C. Substitution of Arg-186 with Leu or Phe. For administration into the human circulatory system, it would be better if the affinity were similar to the human RBC [$P_{50}(O_2)$: 8 Torr, 25 °C]. It is expected that providing a certain degree of hydrophobicity into the distal side of the heme by insertion of a nonpolar residue would reduce the O_2 binding affinity of the rHSA−heme complex. The most suitable position for that introduction might be at Arg-186, which is the entrance of the heme pocket and which is rather close to the central Fe(II) ion. Therefore, rHSA(HL/R186L)−hemin and rHSA(HL/R186F)−hemin were prepared. The O_2 dissociation rate constants of rHSA(HL/R186L)−heme and rHSA(HL/R186F)−heme were 3−4-fold higher than that of rHSA(HF)−heme, which reduced the O_2 binding affinities [larger $P_{50}(O_2)$]. This reduction might be attributable to the increased hydrophobicity in the distal pocket. The O_2 binding affinities of rHSA(HL/R186L)−heme [$P_{50}(O_2)$: 10 Torr] and rHSA(HL/R186F)−heme [$P_{50}(O_2)$: 9 Torr] have become equivalent to those of human RBC. The important structural factor in these mutants is Y161L, which enables the rotation of the isopropyl group of Leu-185 above the O_2 coordination site. Unexpectedly, the $k_{on}(O_2)$ and $k_{on}(CO)$ values of rHSA(HL/R186L)−heme and rHSA(HL/R186F)−heme were 3-fold and 3−4-fold higher than those of rHSA(HL)−heme and in the same range as that of rHSA(HF)−heme. In fact, Leu-161 is small, but the hydrophobic Leu-186 or Phe-186 might be integrated into the heme pocket from the entrance and might push up the neighboring Leu-185 residue (Figure 19e) (*188*).

We have engineered mutant rHSA−heme complexes that can bind O_2. Principal modifications to the heme pocket that are necessary to confer reversible O_2 binding are (i) replacement of Tyr-161 by hydrophobic amino acid (Leu or Phe), and (ii) introduction of His as a proximal base at position Ile-142. Furthermore, (iii) modification of the distal amino acid has a considerable effect on the modulation of O_2 and CO binding affinities.

4. CONCLUSIONS

The structures of our artificial O_2 carriers differ greatly from those of sophisticated RBCs. However, clear advantages of simplified artificial O_2 carriers are readily apparent: the absence of blood-type antigens and infectious viruses, stability for long-term storage at room temperature for any emergency, all of which overwhelm the functionality of RBCs. The shorter half-life of artificial O_2 carriers in the bloodstream (ca. 3 days) limits their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties such as $P_{50}(O_2)$ and viscosity supports their possible development of tailor-made O_2 carriers to suit various clinical indications. The achievements of ongoing research described above give us confidence in advancing the further development with the expectation of its eventual realization.

