TABLE I
Basal Values of Each Group

	Control	rHSA	rHSA-heme
MAP (mmHg)	99 ± 2.8	101 ± 2.8	100 ± 6.1
HR (beats/min)	404 ± 19	435 ± 19	420 ± 13
Respiration rate (breaths/min)	66 ± 0.7	75 ± 4.0	70 ± 2.1
paO_2 (mmHg)	84.7 ± 3.1	84.9 ± 3.7	80.8 ± 2.2
pvO_2 (mmHg)	51.0 ± 1.5	45.7 ± 1.1	51.0 ± 1.7
Body weight (g)	305 ± 4.0	304 ± 2.8	305 ± 4.1

maintained within 36.9–37.4°C during the experiments [Fig. 1(a)].

The MAP time course in the control group demonstrated only a small deviation within 83.8–100.0% for 6 h. In the rHSA and rHSA-heme groups, the observed changes in MAP were almost the same as those of the control group. They ranged within 93.2–100 and 89.3–100%, respectively. It is remarkable that no vasoactive reaction was seen after the infusion of rHSA-heme [Fig. 1(b)].

The HR of the control, rHSA, and rHSA-heme groups remained unaltered for 6 h. The values of the control, rHSA, and rHSA-heme groups were in the range of 98.3–103.9, 96.9–108.8, and 85.7–100.0%, respectively [Fig. 1(c)].

The respiration rates also remained stable during the measurements. No significant difference was recognized among the three groups [Fig. 1(d)].

Blood-gas parameters

No difference in the pH changes was observed in the three groups. The pH values of the control, rHSA, and rHSA-heme groups were constant in the narrow ranges of 7.42–7.45, 7.42–7.44, and 7.42–7.44, respectively [Fig. 2(a)].

The paO_2 values of the control, rHSA, and rHSA-heme groups were also constant in the range of 100–108.1, 100–109.1, and 100–110.8%, respectively, by the end of measurements [Fig. 2(b)].

The pvO_2 of the control, rHSA and rHSA-heme groups demonstrated only small changes within 81.5–100.0, 84.9–108.4, and 81.9–100.0%, respectively [Fig. 2(c)].

Blood cell numbers

The Hct values of the control group were unchanged from 34.8–37.0% during the experiment. On the other hand, the 20% exchange transfusion with the

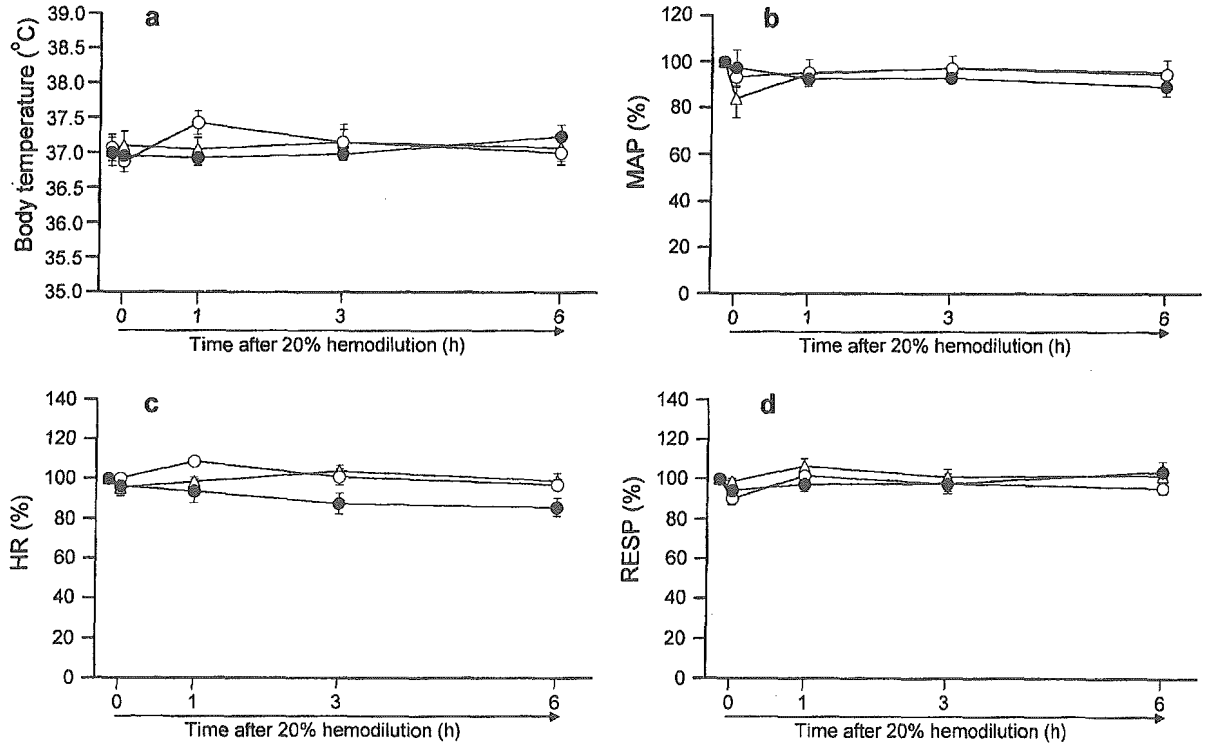

Figure 1. (a) Time courses of body temperature, (b) mean arterial pressure (MAP), (c) heart rate (HR), and (d) respiration rate (RESP) in anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of six rats (triangles, control group without infusion; open circles, rHSA group; solid circles, rHSA-heme group).

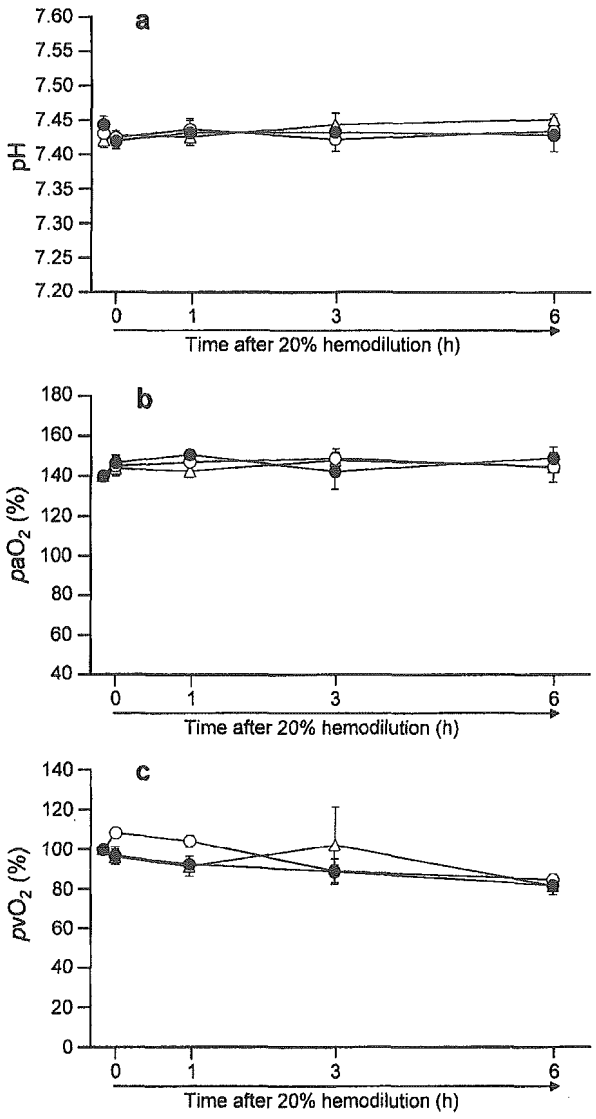

Figure 2. Time courses of (a) blood pH, (b) arterial blood O_2 pressure (paO_2), and (c) venous blood O_2-pressure (pvO_2) in anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of six rats (triangles, control group without infusion; open circles, rHSA group; solid circles, rHSA-heme group).

rHSA or rHSA-heme solution led to rapid decreases in the Hct from 39.0 to 31.7% or from 42.2 to 33.8%, respectively. These declined values were constant for 6 h [Fig. 3(a)]. Concomitantly, the RBC numbers in the rHSA and rHSA-heme groups decreased from 634.8×10^4 to $512.8\times10^4/\mu L$ and from 620.3×10^4 to $497.7\times10^4/\mu L$ by the exchange transfusion. They did not recover for 6 h [Fig. 3(b)].

The WBC numbers in the rHSA and rHSA-heme groups also showed similar declines; however, they appeared to be relatively slow with some deviations [Fig. 3(c)]. The PLT numbers of the rHSA and rHSA-heme groups changed in the range of 57.1–76.9 × $10^4/\mu L$ and 58.4–65.6 × $10^4/\mu L$, respectively [Fig. 3(d)].

Blood biochemical tests

In order to evaluate liver function, kidney function and electrolyte balance after the infusion of rHSA-heme, total 30 analytes of the blood biochemical tests were selected for rat plasma (Fig. 4). In the rHSA group, the A/G ratio increased, and TCho, Echo, and HDL-C decreased compared to the control group.

In the rHSA-heme group, the A/G ratio and fatty acid increased, and LDH, ALP, TCho, Echo, HDL-C, and PhL decreased in comparison to the control group. However, the other analytes showed almost the same values as those of the control group. With respect to the rHSA group, no significant difference was found except for the increases in the amylase, free fatty acid, and iron concentration, and the decrease of ALP and ECho.

DISCUSSION

After the 20% exchange transfusion with the 5 g/dL rHSA solution, the Hct and RBC numbers decreased approximately 80% of their basal values. This was only because of the 20% dilution and did not imply any acute toxicity of rHSA. The significant increase in the A/G ratio was caused by the slightly high rHSA concentration of 5 g/dL. Because the albumin concentration in rat plasma is generally 3–4 g/dL, the exchange transfusion with this sample led to the increase in the albumin concentration and decrease in the globulin concentration, thus resulting in the elevated A/G ratio. The hemodilution by the 20% replacement of the animal's blood volume could reduce the level of the analytes immediately after the infusion; however, the majority of the data in the rHSA group recovered within 6 h; only LDH, TCho, Echo, and HDL-C showed values that were about 80% of the corresponding ones in the control group.

Because FCho was almost the same as that in the control group, the decreases in TCho, ECho, and HDL-C were presumably caused by the decreasing of ECho, which might come from the depressed synthetic function in the livers by administration of the external protein. The circulation parameters and blood gas parameters in the rHSA group moved within the narrow range for 6 h—almost the same as those observed in the control group. Based on these data, the authors are certain that the administration of rHSA

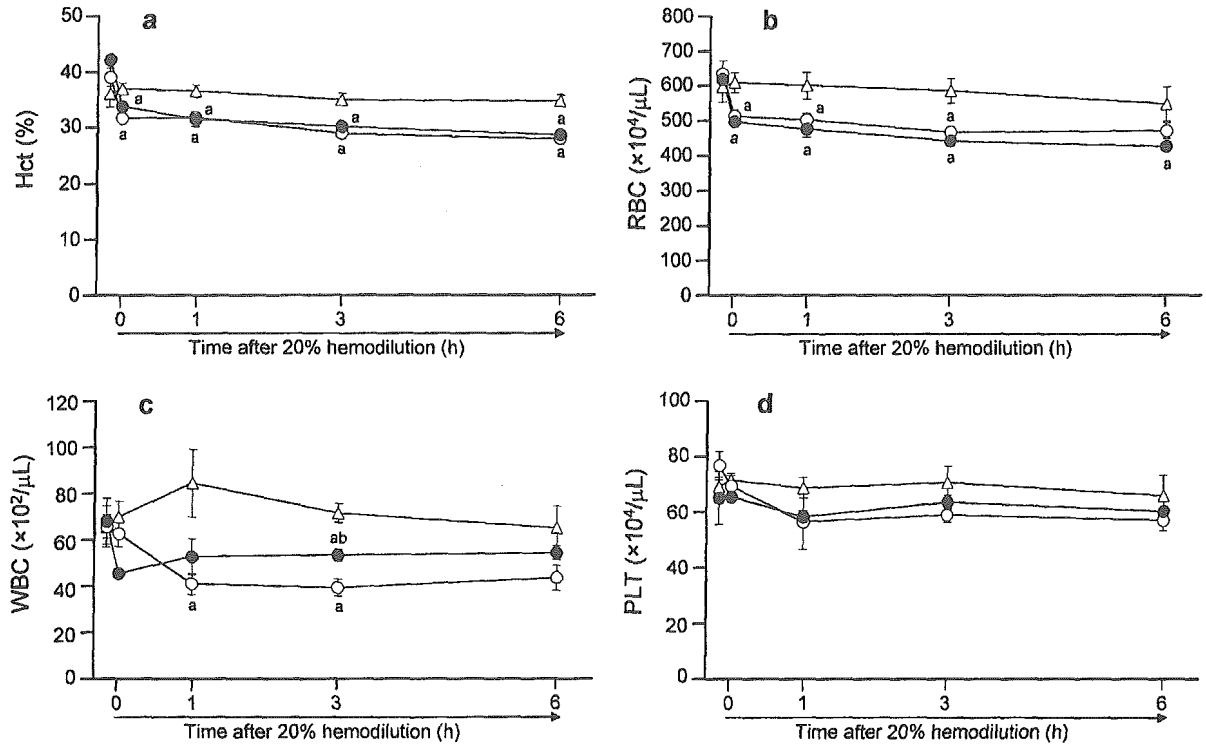

Figure 3. Time courses of hematocrit (Hct) value (a), red blood cell (RBC) numbers (b), white blood cell (WBC) numbers (c) and platelet (PLT) numbers (d) in whole blood of anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of 6 rats (triangles, control group without infusion; open circles, rHSA group; solid circles, rHSA-heme group). $^{a}p < 0.05$ versus control group (Tukey-Kramer test).

into anesthetized rats did not induce any toxic reaction under the present experimental conditions.

