. Rev. 33,* 17−53.

(3) Curry, S., Brick, P., and Franks, N. P. (1999) Fatty acid binding to human serum albumin: new insights from crystallographic studies. *Biochim. Biphys. Acta 1441,* 131−140.

(4) (a) Komatsu, T., Hamamatsu, K., Wu, J., and Tsuchida, E. (1999) Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*-pivamido)phenylporphyrinatoiron(II) Derivatives. *Bioconjugate Chem. 10,* 82−86. (b) Tsuchida, E., Komatsu, T., Matsukawa, Y., Hamamatsu, K., and Wu, J. (1999) Human serum albumin incorporating tetrakis(*o*-pivalamido)phenylporphinatoiron(II) derivative as a totally synthetic O_2-carrying hemoprotein. *Bioconjugate Chem. 10,* 797−802. (c) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2000) Kinetics of CO- and O_2-binding to human serum albumin-heme hybrid. *Bioconjugate Chem. 11,* 772−776. (d) Komatsu, T., Matsukawa, Y., and 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. *Bioconjugate Chem. 13,* 397−402. (e) Tsuchida, E., Komatsu, T., Hamamatsu, K., Matsukawa, Y., Tajima, A., Yoshizu, A., Izumi, Y., and Kobayashi, K. (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. *Bioconjugate Chem. 11,* 46−50. (f) Kobayashi, K., Komatsu, T., Iwamaru, A., Matsukawa, Y., Watanabe, M., Horinouchi, H., and Tsuchida, E. (2003) Oxygenation of hypoxia region in solid tumor by administration of human serum albumin incorporating synthetic hemes. *J. Biomed. Mater. Res. 64A,* 48−51. (g) Tsuchida, E., Komatsu, T., Matsukawa, Y., Nakagawa, A., Sakai, H., Kobayashi, K., and Suematsu, M. (2003) Human serum albumin incorporating synthetic heme: red blood cell substitute

without hypertension by nitric oxide scavenging. *J. Biomed. Mater. Res. 64A*, 257–261.
(5) Russo, S. M., Pepe, J. Y., Donohue, S., Cable E. E., Lambrecht, R. W., and Bonkovsky, H. L. (1995) Tissue distribution of zinc-mesoporphyrin in rats: relationship to inhibition of heme oxygenase. *J. Pharmacol. Exp. Ther. 272*, 766–774.
(6) Tsuchida, E., Komatsu, T., Ando, K., Kumamoto, S., and Nishide, H (1995) Synthesis and O_2-binding properties of tetraphenylporphyrinatoiron(II) derivatives bearing a proximal imidazole covalently bound at the β-pyrrolic position. *J. Chem. Soc., Perkin Trans. 2* 1995, 747–753.
(7) The synthetic details and spectroscopic data of the porphyrins can be obtained from the Supporting Information.
(8) Tsuchida, E., Komatsu, T., Arai, K., and Nishide, H. (1993) Synthesis and dioxygen-binding properties of double-sided porphyrinatoiron(II) complexes bearing covalently bound axial imidazole. *J. Chem. Soc., Dalton Trans.* 2465–2469.
(9) (a) Collman, J. P., Brauman, J. I., Collins, T. J., Iverson, B. L., Lang, G., Pettman, R. B., Sessler, J. L., and Walters, M. A. (1983) Synthesis and characterization of the "Pocket" porphyrins. *J. Am. Chem. Soc. 105*, 3038–3052. (b) Collman, J. P., Brauman, J. I., Iverson, B. L., Sessler, J. L., Morris, R. M., and Gibson, Q. H. (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.
(10) Tsuchida, E., Komatsu, T., and Yanagimoto, T. (2000) Molecular environment effect on O_2-binding to lipidporphyrinatoiron(II) complexes in aqueous media, *J. Porphyr. 4*, 81–87.
(11) The esff force field simulation was performed using an Insight II system (Molecular Simulations Inc.). The structure was generated by alternative minimizations and annealing dynamic calculations from 1,000 K to 100 K.
(12) Severinghaus, J. W. (1966) Blood gas calculator. *J. Appl. Physiol. 21*, 1108–1116.
(13) Sawicki, C. A., and Gibson G. H. (1977) Properties of the T State of Human Oxyhemoglobin Studied by Laser Photolysis. *J. Biol. Chem. 252*, 7538–7547.
(14) Sharma, V. S., Schmidt, M. R., and Ranney, H. M. (1976) Dissociation of CO from Carboxyhemoglobin. *J. Biol. Chem. 251*, 4267–4272.
(15) Steinmeier, R. C., and Parkhurst, L. J. (1975) Kinetic Studies on the Five Principle Components of Normal Adult Human Hemoglobin. *Biochemistry 14*, 1564–1573.