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LITERATURE CITED

(1) Perutz, M. F. (1970) Stereochemistry of cooperative effects in haemoglobin. *Nature 228*, 726–739.
(2) Jones, R. D., Summerville, A., and Basolo, F. (1979) Synthetic oxygen carriers related to biological systems. *Chem. Rev. 79*, 139–179.
(3) Collman, J. P. (1977) Synthetic model for the oxygen-binding hemoproteins. *Acc. Chem. Res. 10*, 265–272.
(4) Momenteau, M., and Reed, C. (1994) Synthetic heme dioxygen complexes. *Chem. Rev. 94*, 659–698.
(5) Collman, J. P., Gagne, R. R., Halbert, T. R., Marchon, J.-C., and Reed, C. A. (1973) Reversible oxygen adduct formation in ferrous complexes derived from "picket fence" porphyrins. A model for oxymyoglobin. *J. Am. Chem. Soc. 73*, 7868–7870.
(6) Collman, J. P., Gagne, R. R., Reed, C. A., Halbert, T. R., Lang, G., and Robinson, W. T. (1975) "Picket fence porphyrins." Synthetic models for oxygen binding hemoproteins. *J. Am. Chem. Soc. 97*, 1427–1439.
(7) Matsushita, Y., Hasegawa, E., Eshima, K., and Tsuchida, E. (1983) Synthesis of amphiphilic porphinatoiron complexes having phosphocholine groups. *Chem. Lett. 1983*, 1387–1389.
(8) Tsuchida, E. (1985) Liposome-embedded iron-porphyrins as an artificial oxygen carrier. *Ann. N.Y. Acad. Sci. 446*, 429–442.
(9) Tsuchida, E., and Nishide, H. (1986) Hemoglobin model - artificial oxygen carrier composed of porphinatoiron complexes. *Top. Curr. Chem. 1132*, 63–99.
(10) Komatsu, T., Moritake, M., Nakagawa, A., and Tsuchida, E. (2002) Self-organized lipid-porphyrin bilayer membranes in vesicular form: nanostructure, photophysical properties and dioxygen coordination. *Chem. Eur. J. 8*, 5469–5480.
(11) Komatsu, T., Ando, K., Kawai, N., Nishide, H., and Tsuchida, E. (1995) O_2-transport albumin - a new hybrid hemoprotein incorporating tetraphenylporphinatoiron(II). *Chem. Lett.* 813–814.
(12) Savitsky, J. P., Doczi, J., Black, J., and Arnold, J. D. (1978) A clinical safety trial of stroma-free hemoglobin. *Clin. Pharm. Ther. 23*, 73–80.
(13) Greenwald, R. B., Pendri, A., Martinez, A., Gilbert, C., and Bradley, P. (1996) PEG thiazolidine-2-thione, a novel reagent for facile protein modification: conjugation of bovine hemoglobin. *Bioconjugate Chem. 7*, 638–641.
(14) Kluger, R., and Li, X. (1997) Efficient chemical introduction of a disulfide cross-link and conjugation site into human hemoglobin at β-lysine-82 utilizing a bifunctional aminoacyl phosphate. *Bioconjugate Chem. 8*, 921–926.
(15) Sakai, H., Yuasa, M., Onuma, H., Takeoka, S., and Tsuchida, E. (2000) Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjugate Chem. 11*, 56–64.
(16) Hai, T. T., Pereira, D. E., Nelson, D. J., Catarello, J., and Smak, A. (2000) Surface modification of diaspirin cross-linked hemoglobin (DCLHb) with chondroitin-4-sulfate derivatives. Part 1. *Bioconjugate Chem. 11*, 705–713.
(17) Manjula, B. N., Tsai, A., Upadhya, R., Perumalsamy, K., Smith, P. K., Malavalli, A., Vandegriff, K., Winslow, R. M., Intaglietta, M., Prabhakaran, M., Friedman, J. M., and Acharya, A. S. (2003) Site-specific PEGylation of hemoglobin at Cys-93(β): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate Chem. 14*, 464–472.
(18) Murray, J. A., Ledlow, A., Launspach, J., Evans, D., Loveday, M., and Conklin, J. L. (1995) The effects of recombinant human

hemoglobin on esophageal motor function in humans. *Gastroenterology 109*1241–1248.

(19) Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, S. L., Arnone, A., and Walder, J. A. (1986) Isolation and characterization of a new hemoglobin derivative cross-linked between the a chains (lysine $99\alpha_1 \gg$ lysine $99\alpha_2$). *J. Biol. Chem. 261*, 9929–9937.

(20) Iwashita, Y. (1991) Pyridoxalated hemoglobin-polyoxyethylene conjugate (PHP) as an O_2 carrier. *Artif. Organs Today 1*, 89–114.

(21) Riess, J. G. (2001) Oxygen carriers ("blood substitutes") - raison d'etre, chemistry, and some physiology. *Chem. Rev. 101*, 2797–2920.

(22) de Figueiredo, L. F., Mathru, M., Solanki, D., Macdonald, V. W., Hess, J., and Kramer, G. C. (1997) Pulmonary hypertension and systemic vasoconstriction might offset the benefits of acellular hemoglobin blood substitutes. *J. Trauma 42*, 847–854.