After the exchange transfusion with the rHSA-heme solution, no significant difference was seen in the circulation parameters and blood gas parameters. Previous studies to elucidate the influence of albumin-heme on the MAP changes and microcirculation in the capillaries demonstrated that neither vasoconstriction nor hypertension occurred, because of the low permeability of the albumin vehicle through the vascular endothelium.[9] The latest exchange transfusion experiment with rHSA-heme after isovolemic hemodilution also supported this hypothesis.[10]

By careful inspection of the results from the blood biochemical tests, it was found that the rHSA-heme group showed higher values of amylase and free fatty acid, and lower values of ALP and ECho compared to those of the rHSA group. In general, amylase has two isozymes, which are secreted by the pancreatic parenchyma and salivary gland. When the concentrations of the amylase and lipase (pancreatic parenchyma enzyme) simultaneously increase, it may be a sign of pancreatitis and other pancreas disorders.[12–14] However, the slight increase in the lipase concentration in the current protocol was not significant. Therefore, the possibility of pancreas disorders caused by the rHSA-heme infusion is considered negligible. The high amylase value is probably due to the isozyme secreted by the salivary gland.

With the increase in the free fatty acid, the TG concentration also tended to elevate to some degree. The increase in TG may activate the lipase, which acts as its decomposition enzyme, and results in the increasing free fatty acid as a metabolite. The relation between rHSA-heme and the TG increase is still not clear.

CONCLUSIONS

The appearance of the all animals showed absolutely no change for 6 h after the 20% exchange transfusion with albumin-heme. The physiological responses of the blood circulation, gas equilibria, and blood cell numbers in the rHSA-heme group were almost the same as those of the control and rHSA groups. MAP and HR did remain constant after the injection of the rHSA-heme, suggesting again that the albumin-based O_2-carrier does not induce the vasoconstriction. The blood biochemical tests of the withdrawn plasma of rHSA-heme group showed results similar to those of the control and rHSA groups, implying no acute toxicity by the exchange transfusion

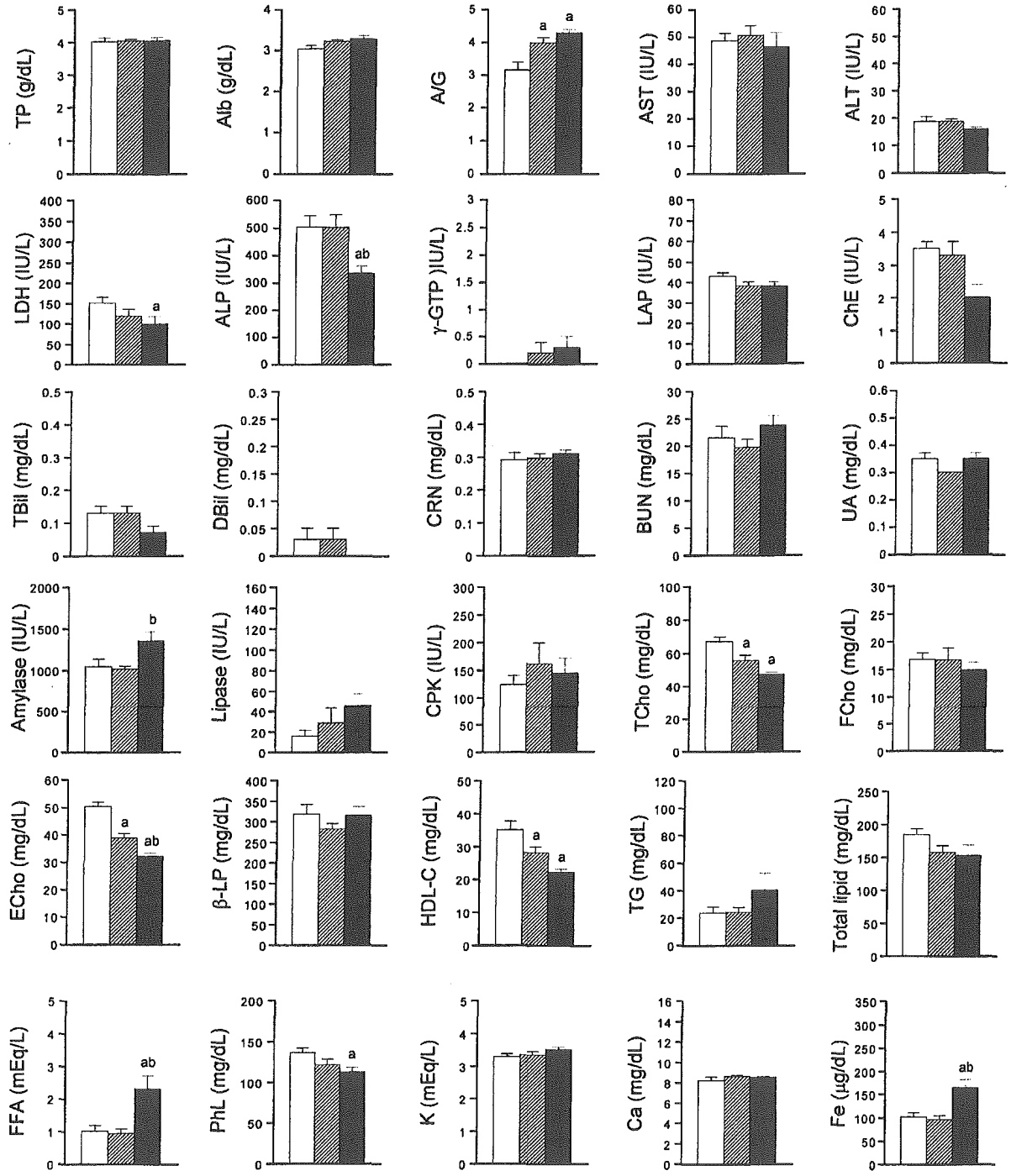

Figure 4. Blood biochemical tests of rat plasma after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of six rats (white bar, control group without infusion; diagonal bar, rHSA group; black bar, rHSA-heme group). [a]$p < 0.05$ versus control group (Tukey-Kramer test); [b]$p < 0.05$ versus rHSA group (Tukey-Kramer test).

with rHSA-heme. These results showed the initial clinical safety of the rHSA-heme solution, which allows us to undergo further advanced preclinical testing of this synthetic O_2-carrying hemoprotein as a new class of red-cell substitutes. Biochemical tests and histopathological observations for 7 days after the exchange transfusion with rHSA-heme will be reported in a forthcoming article.

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References

1. Chang TMS. Recent and future developments in modified hemoglobin and microencapsulated hemoglobin as red blood cell substitutes. Artif Cells Blood Substit Immobil Biotechnol 1997;25:1–24.
2. Tsuchida E. Perspectives of blood substitutes. In: Tsuchida E, editor. Blood substitutes: Present and future perspectives. Lausanne: Elsevier; 1998. p 1–14.
3. Winslow RM. New transfusion strategies: red cell substitutes. Annu Rev Med 1999;50:337–353.
4. Squires JE. Artificial blood. Science 2002;295:1002–1005.
5. 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. Bioconjug Chem 1999;10:82–86.
6. 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. Bioconjug Chem 1999;10:797–802.
7. 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. Bioconjug Chem 2002;13:397–402.
8. 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. Bioconjug Chem 2000;11:46–50.
9. 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.
10. Komatsu T, Yamamoto H, Huang Y, Horinouchi H, Kobayashi K, Tsuchida E. *Physiological responses to exchange transfusion* with synthetic oxygen-carrier "albumin-heme" in acute anemia *after 70% hemodilution.* J Biomed Mater Res. Submitted for publication.
11. 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 2003;66A:292–297.
12. Gorelick FS. Acute pancreatitis. In: Yamada T, editor. Textbook of gastroenterology (2nd ed.). Philadelphia: Lippincott; 1955. p 2064–2091.
13. Agarwal N, Pitchumoni CS, Sivaprasad AV. Evaluating tests for acute pancreatitis. Am J Gastroenterol 1990;85:356–361.
14. Clavin PA, Burgan S, Moossa AR. Serum enzyme and other laboratory tests in acute pancreatitis. Br J Surg 1989;76:1234–1238.

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COMMUNICATIONS

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Dioxygenation of Human Serum Albumin Having a Prosthetic Heme Group in a Tailor-Made Heme Pocket

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Human serum albumin (HSA, MW = 66.5 kD) is the most abundant plasma protein in our bloodstream and serves as a transporter for small hydrophobic molecules such as fatty acids, bilirubin, and steroids.[1,2] Hemin dissociated from methemoglobin is also bound within a narrow D-shaped cavity in subdomain IB of HSA with an axial coordination of Tyr-161 and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (Figure 1).[3,4] In terms of the general hydrophobicity of the α-helical pocket, HSA potentially has features similar to the heme-binding site of myoglobin (Mb) or hemoglobin (Hb). However, even if one reduces the ferric HSA−hemin to obtain a ferrous complex, it is immediately oxidized by O_2. This is due to the fact that HSA lacks a proximal histidine, which enables the heme group to bind O_2.[3−5] We have shown that HSA incorporating tetraphenylporphinatoiron derivatives having a covalently linked axial-base can absorb O_2 under physiological conditions with a O_2 binding affinity similar to that of Hb.[6]

In this paper, we report for the first time the introduction of a proximal histidine into the subdomain IB of HSA by site-directed mutagenesis to construct a tailor-made heme pocket, which allows a reversible O_2 binding to the prosthetic heme group. Laser flash photolysis experiments revealed that this artificial hemoprotein appears to have two different geometries of the axial-imidazole coordination and shows rather low O_2 binding affinity.

We designed two recombinant HSA (rHSA) mutants, in which single or double mutations were introduced into subdomain IB: I142H [rHSA(A)] and I142H/Y161L [rHSA(B)] (Figure 1). Replacement of Y161 by histidine was not done because modeling experiments indicated that the distance from N_ϵ(H142) to Fe(heme) would be too great (>4 Å). In our mutants, the N_ϵ(H142)−Fe distance was estimated to be 2.31 Å (compared to 2.18 Å in Mb). The specific mutations were introduced into the HSA coding region in a plasmid vector (pHIL-D2 HSA) using the QuikChange (Stratagene) mutagenesis kit, and the mutants were expressed in the yeast species *Pichia pastoris*.[7] The rHSA(wild-type or mutants)−hemin complexes were prepared essentially according to our previously reported procedures, except that myristate was not added.[4] The resulting hemoproteins exhibited only a single band in SDS-PAGE.