BC049859M

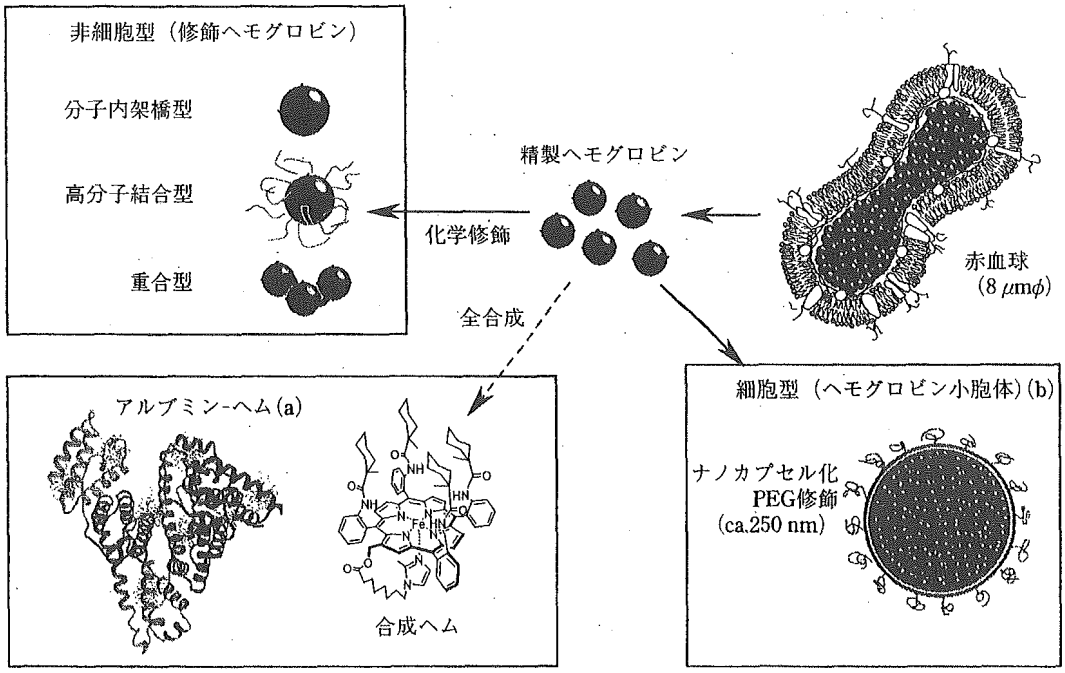
第12節　人工赤血球

1. 酸素輸液の必要性

輸血システムの確立により，輸血用血液が普遍的に常備されるようになり，医療の進展に大きな貢献をしてきた。輸血にともなう感染の確率は，核酸増幅法など献血液の厳重な検査と管理によって著しく低下してはいる。だが，未知の感染源の可能性も考えると，完全に零とはいえない。加えて赤血球の保存期限はわずか3週間である。献血1回当たりの採血量は400 mlになったが，人口の高齢化にともない健康な献血者総数は低下しつづけている。わが国のように自然災害が危惧される場合，緊急需要に対応した大量保存は重要な国家的施策でもある。血液型に関係なく要請に即応して，いつでもどこでもただちに供与できる，酸素輸液(人工赤血球)の出現が強く期待されている(図1)。

2. ヒトヘモグロビン小胞体の構成

酸素輸液の開発は歴史的には粗製ヘモグロビン(Hb)の投与(1916年)に始まる。現在は修飾Hb(分子内架橋型，重合型，高分子結合型など直接Hbを加工)の臨床試験が進行中である。しかし，修飾Hb溶液系では血管収縮による血圧亢進，代謝異常，軽度の神経毒性など，赤血球とかけ離れた構造に起因する副作用が指摘されている。Hb