(23) Tsai, A. G., Kerger, H., and Intaglietta, M. (1995) Microcirculatory consequences of blood substitution with αα-hemoglobin. In *Blood Substitutes: Physiological Basis of Efficacy* (Winslow, R. M., Vandegriff, K., Intaglietta, M., Eds.) pp155−174, Birkhauser, Boston.

(24) Goda, N., Suzuki, K., Naito, M., Takeoka, S., Tsuchida, E., Ishimura, Y., Tamatani, T., and Suematsu, M. (1998) Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J. Clin. Invest. 101*, 604–612.

(25) Sakai, H., Hara, H., Yuasa, M., Tsai, A. G., Takeoka, S., Tsuchida, E., and Intaglietta, M. (2000) Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol. 279*, H908−H915.

(26) Burhop, K., Gordon, D., and Estep, T. (2004) Review of hemoglobin-induced myocardial lesions. *Artif. Cells Blood Substit. Immobil. Biotechnol. 32*, 353–374.

(27) Neragi-Miandoab, S., and Vlahakes, G. J. (2006) Elevated troponin I level with hemoglobin based oxygen carrying solutions (HBOCs) as a priming solution despite improved left ventricular function. *Interact. Cardiovasc. Thorac. Surg. 5*, 135–138.

(28) Natanson, C., Kern, S. J., Lurie, P., Banks, S. M., and Wolfe, S. M. (2008) Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA 299*, 2304–2312.

(29) Balla, J., Jacob, H. S., Balla, G., Nath, K., Eaton, J. W., and Vercellotti, G. M. (1993) Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc. Natl. Acad. Sci. U.S.A. 90*, 9285–9289.

(30) Sakai, H., Sato, A., Masuda, K., Takeoka, S., and Tsuchida, E. (2008) Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J. Biol. Chem. 283*, 1508–1517.

(31) Sakai, H., Sato, A., Sobolewski, P., Takeoka, S., Frangos, J. A., Kobayashi, K., Intaglietta, M., and Tsuchida, E. (2008) NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers. *Biochim. Biophys. Acta 1784*, 1441−1447.

(32) Martini, J., Cabrales, P., Tsai, A. G., and Intaglietta, M. (2006) Mechanotransduction and the homeostatic significance of maintaining blood viscosity in hypotension, hypertension and haemorrhage. *J. Intern. Med. 259*, 364–372.

(33) Sakai, H., Suzuki, Y., Kinoshita, M., Takeoka, S., Maeda, N., and Tsuchida, E. (2003) O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs. *Am. J. Physiol. Heart Circ. Physiol. 285*, H2543−H2555.

(34) Vandegriff, K. D., and Olson, J. S. (1984) The kinetics of O_2 release by human red blood cells in the presence of external sodium dithionite. *J. Biol. Chem. 259*, 12609–12618.

(35) Chang, T. M. S. (2005) Therapeutic applications of polymeric artificial cells. *Nat. Rev. Drug Discov. 4*, 221–235.

(36) Toyoda, T. (1965) Artificial blood. *Kagaku 35*, 7–13 (in Japanese).

(37) Kimoto, S., Hori, M., Toyoda, T., and Sekiguchi, W. (1968) Artificial red cells. *Gekachiryou (Surgical Therapy) 19*, 324–332 (in Japanese).

(38) Bangham, A. D., and Horne, R. W. (1964) Negative staining of phospholipids and their structure modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol. 8*, 660–668.

(39) Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol. 13*, 238–252.

(40) Djordjevich, L., and Miller, I. F. (1977) Lipid encapsulated hemoglobin as a synthetic erythrocyte. *Fed. Proc. 36*, 567–571.

(41) Farmer, M. C., Rudolph, A. S., Vandegriff, K. D., Hayre, M. D., Bayne, S. A., and Johnson, S. A. (1988) Liposome-encapsulated hemoglobin: oxygen binding properties and respiratory function. *Biomater. Artif. Cells Artif. Organs 16*, 289–299.