In the absorption spectrum of the rHSA(wt)−hemin solution, the distinct charge-transfer (CT) band of Fe^{3+}−phenolate appeared at 625 nm.[8] A magnetic circular dichroism (MCD) spectrum showed a W-shaped feature in the Soret-band region.[9] These results imply the formation of a high-spin Fe^{3+} complex with the phenolate oxygen ligand of Y161, which is quite consistent with that found in the crystal structure.[3,4]

rHSA(B)−hemin did not exhibit the CT band because of the Y161L mutation and was easily reduced to the corresponding ferrous complex by adding a small molar excess amount of aqueous $Na_2S_2O_4$ under an Ar atmosphere. A single broad absorption band (λ_{max} = 559 nm) in the α,β region was very similar to that of deoxy Mb and indicated the formation of a five-N-coordinate Fe^{2+} complex (Figure 2).[10] The spectral pattern was unaltered in the temperature range of 0−25 °C. The shape of the asymmetric MCD spectrum also resembled that of deoxy Mb.[11] This suggests that the heme is accommodated into the tailor-made heme pocket with an axial coordination involving His-142.

Upon exposure of the rHSA(B)−heme solution to O_2 gas, the UV−vis absorption changed to that of the dioxygenated complex (λ_{max} = 412, 537, 573 nm) at 0−25 °C[10] (lifetime of the O_2−adduct: ca. 10 min). After exposure to flowing CO, the heme produced a typical carbonyl complex (λ_{max} = 419, 538, 565 nm).

On the contrary, rHSA(A)−heme could not bind O_2 even at low temperature (~0 °C). It can be thought that the polar phenolate residue at the top of the porphyrin platform is likely to accelerate the proton-driven oxidation of the Fe^{2+} center. Replacing Y161 by

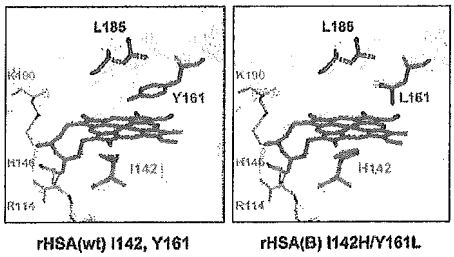

Figure 1. Prosthetic heme group complexed within the heme pocket in subdomain IB of rHSA(wt) and rHSA(B) mutant produced on the basis of the crystal structure coordinate of the rHSA−hemin complex (ref 4).

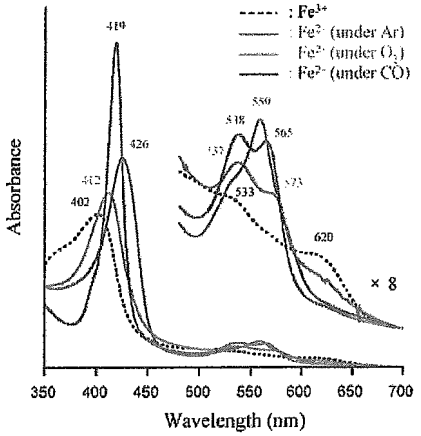

Figure 2. UV−vis absorption spectral changes of rHSA(B)−heme in phosphate buffered solution (pH 7.0, 50 mM) at 8 °C.

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Table 1. O_2 and CO binding Parameters of rHSA(B)–heme in Phosphate Buffered Solution (pH 7.0, 50 mM) at 22 °C

hemoprotein	k_{on}^{CO} (M^{-1} s^{-1})	$k_{on}^{O_2}$ (M^{-1} s^{-1})	$k_{off}^{O_2}$ (s^{-1})	$P_{1/2}^{O_2}$ (Torr)
rHSA(B)–heme[a]	2.0×10^6 (I), 2.7×10^5 (II)	7.5×10^6 (I and II)	221 (I), 1.7×10^3 (II)	18 (I), 134 (II)
Mb[b]	5.1×10^5	1.4×10^7	12	0.51
Hb (*R*-state)[c]	4.6×10^6	3.3×10^7	13	0.24

[a] Number in parentheses (I or II) indicates species I or II. [b] At 20 °C; ref 17. [c] At 20–21.5 °C; ref 18.

hydrophobic leucine greatly enhanced the stabilization of the O_2–adduct complex.

We then employed laser flash photolysis (Nd:YAG SHG; λ = 532 nm; 6 ns pulse width) to evaluate the kinetics of O_2 and CO bindings to rHSA(B)–heme.[12–14] Interestingly, the time dependence of the absorption change accompanying the CO recombination showed double-exponential profiles; the ratio of the amplitude of the fast and slow phases was always 3:2. On the other hand, the rebinding process of O_2 obeyed monophasic decay. On the basis of studies on synthetic model hemes, it has been shown that the proximal-side steric effect is the only primary factor that influences the association rate for CO but not for O_2.[12–14] One possible explanation is that there may be two different geometries of the axial His-142 coordination and that each one shows the individual kinetics of the CO rebinding. Marden and co-workers also found a similar behavior in CO association with HSA–heme and interpreted it as indicating that there are two orientations of the heme plane in the albumin scaffold.[5] In our case, the alternative geometries may arise because the heme molecule binds into the pocket of subdomain IB in two orientations related by a 2-fold rotation about its center (180° rotational isomers). Asymmetric 3,8-divinyl groups at the porphyrin periphery, in particular, would occupy different positions that result in a small divergence of the porphyrin ring and its iron center. The crystal structure analyses could not resolve this ambiguity, because the two configurations exist as a mixture.[3,4]

Our hypothesis is consistent with infrared spectroscopy data. The CO coordinated with rHSA(B)–heme showed a broad ν_{CO} at 1962 cm^{-1} with a shoulder at 1942 cm^{-1}. We therefore suggest that there are two different modes of π-back-donation from the central Fe^{2+} to the bound CO. It is remarkable that the lower stretching frequency is very close to that of Mb (ν_{CO} = 1943 cm^{-1}).[15] Attempts to determine the ratio of the two hemin orientations by ^1H NMR spectroscopy unfortunately failed.[16] The downfield spectra of rHSA(B)–hemin in met and met-azido forms did not show sharp resonances of the four porphyrin CH_3 groups.

The two geometries of the His-142 ligation in rHSA(B)–heme should yield two different O_2 binding affinities. By analyzing CO/O_2 competitive binding following laser flash photolysis, we obtained the association and dissociation rate constants for O_2 ($k_{on}^{O_2}$, $k_{off}^{O_2}$), and the O_2 binding affinities ($P_{1/2}^{O_2}$) for these two species (I and II) (Table 1).[12–14] The $P_{1/2}^{O_2}$ values were determined to be 18 and 134 Torr, respectively; this means that the O_2 binding affinities were 2.8 and 0.4%, respectively, of that observed for Mb.[17,18] Kinetically, for species I with $P_{1/2}^{O_2}$ of 18 Torr, an 18-fold increase in the $k_{off}^{O_2}$ value leads to the low affinity for O_2. Repeated studies with synthetic model hemes and engineered mutants of Mb or Hb have shown that low polarity of the microenvironment around the heme site decreases the O_2-binding affinity by increasing the dissociation rate constant.[12–14,17] In this albumin-based hemoprotein, the porphyrin ring is buried in the core of the pocket entirely made of hydrophobic residues. Therefore, the O_2 binding affinity becomes significantly lower than those of Mb and Hb. In species II with $P_{1/2}^{O_2}$ of 134 Torr, the proximal pull effect could further increase the $k_{off}^{O_2}$ value and may cause a large decline in the O_2 binding affinity.

We have recently found that heme is accommodated into the different architecture of a tailor-made heme pocket in rHSA (Y161L/L185H) [rHSA(C)] and that the rHSA(C)–heme complex also binds O_2 as well. In this case, the proximal histidine coordinates with the heme plane from the roof side, and the O_2 molecule binds from the floor side (Figure 1). Our combined structural and mutagenic approach allows us to significantly enhance the O_2 binding properties of rHSA–heme complexes and thereby mimic the diverse biological reactivities of natural hemoproteins. From the viewpoint of clinical applications, "O_2-carrying plasma albumin" could be of extreme medical importance not only for red blood cell substitutes but also for O_2-therapeutic reagents. The crystal structure analysis of this new class of artificial hemoproteins is now underway.

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Supporting Information Available: Time dependence of the absorption change accompanying the CO or O_2 rebinding to rHSA(B)–heme after the laser flash photolysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Peters, T. *All about Albumin: Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, 1996.
(2) Curry, S.; Madelkow, H.; Brick, P.; Franks, N. *Nat. Struct. Biol.* **1998**, *5*, 827.
(3) Wardell, M.; Wang, Z.; Ho, J. X.; Robert, J.; Ruker, F.; Rubel, J.; Carter, D. C. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 813.
(4) Zunszain, P. A.; Ghuman, J.; Komatsu, T.; Tsuchida, E.; Curry, S. *BMC Struct. Biol.* **2003**, *3*, 6.
(5) Marden, M. C.; Hazard, E. S.; Leclerc, L.; Gibson, Q. H. *Biochemistry* **1989**, *28*, 4422.
(6) Komatsu, T.; Matsukawa, Y.; Tsuchida, E. *Bioconjugate Chem.* **2002**, *13*, 397.
(7) Peterson, C. E.; Ha, C. E.; Harohalli, K.; Park, D.; Bhagavan, N. V. *Biochemistry* **1997**, *36*, 7012.
(8) Adachi, S.; Nagano, S.; Watanabe, Y.; Ishimori, K.; Morishima, I. *Biochem. Biophys. Res. Commun.* **1991**, *180*, 138.
(9) Pond, A. E.; Roach, M. P.; Sono, M.; Rux, A. H.; Franzen, S.; Hu, R.; Thomas, M. T.; Eilks, A.; Dou, Y.; Ikeda-Saito, M.; Oritz de Montellano, P. R.; Woodruff, W. H.; Boxer, S. G.; Dawson, J. H. *Biochemistry* **1999**, *38*, 7601.
(10) Antonini, E.; Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North-Holland Publishing: Amsterdam, 1971; p 18.
(11) Vickrey, L.; Nozawa, T.; Sauer, K. *J. Am. Chem. Soc.* **1976**, *98*, 343.
(12) Collman, J. P.; Brauman, J. I.; Iverson, B. L.; Sessler, J. L.; Moris, R. M.; Gibson, Q. H. *J. Am. Chem. Soc.* **1983**, *105*, 3052.
(13) Traylor, T. G.; Tsuchiya, S.; Campbell, D.; Mitchel, M.; Stynes, D.; Koga, N. *J. Am. Chem. Soc.* **1985**, *107*, 604.
(14) Komatsu, T.; Arai, K.; Nishide, H.; Tsuchida, E. *J. Chem. Soc., Dalton Trans.* **1993**, 1734.
(15) Maxwell, J. C.; Volpe, J. A.; Barlow, C. H.; Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1974**, *58*, 166.
(16) (a) Jue, T.; Krishnamoorthi, R.; La Mar, G. N. *J. Am. Chem. Soc.* **1983**, *105*, 5701. (b) Yamamoto, Y.; La Mar, G. N. *Biochemistry* **1986**, *25*, 5288.
(17) Olson, J. S.; Mathews, A. J.; Rohlfs, R. J.; Springer, B. A.; Egeberg, K. D.; Sliger, S. G.; Tame, J.; Renaud, J.-P.; Nagai, K. *Nature* **1988**, *336*, 265.
(18) (a) Gibson, Q. H. *J. Biol. Chem.* **1970**, *245*, 1564. (b) Olson, J. S.; Melvin, E.; Andersen, E.; Gibson, Q. H. *J. Biol. Chem.* **1971**, *246*, 5919. (c) Steinmeier, R. C.; Parkhurst, L. J. *Biochemistry* **1975**, *14*, 1564.