精製ヘモグロビン利用の非細胞型(a)と細胞型(b)のヘモグロビン小胞体，また合成ヘム誘導体を利用するアルブミン-ヘムが開発されている。

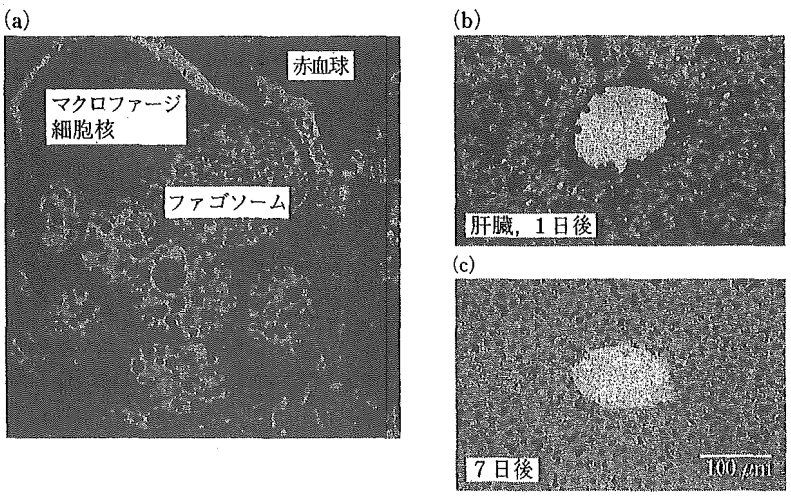
図1　酸素輸液の開発動向

が赤血球内にある理由は，①35％濃厚Hb溶液の高い粘度と膠質浸透圧の抑制，②本来毒性を有するHbの逸脱の抑制，③Hb機能維持のための各種リン酸などエネルギー分子，解糖ならびに還元－酵素系の保持である。また，④血液(血球分散系)は非ニュートン流体で，体内循環とくに末梢血管内における，特色ある流動形式と生理作用の特性を示す。赤血球構造を考えると，Hbを無害なカプセルに入れた細胞型のHb小胞体が有効である[1]。粒径制御とリン脂質が構成する2分子膜で高濃度Hb溶液を被覆する課題は，筆者らによって達成された。高純度高濃度の精製Hb溶液を用い，またextrusion法の採用により，Hb溶液(濃度35％以上)がほぼ1枚膜に包まれた，Hb小胞体の製造に成功した。ウィルス不活化と滅菌の困難も，Hbに一酸化炭素(CO)を結合して安定化させ60～70℃の加熱で完了した。最終段階で光照射励起により，酸素を結合したHbO_2に変換する。またアロステリック因子を共存させて，酸素親和度の制御(9～60 Torr)ができる。Poly(ethyleneglycol)(PEG)結合脂質を粒子表面に配置して，小胞体粒子間の凝集抑制と分散安定度向上の効果を観測し，この表面修飾過程を動力学的に明らかにし，溶液のまま室温にて2年以上保存できる系が構築されている。

3．ヘモグロビン小胞体の投与試験

Hb小胞体の動物投与試験では，90％超の高度交換輸血の場合でも，循環動態保持が確認されている[2),3)]。小胞体表面をPEG修飾すると，小胞体凝集を抑制でき，粘度と膠質浸透圧はほぼ血液と同等の非ニュートン流体になる。80％交換輸血における皮下微小循環動態の検討では，血管内を流動するPEG修飾Hb小胞体に凝集は認められず，血漿相に均一分散している[4)]。他方，未修飾群は細静脈や毛細管内で凝集体が観測されている。血流速度，有効毛細管密度，組織酸素分圧ともにPEG修飾系が，未修飾の系に比較しきわめて高い値に推移し，PEG修飾が不可欠であることが確認できた。

Hb小胞体の安全性についても明らかになっている。Hb小胞体は最終的に脾，肝に移行するが，貪食細胞に捕捉されたHb小胞体は7日以内に分解消失することが，組織病理学的に確認されている[5)]（図2）。細網内皮系の貪食機能は変動するが，可逆的であり，一過性で血液生化学的検査の結果