(42) Rudolph, A. S., Klipper, R. W., Goins, B., and Phillips, W. T. (1991) In vivo biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. *Proc. Natl. Acad. Sci. U.S.A. 88*, 10976–10980.

(43) Phillips, W. T., Klipper, R. W., Awasthi, V. D., Rudolph, A. S., Cliff, R., Kwasiborski, V., and Goins, B. A. (1999) Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. *J. Pharmacol. Exp. Ther. 288*, 665–670.

(44) Usuba, A., Osuka, F., Kimura, T., Sato, R., Ogata, Y., Gotoh, H., Kimura, T., and Fukui, H. (1998) Effect of liposome-encapsulated hemoglobin, neo red cells, on hemorrhagic shock. *Surg. Today 28*, 1027–1035.

(45) Yoshioka, H. (1991) Surface modification of haemoglobin-containing liposomes with polyethylene glycol prevents liposome aggregation in blood plasma. *Biomaterials 12*, 861–864.

(46) Sakai, H., Hamada, K., Takeoka, S., Nishide, H., and Tsuchida, E. (1996) Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol. Prog. 12*, 119–125.

(47) Takeoka, S., Ohgushi, T., Terase, K., Ohmori, T., and Tsuchida, E. (1996) Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir 12*, 1755–1759.

(48) Sou, K., Naito, Y., Endo, T., Takeoka, S., and Tsuchida, E. (2003) Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol. Prog. 19*, 1547–1552.

(49) Sakai, H., Takeoka, S., Yokohama, H., Seino, Y., Nishide, H., and Tsuchida, E. (1993) Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif. 4*, 563–569.

(50) Abe, H., Ikebuchi, K., Hirayama, J., Fujihara, M., Takeoka, S., Sakai, H., Tsuchida, E., and Ikeda, H. (2001) Virus inactivation in hemoglobin solution by heat treatment. *Artif. Cells Blood Substit. Immobil. Biotechnol. 29*, 381–388.

(51) Chung, J., Hamada, K., Sakai, H., Takeoka, S., and Tsuchida, E. (1995) Ligand-exchange reaction of carbonylhemoglobin to oxyhemoglobin in a hemoglobin liquid membrane. *Nippon Kagaku Kaishi 2*, 123–127 (in Japanese).

(52) Takeoka, S., Sakai, H., Kose, T., Mano, Y., Seino, Y., Nishide, H., and Tsuchida, E. (1997) Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. *Bioconjugate Chem. 8*, 539–544.

(53) Teramura, Y., Kanazawa, H., Sakai, H., Takeoka, S., and Tsuchida, E. (2003) Prolonged oxygen-carrying ability of hemoglobin vesicles by coencapsulation of catalase *in vivo*. *Bioconjugate Chem. 14*, 1171–1176.

(54) Sakai, H., Masada, Y., Onuma, H., Takeoka, S., and Tsuchida, E. (2004) Reduction of methemoglobin via electron transfer from photoreduced flavin: restoration of O_2-binding of concentrated hemoglobin solution coencapsulated in phospholipid vesicles. *Bioconjugate Chem. 15*, 1037–1045.

(55) Atoji, T., Aihara, M., Sakai, H., Tsuchida, E., and Takeoka, S. (2006) Hemoglobin vesicles containing methemoglobin and L-tyrosine to suppress methemoglobin formation *in vitro* and *in vivo*. *Bioconjugate Chem. 17*, 1241–1245.

(56) Sakai, H., Takeoka, S., Seino, Y., and Tsuchida, E. (1994) Suppression of methemoglobin formation by glutathione in a concentrated hemoglobin solution and in a Hb-vesicle. *Bull. Chem. Soc. Jpn. 67*, 1120–1125.

(57) Rameez, S., Alosta, H., and Palmer, A. F. (2008) Biocompatible and biodegradable polymersome encapsulated hemoglobin: a potential oxygen carrier. *Bioconjugate Chem. 19*, 1025–1032.