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Exchange transfusion with synthetic oxygen-carrying plasma protein "albumin-heme" into an acute anemia rat model after seventy-percent hemodilution

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Abstract: Recombinant human serum albumin (rHSA) incorporating the synthetic heme "albumin-heme" is an oxygen-carrying plasma protein that has the potential to be a red blood cell substitute. The physiological responses to a 30% exchange transfusion with two types of albumin-heme (rHSA-FecycP, rHSA-FepivP) solutions after 70% isovolemic hemodilution with 5 g/dL rHSA were investigated using anesthetized rats. The circulation parameters, blood parameters, renal cortical oxygen pressure (pO_2), and muscle tissue pO_2 were carefully monitored for 60 min after the injection. The declined mean arterial pressure and the mixed venous partial pO_2 significantly recovered to 70.8 and 91.9% of the basal values by intravenous infusion of albumin-hemes, respectively. The lowered renal cortical pO_2 also increased, indicating oxygen transport by this synthetic hemoprotein. The administration of albumin-heme into the acute anemia rat model after hemorrhage improved the circulatory volume and resuscitated the shock state. Both rHSA-FecycP and rHSA-FepivP transported oxygen through the body. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 644–651, 2004

Key words: exchange transfusion; entirely synthetic oxygen-carrier; hemoprotein; albumin-heme; red blood cell substitute

INTRODUCTION

Because the risk of transmission of viral illness by transfused blood has become extremely low, transfusion of donor blood has nowadays become a routine procedure. However, this level of safety has been achieved at great cost, and the hepatitis virus or unknown infectious pathogens cannot be completely excluded by the nucleic acid test system. Furthermore, (i) the transfusion of donor blood requires cross-matching and compatibility tests to avoid the consequences of a hemolytic reaction in the recipient, and (ii) the purified red blood cells (RBCs) should be stored at 4°C. These requirements substantially limit the availability of blood in a disaster or emergency. Under this background, several types of hemoglobin (Hb)-based oxygen carriers have been studied as an RBC substitute or oxygen therapeutic agent.[1–4] Nevertheless, these materials do not fulfill all the requirements of blood replacement compositions. The first concern is the source of human Hb, which is limited by the availability of outdated human blood. Animal blood will raise anxiety of transmission of animal pathogens; for instance, bovine Hb may harbor prions. The Hb products potentially carry risks because of the biological origin of the raw materials. Additional problems of Hb-based oxygen carriers (i.e., modified Hb) are the high colloid osmotic pressure[5] and its vasopressor effect, which increase blood pressure and decrease cardiac output.[6–8] Although diaspirin intramolecularly crosslinked Hb is no longer developed, half of the products in advanced clinical trials still showed vasoconstriction.[4] The precise mechanism of

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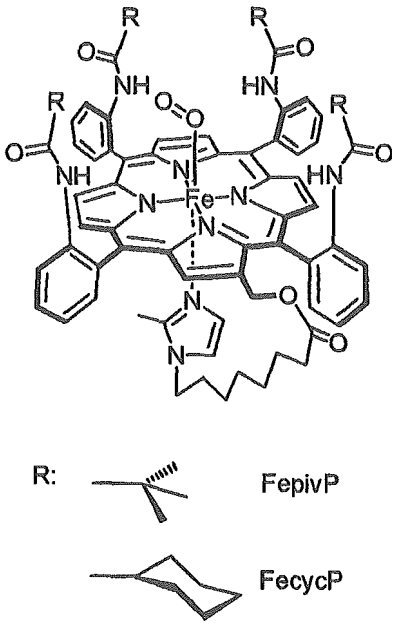


Chart 1

this hypertension is controversial, but many investigators suspect that the small Hb molecules penetrate the vascular endothelium and capture the endothelial-derived relaxing factor, namely, nitric oxide. In fact, glutaraldehyde crosslinked human polyHb does not show vasoactivity.[9] Others believe that the excessive delivery of oxygen to arteriolar vascular walls induces autoregulatory vasoconstriction.[10–13]

We have prepared entirely synthetic oxygen carriers without Hb. The incorporation of synthetic heme, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphinatoiron(II) (FepivP, Chart 1), into recombinant human serum albumin (rHSA) provides an artificial hemoprotein (rHSA-heme; rHSA-FepivP) that can reversibly bind and release oxygen under physiological conditions (pH 7.3, 37°C) like Hb.[14–19] An FepivP analog, 2-[8-{*N*-(2-methylimidazolyl)}-octanoyloxymethyl]-5,10,15,20-tetrakis{α,α,α,α-*o*-(1-methylcyclohexanamido)phenyl}porphinatoiron(II) (FecycP, Chart 1), which contains more hydrophobic 1-methylcyclohexanoyl fences, is also included into rHSA as well and the obtained rHSA-FecycP hybrid showed the same oxygen-binding equilibrium and a longer lifetime ($\tau_{1/2}$) of the oxy species compared with that of rHSA-FepivP.[20] The physicochemical properties and oxygen-transporting ability of these rHSA-based oxygen carriers, which are free of infectious pathogens, satisfy the initial clinical requirements as an RBC substitute. It is remarkable that the colloid osmotic pressure of 5 g/dL rHSA, rHSA-FecycP, and rHSA-FepivP are all the same.[15] We herein report the systematic evaluations of the physiological responses to exchange transfusions with two types of albumin-heme solutions, rHSA-FecycP and rHSA-FepivP, in anesthetized rats. The animals were first placed into an acute anemia induced by 70% hemodilution and then underwent a 30% exchange transfusion with albumin-hemes. Circulation parameters and blood parameters were monitored for 60 min after the injection. The *in vivo* oxygen deliveries to the renal cortex and the muscle tissue were also measured.

MATERIALS AND METHODS

Preparation of rHSA-heme solutions

The rHSA (Albrec®, 25 wt %) was obtained from NIPRO Corp. (Osaka, Japan). The 5 g/dL rHSA was made by diluting Albrec® with saline solution (Otsuka Pharmaceutical Co., Ltd.). Two types of albumin-hemes (rHSA-FecycP and rHSA-FepivP [rHSA]: approximately 5 g/dL) used for the exchange transfusions were prepared according to our previously reported procedure with some modifications.[19] The characteristics of these oxygen-carrying fluids are almost identical (Table I) except for the stability of the oxygenated species; oxy rHSA-FecycP showed a 4.5-fold longer lifetime against autooxidation than that of oxy rHSA-FepivP at 37°C *in vitro*. These red-colored albumin-heme solutions were filtered using a DISMIC 25CS045AS just before use.

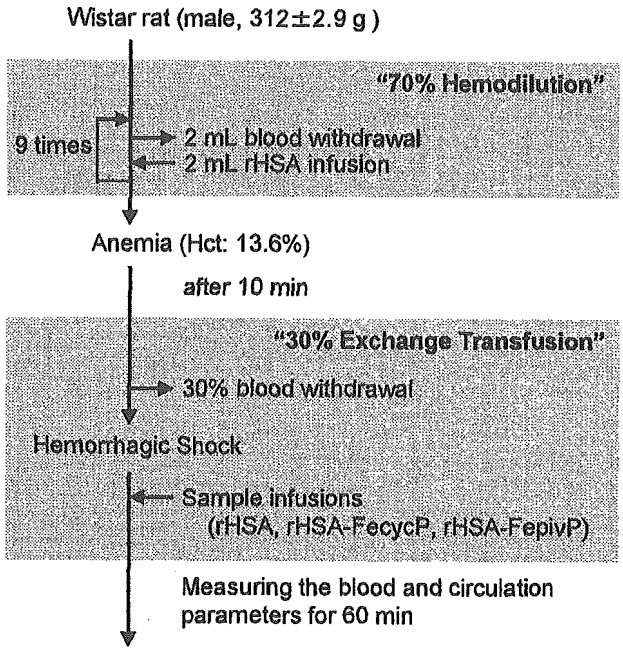
Hemodilution and exchange transfusion

The investigations were performed according to our previously reported experimental protocols with 24 male Wistar rats (312 ± 2.9 g) (Scheme 1).[21] All animal handling and care were in accordance with the National Institutes of Health guidelines. The protocol details were approved by the Animal Care and Use Committee of Keio University.

The animals were placed on a heating pad under an inhalation anesthesia with sevoflurane; its concentration was kept at 2.0% for the operations and 1.5% for the experiments. After an incision was made in the neck, the heparinized catheter (Natume Seisakusho SP-55) was introduced into the right common carotid artery for blood withdrawal. Other catheters (SP-31) were also inserted into the left femoral artery for continuous mean arterial pressure (MAP) monitoring, and the right femoral vein for sample injection.

TABLE I
Characteristics of the Albumin-Heme Solutions

	rHSA-FecycP	rHSA-FepivP
pH	7.45	7.46
Viscosity (cP)	1.1	1.1
Heme (m*M*)	2.8	2.7
Met-heme content (%)	0	0
rHSA (%)	4.9	4.9
*p*50 (mmHg) at 37°C	37	32



Scheme 1. Diagram illustrating the exchange, bleeding procedures, and materials.

Polarographic oxygen-electrodes (Intermedical POE-10N and POE-40PS) were inserted into the left renal cortex and muscle in the abdomen. Core temperature was measured with a rectal thermometer (Technol Seven D617-1).

After stabilization of the animal's condition, isovolemic 70% hemodilution was performed using 5 g/dL rHSA solution. Blood withdrawal via the common carotid artery (2 mL) and rHSA infusion from the femoral vein (2 mL) (each 1 mL/min) were repeated for nine cycles until the hematocrit (Hct) was reduced to 13.6% (32% of the initial Hct value: 42.6%). After 10 min, a 30% volume of the circulatory blood was withdrawn, producing a severe hemorrhagic shock state. The same volume of the samples (rHSA-FecycP and rHSA-FepivP) was then intravenously (iv) injected (n = 6 each). As negative- or positive-control groups, rats were infused with the 5 g/dL rHSA solution or the shed rat blood ([heme] = 5.3 mM) (rHSA group and whole blood group, n = 6 each). A blood-taking from the artery (0.3 mL) and vein (0.2 mL) was performed at the following six points: (i) before the 70% hemodilution, (ii) immediately after the hemodilution, and (iii) 10 min after the hemodilution, (iv) immediately after the 30% bleeding, (v) immediately after the sample infusion, and (vi) 60 min after the sample infusion. The animals were sacrificed after the experiments by sodium pentobarbital overdose. MAP was monitored through the femoral artery catheter connected to a transducer (NIHON KODEN TP-400 T or Becton Dickinson P23XL) with a pressure coupler and an amplifier (NIHON KODEN PP-101H and AP-100H). An electrocardiogram was measured using a bioelectric coupler and an amplifier (NIHON KODEN PC-101H and AC-100H), and heart rate (HR) was obtained from its R wave. MAP and HR were recorded by a Polygraph System (NIHON KODEN LEG-1000 version 01-02 or PEG-1000 version 01-01) at the following eight points: (i) before the 70% hemodilution, (ii) immediately after the hemodilution, (iii) 10 min after the hemodilution, (iv) immediately after the 30% bleeding, (v) immediately after the sample infusion, (vi) 5 min, (vii) 30 min, and (viii) 60 min after the sample infusion. The renal cortical oxygen-tension [ptO_2(R)] and muscle tissue oxygen-tension [ptO_2(M)] were simultaneously measured with a tissue oxygen-pressure monitor (Inter Medical PO_2-100DW) using an oxygen electrode (described above). Withdrawn blood was rapidly applied to a blood gas system (Radio Meter Trading ABL555) to measure the oxygen pressure (paO_2), pH and carbon dioxide pressure ($paCO_2$) of the arterial blood, and the oxygen pressure (pvO_2) of the venous blood. The RBC numbers were counted for the mixture of arterial blood (30 μL) and ACD-A solution using a multisystem automatic blood cell counter (Sysmex KX-21).

Data analysis

MAP, HR, respiration rate, paO_2, pvO_2, ptO_2(R), ptO_2(M), and $paCO_2$ are represented by percent ratios of the basal values as mean ± standard error of mean (SEM). Body temperature, pH, RBC numbers, and body weight are denoted by mean ± SEM.

Statistical analyses were performed using the Tukey-Kramer multiple comparison test for more than three groups [pH, RBC numbers, body weight, MAP, HR, respiration rate, paO_2, pvO_2, ptO_2(R), ptO_2(M), and $paCO_2$], and by repeated-measures analysis of variance followed by paired t test for comparison with a basal value (body temperature). Values of $p < 0.05$ were considered significant. The statistical analytic software used was StatView (SAS Institute Inc.).

RESULTS

Blood parameters

The basal values of several measurements, for which data are shown in percent ratios, are summarized in Table II. There are no significant differences in the four groups.

The 70% hemodilution decreased the Hct to 12.8–14.3% and RBC numbers to 202.2–223.2 × 10^4/μL, leading to acute anemia [Fig. 1(a,b)]. By a further 30% exchange transfusion with the whole blood of rat, both parameters slightly increased to 14.3% and 229.7 × 10^4/μL, respectively. They remained constant during the experimental period. In the rHSA-FecycP and rHSA-FepivP groups, both parameters decreased to 7.8–9.6% and 129.2–149.2 × 10^4/μL by the sample replacement. They corresponded well to the calculated values.