(a) Hb小胞体投与1日後のラット脾臓マクロファージの透過型電子顕微鏡写真。食胞（ファゴソーム）中にHb小胞体の粒子が多数認められる。この後3日目には消失する。(b)(c) Hb小胞体投与後のラット肝臓の抗ヒトHb抗体染色後の顕微鏡写真。色の濃い部分がヒトHbの存在部位。投与1日後では多量のHbの存在（Kuppfer細胞内）が認められるが，投与7日後にはほとんど消失，蓄積を認めない。

図2　ヘモグロビン小胞体の代謝過程

からも臓器の異常は認められていない。

修飾Hb投与に際し血圧の異常亢進や，血管床への血小板沈着などの副作用が認められている。これはNOとの高い親和性に起因する。とくに分子内架橋Hbはきわめて小さい(7 nm)ため，血管内平滑筋近傍に接近し，内皮細胞由来NOを捕捉し，血管弛緩機能を低下させる。血管収縮は末梢循環と組織酸素化を阻害する。他方，直径250 nmのHb小胞体では血管外漏出はなく，また血管平滑筋まで接近せず，NO結合反応は抑制され，血管収縮も血圧亢進も起こらない[6]。

肝臓中ではhemeoxygenaseが産生する一酸化炭素(CO)が，血管弛緩因子として作用する。摘出肝灌流実験では，Hb溶液はDisse腔に侵入し，Hbの代謝異常によるbilirubin排泄の亢進，またCOが血中Hbに捕捉され20％の血管抵抗増大を示し，同時に類洞の不連続的狭窄と流動停止領域の存在を確認している。他方，Hb小胞体はDisse腔に侵入できないため，この現象は生起しない[7,8]。

4. ヘムタンパク質系酸素輸液

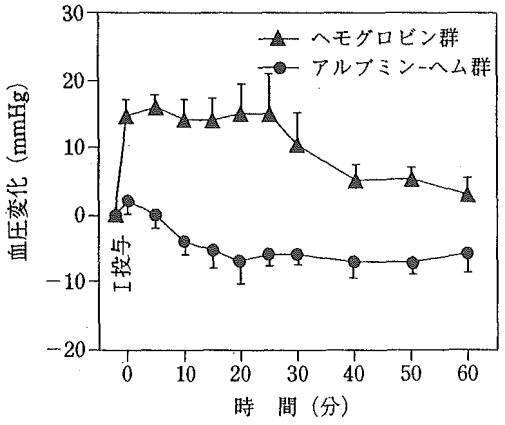
他方，全合成型の酸素輸液の開発も進んでいる[9,10]。血清アルブミン(Mw. 66.5 kD)は血漿タンパク質の約70％を占める単純タンパク質であり，コロイド浸透圧の維持，各種内因性物質・薬物の運搬，血液pHの調整などの役割を果たしている。わが国では世界に先駆けて，遺伝子組み換えヒト血清アルブミン(rHSA)の，世界初の上市を間近に迎える状況にある。筆者らはこのアルブミンの非特異的多分子結合能を利用し，rHSAに酸素配位能を有するヘム誘導体を包接させる方法により，新しいアルブミン－ヘム(合成ヘムタンパク質)の創製に成功した。アルブミン－ヘムは，①完全合成系酸素輸液であり，感染の危険性がまったくない，②一切のヒト(および動物)由来の血液資源を必要としない，③酸素親和度(P_{50})はヘム構造の調整により調節可能，④アルブミンが血管内皮を透過しないため，NO捕捉にともなう血管収縮・血圧亢進は惹起されない，などの優れた特徴をもつ。すでに酸素輸液としての酸素結合能，溶液物性，血液適合性が実証されており，安全性と効果の解明を中心とした前臨床評価試験が進められている。

アルブミン1分子当たりに結合するヘムの数は最大8，これはHbの2倍に相当する。包接の駆動力は疎水性相互作用であるため，結合後もアルブミンの二次構造や表面電荷に変化はないと考えられてきた。だが，ごく最近S. Curry (Imperial College)らは，脂肪酸包接rHSAのX線結晶構造解析に初めて成功し，基質結合後のrHSAは幅8 nmから9 nmへ膨張することを明らかにした[11]。この発見はヘムがrHSA内部に包接されると，分子レベルで構造変化が誘起されることを示唆しており，酸素配位特性の変化とも関連して，アルブミン－ヘム結晶構造の解明が待たれている。