(58) Zhao, J., Liu, C. S., Yuan, Y., Tao, X. Y., Shan, X. Q., Sheng, Y., and Wu, F. (2007) Preparation of hemoglobin-loaded nanosized particles with porous structure as oxygen carriers. *Biomaterials 28*, 1414–1422.

(59) Rudolph, A. S. (1988) The freeze-dried preservation of liposome encapsulated hemoglobin: a potential blood substitute. *Cryobiology 25*, 277–284.

(60) Rabinovici, R., Rudolph, A. S., Vernick, J., and Feuerstein, G. (1994) Lyophilized liposome encapsulated hemoglobin: evaluation of hemodynamic, biochemical, and hematologic responses. *Crit. Care Med. 22*, 480–485.

(61) Ringsdorf, H., Schlarb, B., and Venzmer, J. (1988) Molecular architecture and function of polymeric oriented systems - models for the study of organization, surface recognition, and dynamics of biomembranes. *Angew. Chem., Int. Ed. 27*, 113–158.

(62) Kato, A., Arakawa, M., and Kondo, T. (1984) Preparation and stability of liposome-type artificial red blood cells stabilized with carboxymethylchitin. *J. Microencapsul. 1*, 105–112.

(63) Mobed, M., Nishiya, T., and Chang, T. M. (1992) Preparation of carboxymethylchitin-incorporated submicron bilayer-lipid membrane artificial cells (liposomes) encapsulating hemoglobin. *Biomater. Artif. Cells Immobil. Biotechnol. 20*, 365–368.

(64) Li, S., Nickels, J., and Palmer, A. F. (2005) Liposome-encapsulated actin-hemoglobin (LEAcHb) artificial blood substitutes. *Biomaterials 26*, 3759–3769.

(65) Tsuchida, E., Hasegawa, E., Kimura, N., Hatashita, M., and Makino, C. (1992) Polymerization of unsaturated phospholipids as large unilamellar liposomes at low temperature. *Macromolecules 25*, 2007–212.

(66) Sakai, H., Takeoka, S., Yokohama, H., Nishide, H., and Tsuchida, E. (1992) Encapsulation of Hb into unsaturated lipid vesicles and gamma-ray polymerization. *Polym. Adv. Technol. 3*, 389–394.

(67) Akama, K., Awai, K., Yano, Y., Tokuyama, S., and Nakano, Y. (2000) In vitro and in vivo stability of polymerized mixed liposomes composed of 2,4-octadecadienoyl groups of phospholipids. *Polym. Adv. Technol 11*, 280–287.

(68) Sakai, H., Takeoka, S., Park, S. I., Kose, T., Nishide, H., Izumi, Y., Yoshizu, A., Kobayashi, K., and Tsuchida, E. (1997) Surface-modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. *Bioconjugate Chem. 8*, 23–30.

(69) Sakai, H., Tsai, A. G., Kerger, H., Park, S. I., Takeoka, S., Nishide, H., Tsuchida, E., and Intaglietta, M. (1998) Subcutaneous microvascular responses to hemodilution with red cell substitutes consisting of polyethylene glycol-modified vesicles encapsulating hemoglobin. *J. Biomed. Mater. Res. 40*, 66–78.

(70) Sakai, H., Okamoto, M.,; Ikeda E., Horinouchi H., Kobayashi K., and Tsuchida, E. (2008) Histopathological changes of rat brain after direct injection of hemoglobin vesicles (oxygen carriers) and neurological impact in an intracerebral hemorrhage model. *J. Biomed. Mater. Res., Part A* (in press).

(71) Sakai, H., Tomiyama, K., Sou, K., Takeoka, S., and Tsuchida, E. (2000) Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin vesicles as oxygen carriers in a liquid state. *Bioconjugate Chem. 11*, 425–432.

(72) Sou, K., Endo, T., Takeoka, S., and Tsuchida, E. (2000) Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjugate Chem. 11*, 372–379.

(73) Sato, T., Sakai, H., Sou, K., Buchner, R., and Tsuchida, E. (2007) Poly(ethylene glycol)-conjugated phospholipids in aqueous micellar solutions: hydration, static structure, and interparticle interactions. *J. Phys. Chem. B 111*, 1393–1401.