Survival time

After the exchange transfusion with the 5 g/dL rHSA solution (rHSA group), all animals died within 32 min;

TABLE II
Basal Values of Each Group

	rHSA	Whole blood	rHSA-FecycP	rHSA-FepivP
MAP (mmHg)	110 ± 2.9	115 ± 3.4	101 ± 7.3	107 ± 4.8
HR (beats/min)	371 ± 14	379 ± 17	380 ± 22	349 ± 10
Respiration rate (breaths/min)	66 ± 3.2	67 ± 4.4	60 ± 2.3	64 ± 1.7
ptO_2(R) (mmHg)	29.2 ± 2.8	38.5 ± 2.6	31.7 ± 3.6	30.2 ± 4.3
ptO_2(M) (mmHg)	39.8 ± 3.6	38.5 ± 3.7	44.2 ± 4.6	42.7 ± 3.9
paO_2 (mmHg)	83.8 ± 2.3	84.2 ± 2.3	87.8 ± 2.6	81.7 ± 2.2
pvO_2 (mmHg)	47.9 ± 1.6	46.8 ± 1.9	48.2 ± 3.1	47.4 ± 1.1
$paCO_2$ (mmHg)	42.0 ± 0.9	40.2 ± 0.8	39.6 ± 1.2	41.7 ± 1.0
Body weight (g)	312 ± 3.5	312 ± 3.9	311 ± 3.4	314 ± 0.8

the average survival time was 14.9 ± 4.3 min. However, all animals survived >60 min in the rHSA-FecycP, rHSA-FepivP, and whole blood groups. The body temperatures remained constant throughout the measurements (rHSA-FecycP group: 36.1°–36.6°C; rHSA-FepivP group: 36.3°–36.7°C; whole blood groups: 36.2°–36.8°C).

MAP, HR, and respiration rate

The 70% hemodilution decreased MAP to 77.4–81.9% of the basal value, and the further 30% bleeding decreased it to 24.9–37.7% of the baseline [Fig. 2(a)]. In contrast to the fact that no recovery was observed by the iv administration of 5 g/dL rHSA, the lowered MAP was increased to 85.6% of the baseline by the whole blood injection. In the rHSA-FecycP and rHSA-FepivP groups, the decreased MAP was elevated to 70.1 and 71.4% of the basal values (80.7 and 87.1% of the values before the bleedings).

The HR and respiration rate had decreased to 88.6–97.4 and 76.5–82.5% of the baselines by the 30% bleeding [Fig. 2(b,c)]. Both parameters returned to the initial levels by the injection of rHSA-FecycP, rHSA-FepivP, and whole blood within 30 min.

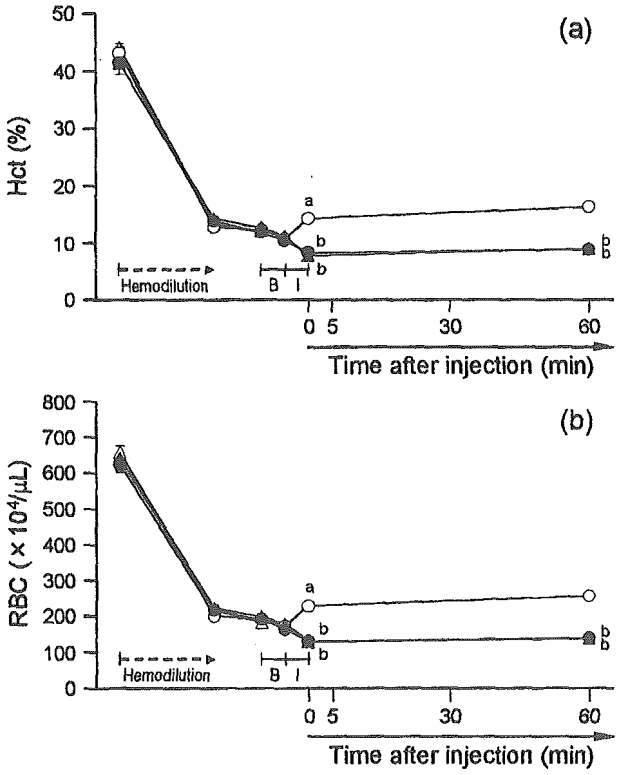


Figure 1. Effect of rHSA-heme solutions on Hct (a), and RBC numbers (b) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^a p < 0.05$ versus rHSA group. $^b p < 0.05$ versus whole blood group.

ptO_2(R) and ptO_2(M)

After the 30% bleeding, ptO_2(R) decreased to 59.9–77.3% of the baseline [Fig. 3(a)]. Whereas the iv administrations of rHSA solution did not show restoration, the injection of the rHSA-FecycP, rHSA-FepivP, or whole blood immediately increased the ptO_2(R) level to 84.1–94.8% of the baselines (102.1–112.3% of the values before the bleeding). They remained unaltered by the end of the measurements.

The ptO_2(M) had also decreased to 49.4–52.9% of the basal value after the isovolemic hemodilution, and further decreased to 12.1–26.7% because of the 30% bleeding [Fig. 3(b)]. The iv administration of the whole blood increased the ptO_2(M) to 62.8% of the baseline after 60 min. In the rHSA-FecycP and rHSA-FepivP groups, the increasing ratios were relatively small: 34.2 and 36.9% of the baselines (76.2 and 66.8% of the values before the bleedings).

Blood gas parameters

The paO_2 increased to 111.3–123.2% of the baseline after the isovolemic hemodilution, and reached 148.0–153.6% after the 30% bleeding [Fig. 4(a)]. The injection of the whole blood showed a significant effect for restoration. Nonetheless, the recoveries by the albu-

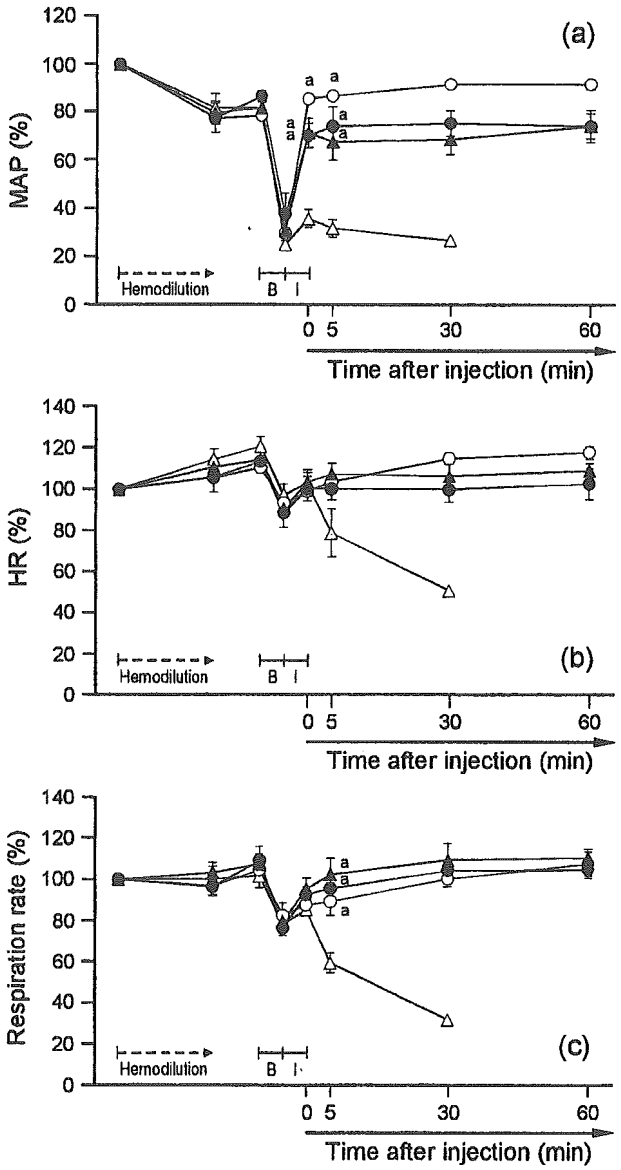


Figure 2. Effect of rHSA-heme solutions on MAP (a), HR (b), and respiration rate (c) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^{a}p < 0.05$ versus rHSA group.

min-heme solutions were almost to the same extent as that seen in the rHSA group.

The pvO_2 decreased to 56.5–69.8% of the basal value after the 30% bleeding. The low value was not improved by the iv administration of 5 g/dL rHSA [Fig. 4(b)]. In contrast, the infusions of rHSA-FecycP, rHSA-FepivP, and whole blood immediately increased pvO_2 to 88.9–94.8%.

The changes in pH showed the same profile in all groups until 60 min after injection [Fig. 4(c)]. The pH of 7.39–7.42 was slightly increased to 7.47–7.49 after the bleeding and reduced to 7.34–7.37 by the administration of the samples, which slowly returned close to the initial level except for the rHSA-FecycP group.

The $paCO_2$ decreased to 66.3–73.9% of the basal value after the 30% bleeding. The iv administration of whole blood immediately increased it to 95.1% [Fig. 4(d)]. The recovery by the infusion of the rHSA-FecycP or rHSA-FepivP solution was relatively small (80.9–88.0%).

DISCUSSION

In this study, animals were anesthetized with sevoflurane, which is widely used in clinical situations. We had evaluated the oxygen-transporting ability of rHSA-FepivP using a similar acute anemia model with pentobarbital as an anesthesia.[21] Most of the parameter changes showed the same extent as those observed in the former experiment, except for the small degree of $ptO_2(R)$ reduction after the bleeding (32%) (50% decrease in the former study). It is probably attributable to the difference in the blood exchanging ratio.

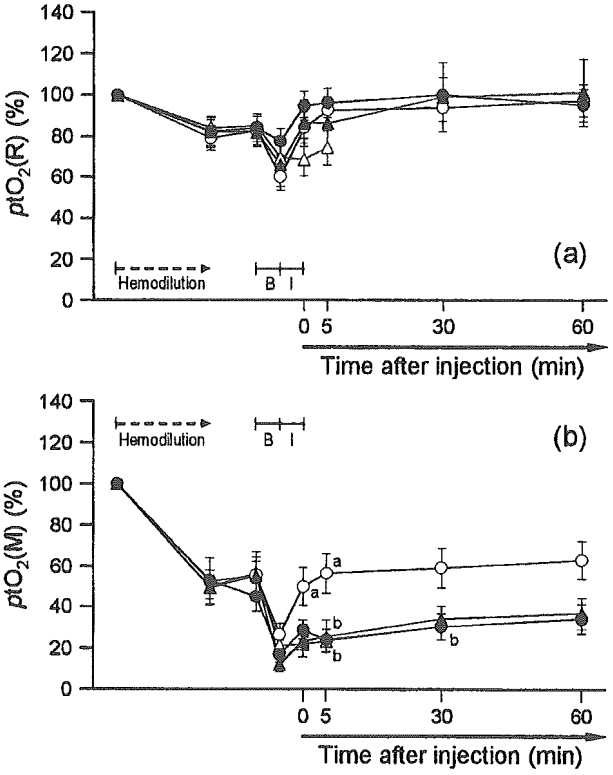


Figure 3. Effect of rHSA-heme solutions on the $ptO_2(R)$ (a) and $ptO_2(M)$ (b) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^{a}p < 0.05$ versus rHSA group. $^{b}p < 0.05$ versus whole blood group.