また酸素配位結合部位であるヘムの化学構造(酸素配位座近傍置換基，分子内軸塩基など)を変化させた一連の誘導体群が合成され，rHSA包接体とした系について，ヘム構造と酸素配位能の相関が定量的に明らかにされている[12,13]。

アルブミン－ヘムのNO親和度はO_2親和度の7.6×10^6倍と高いが[14]，体内へ投与しても，非細胞型Hbに見られる血管内皮からの漏出，NO捕捉にともなう血圧亢進は認められない(図3)。これはアルブミンの表面電荷が負に帯電していることに起因する。

さらにアルブミンのCys^{34}をビスマレイミド誘導体で架橋する方法により，アルブミン二量体が



ヘモグロビン投与ではただちに血圧が上昇するが，アルブミン-ヘムではそのような現象は認められない。

図3　アルブミン-ヘム溶液をラットに投与した後の血圧変化

合成され，それにヘム16分子が結合されたアルブミン－ヘム二量体[15]も完成している。この10 wt％水溶液はコロイド浸透圧を生理条件に保った条件で，血液の1.3倍量の酸素を溶解できる。

5. おわりに

酸素輸液の研究は，このようにこの20年間で具体的対象物についての物性と動的機能の相関が詳しく解明できるようになっている。①型物質や感染源をまったく含まない，②浸透圧や粘度などの溶液物性が血液と同等に調節可能，③毒性がきわめて低く，ヒト血液換算で2～3 lit. 投与の場合でも安全かつ代謝可能，④長期保存(室温，2年間)と安価供給が可能などの特性を有する，酸素輸液の実現が間近といってよい。

【参考・引用文献】

1) E. Tsuchida., ed.：Blood substitutes, present and future perspective. Elsevier, Amsterdam, (1998).
2) Y. Izumi, et al.：*Crit Care Med*, **24**, 1869-1873 (1996).
3) H. Sakai, et al.：*Bioconjugate Chem*, **8**, 23-30 (1997).
4) H. Sakai, et al.：*J. Biomed Mater Res*, **40**, 66-78 (1998).
5) H. Sakai, et al.：*Am. J. Phathol*, **159**, 1079-1088 (2001).
6) H. Sakai, et al.：*Am. J. Physiol Heart Circ Physiol*, **279**, 908-915 (2000).
7) N. Goda, et al.：*J. Clin. Invest*, **101**, 604-612 (1998).
8) T. Kyokane, et al.：*Gastroenterology*, **120**, 1227-1240 (2001).
9) E. Tsuchida, et al.：*Bioconjugate Chem*, **8**, 534-538 (1997).
10) T. Komatsu, et al.：*Bioconjugate Chem*, **10**, 82-86 (1999).
11) S. Curry, et al.：*Nature Struc. Biol*, **5**, 827-835 (1998).
12) T. Komatsu, et al.：*Bull. Chem. Soc. Jpn*, **74**, 1695-1702 (2001).
13) T. Komatsu, et al.：*Bioconjugate Chem*, **13**, 397-402 (2002).
14) T. Komatsu. et al.：*Bioconjugate Chem.*, **12**, 71-75 (2001).
15) T. Komatsu, et al.：*Macromolecules*, **32**, 8388-8391 (1999).

＜土田　英俊／武岡　真司／小松　晃之／酒井　宏水＞

日本表面科学会創立25周年記念
新訂版・表面科学の基礎と応用

発行日　2004年　6月　22日　初版第1刷発行

編者　日本表面科学会「新訂版・表面科学の基礎と応用」編集委員会

発行者　吉田　隆

発行所　株式会社 エヌ・ティー・エス
〒113-8755　東京都文京区湯島2-16-16
TEL:03(3814)3511(代表)　03(3814)9151(営業部)
http://www.nts-book.co.jp/

制作　編集室 アイ・ティ・オウ

印刷　ショウワドウ・イープレス株式会社

製本　牧製本印刷株式会社

ISBN4-86043-051-4　C3050