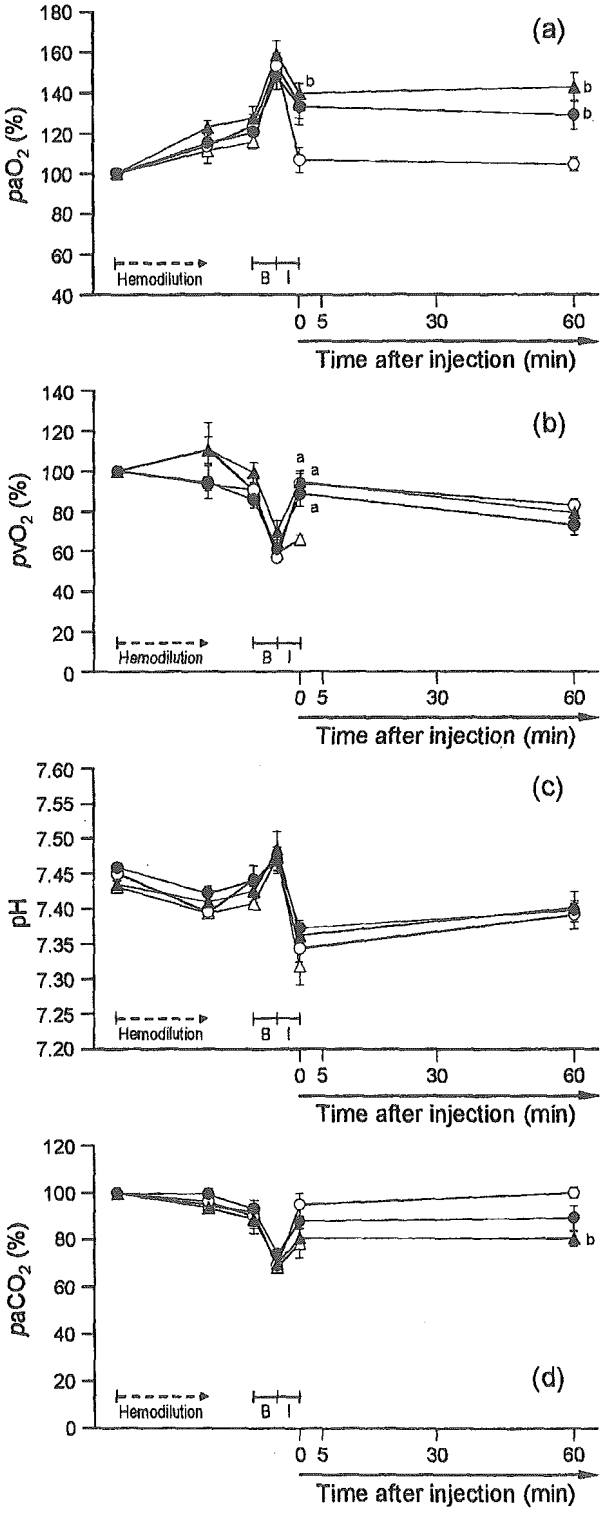


Figure 4. Effect of rHSA-heme solutions on paO_2 (a), pvO_2 (b), pH (c), and $paCO_2$ (d) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^{b}p < 0.05$ versus whole blood group. $^{a}p < 0.05$ versus rHSA group.

In the glass capillaries for the Hct determinations, the supernatant after centrifugation (12 kG, 5 min) was colored red by the homogeneously dispersed albumin-heme molecules without any aggregation. This indicates that the albumin-heme fluids have a good solubility with the blood components.

Acute anemia after 70% hemodilution

The quantitative changes in Hct and RBC numbers showed that the 70% hemodilution and 30% exchange transfusion were precisely controlled. In acute anemia, the oxygen supply centralizes in vital organs, and the muscle tissues become hypoxic by peripheral vasoconstriction. In fact, the ratio of ptO_2 reduction in the muscle tissues after hemodilution was significant compared with that observed in the renal cortex.

Hemorrhagic shock by 30% bleeding

Decreasing the circulation blood volume induced a significant reduction of MAP, ptO_2(R), ptO_2(M), pvO_2, and $paCO_2$. Among the vital organs, renal perfusion is first impaired because of redistribution of the systemic blood flow; therefore, ptO_2(R) is sensitive to a subtle change in the blood circulation and oxygen delivery. The decreased pvO_2 after the 30% bleeding implies a low oxygen saturation of Hb and increased oxygen extraction. The HR and respiration rate had also decreased from the initial values. However, paO_2 was increased to 153.6% (129.6 mmHg) of the baseline, which could be attributable to hyperventilation. The $paCO_2$ decreased to 70.3% of the baseline and the pH was increased to 7.48. A respiratory alkalosis by hyperventilation might overcome the metabolic acidosis effect.

Responses to administration with albumin-heme

In all groups, the pH levels decreased to 7.35 just after sample injections. The rapid increase in blood volume could improve the circulatory flows and wash out the lactate in the tissues.

By administration of the 5 g/dL rHSA solution, the MAP, HR, respiration rate, ptO_2(R), ptO_2(M), paO_2, pvO_2, and $paCO_2$ did not recover, leading to death within 32 min. In contrast, the infusion of whole blood improved these values to their initial levels except for ptO_2(M). In the rHSA-FecycP and rHSA-FepivP groups, the animals survived >60 min after the infusion, and the HR, respiration rate, ptO_2(R), and pvO_2 showed similar recoveries as observed in the whole

blood group. MAP, $ptO_2(M)$, paO_2, pH, and $paCO_2$ also returned, but not adequate relative to the whole blood group. We are certain that the albumin-heme solutions have the potential to (i) resuscitate the hemorrhagic shock, (ii) stabilize the blood circulation, and (iii) transport oxygen throughout the body. Nevertheless, the oxygen delivery to the peripheral tissues is still insufficient. The possible reasons were considered from the viewpoints of the physicochemical properties of albumin-heme.

Oxygen-binding affinity (p_{50}) (oxygen partial pressure in which 50% of Hb or albumin-heme is oxygenated)

It is known that an allosteric effect observed in the oxygen binding to Hb [the Hill coefficient (n) = 3.0] has implications for physiological use; the oxygen-transporting efficacy of RBCs between lungs [oxygen pressure (pO_2): 110 mmHg] and muscle tissues (pO_2: 40 mmHg) is estimated to be 22% at 37°C. The oxygen-equilibrium curve of albumin-heme does not show cooperativity; n is 1.0. However, the oxygen-binding affinity of albumin-hemes are adjusted somewhat low (rHSA-FecycP: p_{50} = 37 mmHg; rHSA-FepivP: p_{50} = 32 mmHg, respectively), so that they are able to show a similar oxygen-transporting efficacy of 22–23%.

Some investigators reported that a low p_{50} value is important to avoid the hypertensive effect; that is, the constrictive response is caused by excessive oxygen delivery to the arterioles.[10–13] McCarthy et al.[22] suggested that the increased oxygen delivery can be limited by increasing the molecular size, oxygen-binding affinity, and viscosity. Indeed, the PEG-conjugated Hb with a molecular weight of 90 kDa had no effect on the MAP.[23] However, Doherty et al.[24] denied this hypothesis based on their systematic experiments using recombinant Hbs (rHbs) with various p_{50} values; the small differences in the oxygen-equilibrium curves of the rHbs did not affect the magnitude of the pressor response. Our previous top-loading experiments using the albumin-heme solution (p_{50}: 32 mmHg) on the MAP changes and microcirculatory observations of the capillaries also showed that neither vasoconstriction nor hypertension occurred, because of its low permeability through the vascular endothelium.[18] Thus, the differences in p_{50} may not be essential.

Heme concentration

The heme concentration of the shed blood to use the exchange transfusion in the whole blood group was 5.3 m*M*. In contrast, the concentrations of the FecycP or FepivP in the rHSA-FecycP or rHSA-FepivP solutions were 2.7–2.8 m*M*. The low heme concentration of albumin-heme is probably related to insufficient oxygen delivery, which may result in the low recovery of $ptO_2(M)$.

Viscosity

It has been reported that an endothelial-derived relaxing factor (nitric oxide) is generated in response to the shear stress on the capillary wall.[25] Recently, maintenance of the plasma viscosity has been proposed as a crucial mechanism to preserve the shear force during microcirculation.[26] The prompt flow by the injection of the albumin-heme solution with low viscosity (1.1 cP) compared with blood (4.4 cP) may reduce the peripheral resistance and induce partial vasoconstriction, which would reduce the blood flow. The correct measurement of the cardiac output is necessary to interpret the pO_2 data and to assay the oxygen delivery.

In conclusion, all physiological responses to the exchange transfusion with albumin-heme in acute anemia reveal that this synthetic RBC substitute has the capability to resuscitate the hemorrhagic shock. A significant difference could not be found between the two types of albumin-hemes, rHSA-FecycP and rHSA-FepivP. This rHSA-based oxygen-carrying plasma expander will become a promising material for a new class of RBC substitutes. The safety and oxygen delivery of albumin-heme will be reported in a forthcoming article.

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References

1. Chang TMS. Recent and future developments in modified hemoglobin and microencapsulated hemoglobin as red blood cell substitutes. Artif Cells Blood Substit Immobil Biotechnol 1997;25:1–24.
2. Tsuchida E. Perspectives of blood substitutes. In: Tsuchida E, editor. Blood substitutes: present and future perspectives. Lausanne, Switzerland: Elsevier Science; 1998. p 1–14.
3. Winslow RM. New transfusion strategies: red cell substitutes. Annu Rev Med 1999;50:337–353.
4. Squires JE. Artificial blood. Science 2002;295:1002–1005.
5. Keipert P, Chang T. Pyridoxylated-polyhemoglobin solution: a low viscosity oxygen-delivery blood replacement fluid with normal oncotic pressure and long term storage feasibility. Biomater Artif Cells Artif Organs 1988;16:185–196.
6. Schultz SC, Grady B, Cole F, Hamilton I, Burhop K, Malcolm DS. A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med 1993;122:301–308.
7. Moisan S, Drapeau G, Burhop KE, Rioux F. Mechanism of the acute pressor effect and bradycardia elicited by diaspirin crosslinked hemoglobin in anesthetized rats. Can J Physiol Pharmacol 1998;76:434–442.

8. Abassi Z, Kotob S, Pieruzzi F, Abouassali M, Keiser HR, Fratantoni JC, Alayash AI. Effects of polymerization on the hypertensive action of diaspirin cross-linked hemoglobin in rats. J Lab Clin Med 1997;129:603–610.
9. Gould SA, Moore EE, Moore FA, Haenel JB, Burch JM, Sehgal H, Sehgal L, DeWoskin R, Moss GS. Clinical utility of human polymerized hemoglobin as a blood substitute after acute trauma and urgent surgery. J Trauma 1997;43:325–332.
10. Guyton AC, Ross JM, Carrier O, Walker JR. Evidence for tissue oxygen demand as the major factor causing autoregulation. Circ Res 1964;14:1–60.
11. Tsai AG, Kerger H, Intaglietta M. Microcirculatory consequences of blood substitution with αα-hemoglobin. In: Winslow RM, Vandegriff KD, Intaglietta M, editors. Blood substitutes: physiological basis of efficiency. Boston, MA: Birkhäuser; 1995. p 155–174.
12. Rohlfs RJ, Bruner E, Chiu A, Gonzales ML, Magde D. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem 1998;273:12128–12134.
13. Winslow RM. αα-Crosslinked hemoglobin: was failure predicted by preclinical testing? Vox Sang 2000;79:1–20.
14. 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. Bioconjug Chem 1999;10:82–86.
15. 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. Bioconjug Chem 1999;10:797–802.
16. Komatsu T, Matsukawa Y, Tsuchida E. Kinetics of CO- and O_2-binding to human serum albumin-heme hybrid. Bioconjug Chem 2000;11:772–776.
17. Komatsu T, Matsukawa Y, Tsuchida E. Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(II) derivatives. Bioconjug Chem 2001;12:71–75.
18. 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.
19. 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 2003;66A:292–297.
20. 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. Bioconjug Chem 2002;13:397–402.
21. 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. Bioconjug Chem 2000; 11:46–50.
22. McCarthy MR, Vandegriff KD, Winslow RM. The role of facilitated diffusion in oxygen transport by cell-free hemoglobin: implication for the design of hemoglobin-based oxygen carriers. Biophys Chem 2001;92:103–117.
23. Vandegriff KD, Malavalli A, Wooldbridge J, Lohman J, Winslow RM. MP4, a new nonvasoactive PEG-Hb conjugate. Transfusion 2003;43:509–516.
24. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, Olson JS, Lemon DD. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. Nat Biotechnol 1998;16:672–676.
25. Malek AM, Izumo S. Control of endothelial cell gene expression by flow. J Biomech 1995;28:1515–1528.
26. Tsai AG, Friesenecker B, McCarthy M, Sakai H, Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. Am J Physiol 1998: 275;H2170–H2180.

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Physicochemical characterization of cross-linked human serum albumin dimer and its synthetic heme hybrid as an oxygen carrier

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Abstract

The recombinant human serum albumin (rHSA) dimer, which was cross-linked by a thiol group of Cys-34 with 1,6-bis(maleimido)hexane, has been physicochemically characterized. Reduction of the inert mixed-disulfide of Cys-34 beforehand improved the efficiency of the cross-linking reaction. The purified dimer showed a double mass and absorption coefficient, but unaltered molar ellipticity, isoelectric point (p*I*: 4.8) and denaturing temperature (65 °C). The concentration dependence of the colloid osmotic pressure (COP) demonstrated that the 8.5 g dL^{-1} dimer solution has the same COP with the physiological 5 g dL^{-1} rHSA. The antigenic epitopes of the albumin units are preserved after bridging the Cys-34, and the circulation lifetime of the ^{125}I-labeled variant in rat was 18 h. A total of 16 molecules of the tetrakis{(1-methylcyclohexanamido)phenyl}porphinatoiron(II) derivative (FecycP) is incorporated into the hydrophobic cavities of the HSA dimer, giving an albumin–heme hybrid in dimeric form. It can reversibly bind and release O_2 under physiological conditions (37 °C, pH 7.3) like hemoglobin or myoglobin. Magnetic circular dichroism (CD) revealed the formation of an O_2–adduct complex and laser flash photolysis experiments showed the three-component kinetics of the O_2-recombination reaction. The O_2-binding affinity and the O_2-association and -dissociation rate constants of this synthetic hemoprotein have also been evaluated.

Keywords: Human serum albumin dimer; Cross-linking; Colloid osmotic pressure; Synthetic heme; Albumin–heme dimer; Oxygen carrier

1. Introduction

Human serum albumin (HSA) is the most abundant plasma protein and contains 35 cysteines, of which 17 couples form intramolecular disulfide bonds to fold a single polypeptide as a unique heart-shape structure [1–4]. Only the first thiol residue in the chain, namely Cys-34, does not participate in the S–S bonding and functions as a binding site for the SH-involving ligands (cysteine, glutathione, and captopril), as well as for the various metal ions and nitric oxide [1,5]. Interestingly, two albumin molecules can associate to produce a dimer through an intermolecular disulfide bridge of Cys-34; approximately 5% of HSA is actually in a dimeric form in our bloodstream [6]. Hughes [7] initially prepared the HSA dimer by the addition of bifunctional $HgCl_2$, which causes Cys-34 to connect through mercury. Subsequent oxidation of this mercury dimer by treatment with iodine gave a disulfide-linked HSA [8]. It can also be directly prepared by oxidation of HSA with ferricyanide [9]. However, electron spin resonance measurements of HSA and the latest crystal structural analysis of the recombinant HSA (rHSA) revealed that Cys-34 locates in a hydrophobic crevice at a depth of 9.5 Å from the surface [2–4,10]. This implies that the intermolecular Cys-34 disulfide bridging might lead to flattening of the pocket. We have linked two rHSA molecules with a flexible bola-shape spacer, 1,6-bis(maleimido)hexane (BMH), which is long (16 Å) enough to connect the Cys-34 residues, to produce a new type of rHSA dimer (Fig. 1) [11].

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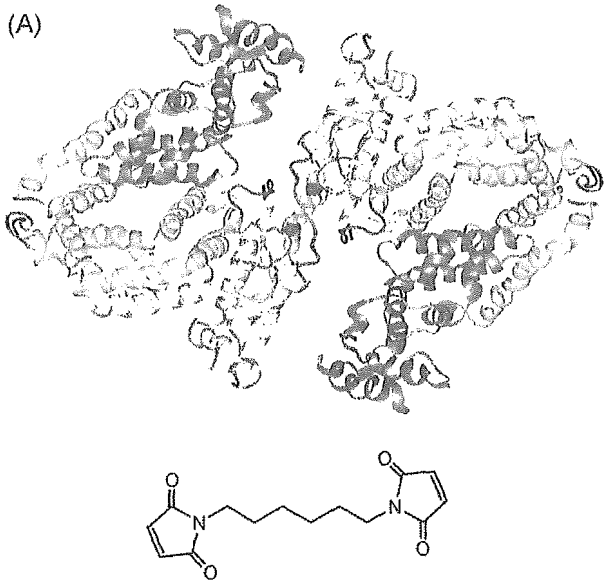
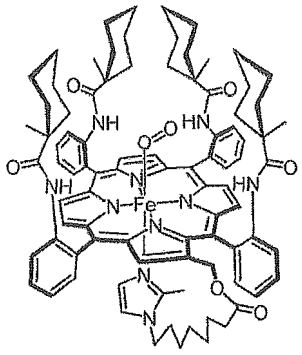
(A)



1,6-Bis(maleimido)hexane (BMH)

(B)



FecycP

Fig. 1. (A) Simulated structure of rHSA dimer cross-linked by Cys-34 with 1,6-bis(maleimido)hexane (BMH). The domains I, II, and III of each rHSA unit are colored white, yellow, and pink, respectively. The cross-linking agent (BMH) is shown in a space-filling representation and colored by atom type (carbon: green, nitrogen: blue, oxygen: red). The figure was made with insight II (Molecular Simulations) on the basis of the 1e78 available at the Brookhaven PDB. (B) Formula of synthetic heme, FecycP.

On the other hand, a maximum of eight molecules of synthetic heme with a covalently bound proximal base is incorporated into the hydrophobic cavities of rHSA, giving an albumin–heme hybrid, which can reversibly bind and release O_2 under physiological conditions (pH 7.3, 37 °C) like hemoglobin (Hb) or myoglobin (Mb) [12]. We have shown that this O_2-carrying plasma protein acts as a red blood cell (RBC) substitute in vitro and in vivo [13]. The only fault of this system is its relatively low heme concentration, which reflects the O_2 solubility in the medium. For instance, the albumin–heme solution with a physiological HSA concentration (≈0.75 mM) involves 6 mM of heme, which corresponds to only 65% of the amount in human blood ([heme]=9.2 mM). A highly condensed solution can dissolve more heme, however, the colloid osmotic pressure (COP) increases in proportion to the albumin concentration. We have found that a total of 16 molecules of 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis{α,α,α,α-*o*-(pivalamido)phenyl}porphinatoiron(II) (FepivP) was incorporated into the BMH-bridged HSA dimer, and this solution with 0.75 mM HSA includes 12 mM of heme [11]. The tertiary structures of the two protein-units might be intact after the cross-linking, and the ligand-binding capacity of the dimer became twofold in excess relative to that of the monomer. Consequently, the saline solution of the albumin–heme dimer can transport a large volume of O_2 in comparison to the human blood while maintaining its COP on a physiological level. A long persistence in circulation due to the large molecular size is also expected. In this paper, we report the efficient synthesis, physicochemical characterization, and preliminary pharmacokinetics of the BMH-bridged rHSA dimer. Furthermore, the O_2-binding properties of the albumin-heme dimer incorporating the FepivP analogue, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis{α,α,α,α-*o*-(1-methylcyclohexanamido)phenyl}porphinatoiron(II) (FecycP, Fig. 1), are evaluated by magnetic circular dichroism and laser flash photolysis.

2. Material and methods

2.1. Materials

An rHSA (Albrec®, 25 wt.%) was provided from the NIPRO (Osaka). Ethanol, dithiothreitol (DTT), 2,2′ -dithiopyridine, and warfarin (all high-purity grades) were purchased from Kanto Chemical, (Tokyo) and used without further purification. 1,6-Bis(maleimido)hexane was purchased from Pierce Biotechnology (Rockford, USA). Diazepam was purchased from Wako Pure Chemical Ind., (Tokyo). 2-[8-{*N*-(2-Methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis{α,α,α,α-*o*-(1-methylcyclohexanamido)phenyl}porphinatoiron(II) (FecycP) was prepared according to our previously reported procedure [14].

2.2. Synthesis of rHSA dimer

Aqueous DTT (1.0 M, 0.24 mL) was added to the phosphate buffer solution (pH 7.0, 10 mM) of rHSA (0.75 mM, 80 mL) under nitrogen, and the solution was quickly mixed by a vortex mixer, followed by an incubation for 30 min at room temperature. The obtained rHSA in reduced form was washed with a total of 880-mL phosphate buffer (pH 7.0, 2.25 mM) using an ADVANTEC UHP-76K ultrafiltration system with a Q0500 076E membrane (cutoff Mw 50 kDa) and finally condensed to 26.7 mL ([rHSA]=2.25 mM). The mercapto-ratio of the Cys-34

was confirmed by the reaction with 2,2′-ditiopyridine (2,2′-DTP), which immediately coupled with the free thiol group to give 2-thiopyridinone (2-TP) with an absorption at 343 nm [molar absorption coefficient (ε_{343}): 8.1×10^3 M^{-1} cm^{-1}]. Quantitative assay of the produced 2-TP showed that the mercapto-ratio of Cys-34 was 100%. Ethanolic BMH (6.38 mM, 4.76 mL) divided into three portions was then slowly added dropwise into the rHSA solution within 1 h under an N_2 atmosphere, and gently stirred overnight at room temperature. The reaction kinetics was observed by the HPLC measurements. The HPLC system consisted of a Shimadzu LC-8A pump and a Shimadzu SPD-10A UV detector. A Shodex Protein KW-803 column was used and the mobile phase was phosphate buffered saline (PBS, pH 7.4) at 25 °C (1.0 mL min^{-1}). The dimer was purified by gel column chromatography with Sephacryl S-200 HR (Pharmacia, 5 cmϕ×40 cm) and PBS (pH 7.4) as the eluant (5.0 mL min^{-1}). These separations were performed using a BIO-RAD EGP Combo Rec system. The elution was monitored by absorption at 280 nm. The purity of the dimer was measured by the HPLC technique described above. The albumin concentrations were assayed by general bromcresol green (BCG) methods using a Wako AlbuminB-Test [15].

2.3. Physicochemical properties

The UV–Vis absorption spectra were recorded on a JASCO V-570 spectrophotometer. The measurements were normally carried out at 25 °C. Circular dichroism (CD) spectra were obtained using a JASCO J-725 spectropolarimeter. The rHSA samples' concentration was 2 μM in PBS, and quartz cuvettes with a 1-mm thickness were used for the measurements over the range of 195–250 nm. The matrix associated laser desorption ionization time-of-flight mass spectra (MALDI-TOF MS) were obtained using a Shimadzu AXIMA-CFR Kompact MALDI, which was calibrated by BSA (Sigma A-0281) and HSA (Sigma A-3782). The specimens were prepared by mixing the aqueous sample solution (10 μM, 1 μL) and matrix (10 mg mL^{-1} sinapinic acid in 40% aqueous CH_3CN, 1 μL) on the measuring plate and drying in air. The viscosity and density of the rHSA solutions (PBS, pH 7.4) were obtained using an Anton PAAR DSC 300 capillary viscometer at 37 °C. The isoelectric points and molecular weights were obtained by a Pharmacia Phastsystem using isoelectric focusing (IEF) in Phast Gel IEF 3-9 and Native-PAGE in Phast Gel Gradient 8–25, respectively. The colloid osmotic pressures of the rHSA solutions (PBS, pH 7.4) were measured by a WESCOR 4420 Colloid Osmometer at 25 °C. A membrane filter with a 30-kDa cutoff was used. Differential scanning calorimetry (DSC) was measured on a SEIKO Instruments DCS120 differential scanning calorimeter at the scan rate of 1 °C min^{-1} in the temperature range between 10 and 95 °C. The concentrations of the rHSA samples were 75 μM in PBS (pH 7.4).

2.4. Ligand binding constants

The PBS solution of ligand (warfarin or diazepam, 20 μM, 2 mL) was mixed with the rHSA sample in PBS (20 μM, 2 mL), and the unbound ligand fractions were separated by centrifugation (2000 rpm, 25 °C, 20 min) using a Millipore Centriplus YM-50. Adsorption of the ligand molecules onto the filtration membranes was negligible. The unbound ligand concentrations were determined by UV–Vis spectroscopic measurements.

2.5. Compatibility with blood components in vitro

Fresh whole blood was obtained from Wistar rats (300 g, male, Saitama Experimental Animals Supply, Japan) and stored in heparinized glass tubes. The rHSA samples (PBS, pH 7.4) were then slowly added to the blood at 50 vol.% concentrations (whole volume 2 mL). After 30 min, 30 μL of the sample was mixed with 100 μL of a Terumo ACD-A solution, which was diluted in advance with pure water by 1:10 (v/v). The blood cell numbers of the obtained samples were counted using a Sysmex KX-21 blood cell counting device. Furthermore, one drop of the incubated sample of the blood with the rHSA dimer was microscopically observed using an Olympus IX50 microscope with an IX70 CCD camera.

2.6. Immunogenicity

The Tris–HCl buffer solutions (TBS, pH 7.4, 50 mM, 50 μL) of the rHSA samples (10 μg mL^{-1}) were injected into a Nunc immunoplate and incubated at 4 °C overnight. The rHSA solutions in the wells were washed with TBS, and 2% skimmed milk was added to avoid the nonspecific binding of the antibody. After washing with TBS including 0.1% Tween 20 (Tween 20–TBS), anti-HSA polyclonal antibody (50 μL per well) was added and incubated for 2 h at 25 °C. The antibody was removed by aspiration, and 50 μL of horseradish peroxidase-labelled rabbit anti-IgG polyclonal antibody diluted 1/5000 by Tween 20–TBS was injected, following an incubation for 1 h at 25 °C. Finally, 100 μL of *o*-phenylenediamine substrate solution (400 mg mL^{-1} in 0.15 M citrate-phosphate buffer (pH 5.0) involving 0.1% H_2O_2) was put into each well. H_2SO_4 (2 M; 50 μL) was then added to stop the reaction. The resulting absorbance in each well was measured at 490 nm using a Japan InterMed Imunomini NJ-2300.

2.7. Circulation lifetime in vivo

The 125-Iodinated rHSA monomer and dimer were prepared by our previously reported procedures, and purified using a Pharmacia Bio-Gel PD-10 column [16]. The recovered ^{125}I-albumin had a specific activity of 2.0×10^7 cpm μg^{-1}, and was diluted by non-labeled albumin before intracardial administration into anesthetized Wistar rats

(200–210 g, male). The kinetics of the albumin clearance from the circulation was monitored by measuring the radioactivity in the plasma phase of blood taken from the lateral tail veins using an Aloka ARC 2000 Autowell Gamma Counter. Acid precipitability of the recovered radioactivity was also measured. The aqueous trichloroacetic acid (TCA, 25%, 0.1 mL) was first added to the plasma (20 μL) diluted with 5 g dL^{-1} rHSA (80 μL), followed by centrifugation (3000 rpm, 10 min). The precipitate was then washed with 12.5% TCA (0.2 mL) and the radioactivity of the pellet was measured. The rats were sacrificed at the end of the experiments by hemorrhage. The radioactivity of the excised organs was also measured as well. The care and handling of the animals were in accordance with NIH guidelines.

2.8. Preparation of albumin–heme dimer

The preparation of rHSA–FecycP dimer was carried out by mixing the EtOH solution of carbonyl-FecycP and an aqueous phosphate buffer solution of rHSA according to our previously reported procedures ([FecycP]/[rHSA]=16:1 (mol/mol)) [13]. The albumin concentrations were assayed by the general BCG methods described above, and the amount of FecycP was determined by the assay of the iron ion concentration using inductively coupled plasma spectrometry (ICP) with a Seiko Instruments SPS 7000A spectrometer. .

2.9. Magnetic circular dichroism (MCD)

MCD spectra for the phosphate buffer solution of the rHSA–FecycP dimer (10 μM) under N_2, CO, and O_2 atmospheres were measured using a JASCO J-820 circular dichrometer fitted with a 1.5-T electromagnet. The accumulation times were normally three, and from each data point was subtracted the spectra without an electromagnetic (at 0 T) as the baseline.

2.10. O_2-Binding equilibrium and kinetics

O_2-Binding to FecycP was expressed by Eq. (1).

$$\text{FecycP} + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{FecycP}(O_2) \quad (K = k_{on}/k_{off}) \tag{1}$$

The O_2-binding affinity (gaseous pressure at half O_2-binding for heme, $P_{1/2}=1/K$) was determined by spectral changes at various partial pressure of O_2 as in previous reports [12,a,d,14]. The FecycP concentrations of 20 μM were normally used for the UV–Vis absorption spectroscopy. The spectra were recorded within the range of 350–700 nm. The half lifetime of the dioxygenated species of the rHSA–FecycP dimer was determined by the time dependence of the absorption intensity at 549 nm, which is based on the O_2–adduct complex. The association and dissociation rate constants for O_2 (k_{on}, k_{off}) were measured by a competitive rebinding technique using a Unisoku TSP-600 laser flash photolysis apparatus [12,17–19]. The absorption decays accompanying the O_2 association to the rHSA–FecycP dimer obeyed three-component kinetics. We employed triple-exponentials to analyze the absorption decays; $\Delta A(t)$ [12,a,b,],

$$\Delta A(t) = C_1\exp(-k_1 t) + C_2\exp(-k_2 t) + C_3\exp(-k_3 t) \tag{2}$$

where k_1, k_2, k_3 are apparent rate constants for the each reaction. The data were fit to this equation using a Solver in Excel 2003.

3. Results and discussion

3.1. Synthesis of rHSA dimer

In the neutral pH range (5.0–7.0), DTT selectively reduces the mixed-disulfide of Cys-34 in HSA or BSA [20–22]. In fact, the addition of the small molar excess DTT into the rHSA solution (phosphate buffer, pH 7.0, 10 mM) under an N_2 atmosphere led to complete reduction of Cys-34 (mercapto-ratio became 100%). After removing DTT, ethanolic BMH was dropwise added to the reduced rHSA to initiate the cross-linking reaction. The pretreatment with DTT significantly increased the yield of the dimer, and the rHSA concentration of 15 g dL^{-1} gave the highest yield of 45%, which is significantly improved from our previous result (Fig. 2) [11]. Several attempts to facilitate the dimerization

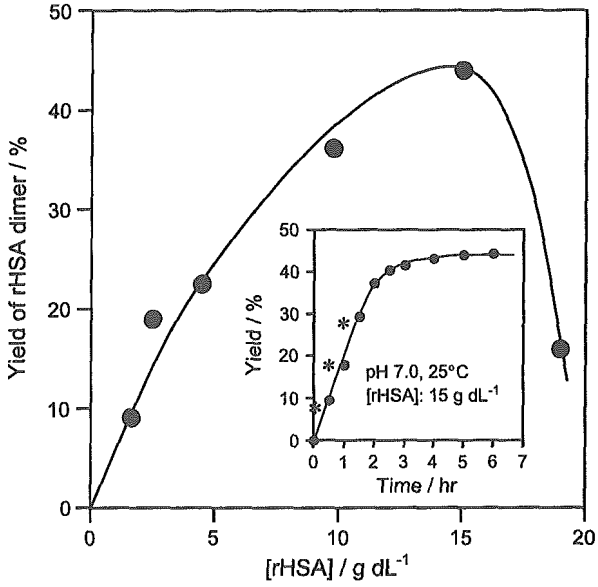


Fig. 2. The relationship between the rHSA concentration and yield of the rHSA dimer. The yields were determined based on the peak area in the HPLC elution curves. Inset shows the time course of the dimerization yield when the rHSA concentration was set at 15 g dL^{-1}. The asterisks indicate the time points when the EtOH solution of BMH was dropwise added. The total EtOH content in the reaction mixture was 15 vol.%.

unfortunately failed: (i) gentle heating (25–50 °C), (ii) changing the co-solvent from EtOH to DMF for dissolving the BMH, and molar ratio [BMH]/[rHSA]: 0.5–1.5, (iii) increasing the concentrations of co-solvents (<30%), and (iv) the further addition of the reactive rHSA monomer.

The HPLC elution curve of the reactant demonstrated only two peaks (rHSA monomer and dimer), which means that the bifunctional BMH was successfully bound to Cys-34 which led to dimer formation and not polymerization (Fig. 3). The yield reached a peak within 4 h (Fig. 2 inset). The addition of EtOH to the mixture (40 vol.%) immediately formed a white precipitate; this is similar to the well-known Cohn's methods [1,23]. However, the precipitate still contained the monomer component. In contrast, separation using gel column chromatography with Sephacryl S-200 HR gave the dimer with 99% purity and 80% recovery. Native-PAGE showed a single band in the molecular weight range of 13 kDa (Fig. 3, above). We could not detect the free thiol in the isolated rHSA dimer (mercapto-ratio: 0%), which is now available in gram quantities. The appearance of the obtained dimer solution (in PBS, 20 g dL^{-1}) did not change over 1 year at room temperature and underwent no aggregation and precipitation.

3.2. Physicochemical properties

The matrix associated laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) of the BMH-bridged rHSA dimer showed a distinct sharp signal at *m/z* 132,741.3, which is in good agreement with the calculated mass (Mw. 133,179.6); the difference was only 0.3% (Table 1). The magnitudes of its UV–Vis absorption (λ_{max}: 280 nm) significantly increased compared to that of

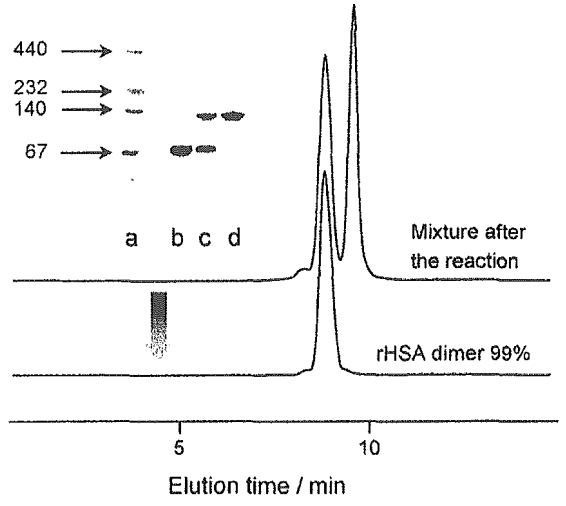


Fig. 3. HPLC elution curves of the rHSA dimer at 25 °C. The upper profile after the reaction indicated that the reactant consists of only the monomer and dimer. After gel column chromatography, the rHSA dimer was isolated with the purity of 99%. The left upper pattern is the native-PAGE electrophoresis of the rHSA dimer. a: markers, b: rHSA, c: mixture after the reaction, d: purified rHSA dimer.

Table 1
Physicochemical properties of rHSA dimer

	rHSA	rHSA dimer
Mw (Da)	66,331[a]	132,741[a]
	66×10^3 [b]	136×10^3 [b]
[calculated value]	66,451	133,180
Cys-34 mercapto ratio (%)	17	0
p*I*	4.8	4.8
ε_{280} (cm^{-1} M^{-1})	3.4×10^4	6.8×10^4
$[\theta]_{208}$ (deg cm^2 $dmol^{-1}$)	1.9×10^4	1.9×10^4
$[\theta]_{222}$ (deg cm^2 $dmol^{-1}$)	1.8×10^4	1.8×10^4

[a] Determined by MALDI-TOF/MS.
[b] Determined by [C] vs. COP/[C] (Fig. 5, inset).

rHSA with the same molar concentrations (Fig. 4(A)). The concentration of the albumin was carefully assayed by (i) BCG method [15] and (ii) weighing method with the weight of the freeze-dried sample and its molecular weight. While the molar absorption coefficient at 280 nm (ε_{280}: 6.8×10^4 M^{-1} cm^{-1}) became exactly twice the monomer's value (3.4×10^4 M^{-1} cm^{-1}), the CD spectral pattern (λ_{min}: 208, 222 nm) and the molar ellipticities at 208 and 222 nm ($[\theta]_{208}$: 1.9×10^4 deg cm^2 $dmol^{-1}$, $[\theta]_{222}$: 1.8×10^4 deg cm^2 $dmol^{-1}$) were identical to those of the monomer (Fig. 4(B), Table 1) [24,25]. It is appropriate to consider that the α-helix content of the each rHSA unit (67%) was unaltered [1–4]. The isoelectric point of the dimer (*p*I: 4.8) was also the same as that of rHSA. All these observations suggested that the secondary/tertiary structure and surface net charges of the rHSA units in the dimer did not change after the S–S disulfide bridging of Cys-34.

The DSC thermogram of this rHSA dimer showed an exothermic peak at 65 °C, which corresponds to its denaturing temperature (T_d). It has been shown that the T_d of HSA is largely dependent on the content of the

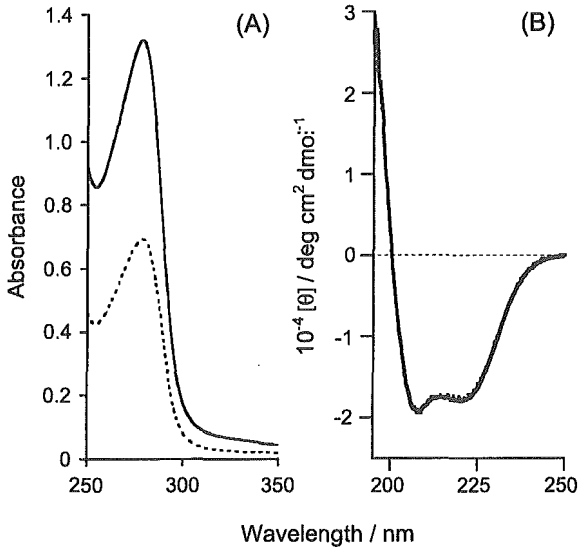


Fig. 4. (A) UV–Vis absorption spectra and (B) CD spectra of rHSA monomer (dotted line) and rHSA dimer (solid line) in PBS solution (pH 7.4) at 25 °C.