(74) Szebeni, J. (2005) Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. *Toxicology 216*, 106–121.

(75) Van de Velde, M., Wouters, P. F., Rolf, N., Van Aken, H., and Vandermeersch, E. (1998) Comparative hemodynamic effects of three different parenterally administered lipid emulsions in conscious dogs. *Crit. Care Med. 26*, 132–137.

(76) Vercellotti, G. M., Hammerschmidt, D. E., Craddock, P. R., and Jacob, H. S. (1982) Activation of plasma complement by perfluorocarbon artificial blood: probable mechanism of adverse pulmonary reactions in treated patients and rationale for corticosteroids prophylaxis. *Blood 59*, 1299–1304.

(77) Phillips, W. T., Klipper, R., Fresne, D., Rudolph, A. S., Javors, M., and Goins, B. (1997) Platelet reactivity with liposome-encapsulated hemoglobin in the rat. *Exp. Hematol. 25*, 1347–1356.

(78) Szebeni, J., Fontana, J. L., Wassef, N. M., Mongan, P. D., Morse, D. S., Dobbins, D. E., Stahl, G. L., Bunger, R., and Alving, C. R. (1999) Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. *Circulation 99*, 2302–2309.

(79) Izumi, Y., Sakai, H., Kose, T., Hamada, K., Takeoka, S., Yoshizu, A., Horinouchi, H., Kato, R., Nishide, H., Tsuchida, E., and Kobayashi, K. (1997) Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J. 43*, 289–297.

(80) Loughrey, H. C., Bally, M. B., Reinish, L. W., and Cullis, P. R. (1990) The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. *Thromb. Haemost. 64*, 172–176.

(81) Chonn, A., Cullis, P. R., and Devine, D. V. (1991) The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol. 146*, 4234–4241.

(82) Sou, K., and Tsuchida, E. (2008) Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: effect of the structure of acidic groups. *Biochim. Biophys. Acta - Biomembr. 1778*, 1035–1041.

(83) Abe, H., Azuma, H., Yamaguchi, M., Fujihara, M., Ikeda, H., Sakai, H., Takeoka, S., and Tsuchida, E. (2007) Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. *Artif. Cells Blood Substit. Biotechnol. 35*, 157–172.

(84) Abe, H., Fujihara, M., Azuma, H., Ikeda, H., Ikebuchi, K., Takeoka, S., Tsuchida, E., and Harashima, H. (2006) Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: effects on coagulation, kallikrein-kinin, and complement systems. *Artif. Cells Blood Substit. Immobil. Biotechnol. 34*, 1–10.

(85) Wakamoto, S., Fujihara, M., Abe, H., Yamaguchi, M., Azuma, H., Ikeda, H., Takeoka, S., and Tsuchida, E. (2005) Effects of hemoglobin vesicles on resting and agonist-stimulated human platelets *in vitro*. *Artif. Cells Blood Substit. Immobil. Biotechnol. 33*, 101–111.

(86) Wakamoto, S., Fujihara, M., Abe, H., Sakai, H., Takeoka, S., Tsuchida, E., Ikeda, H., and Ikebuchi, K. (2001) Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on agonist-induced platelet aggregation and RANTES release *in vitro*. *Artif. Cells Blood Substit. Immobil. Biotechnol. 29*, 191–201.

(87) Takeoka, S., Mori, K., Ohkawa, H., Sou, K., and Tsuchida, E. (2000) Synthesis and assembly of poly(ethylene glycol)-lipids with mono-, di-, and tetraacyl chains and a poly(ethylene glycol) chain of various molecular weights. *J. Am. Chem. Soc. 122*, 7927–7935.

(88) Okamura, Y., Maekawa, I., Teramura, Y., Maruyama, H., Handa, M., Ikeda, Y., and Takeoka, S. (2005) Hemostatic effects of phospholipid vesicles carrying fibrinogen gamma chain dodecapeptide *in vitro* and *in vivo*. *Bioconjugate Chem. 16*, 1589–1596.

(89) Ohkawa, H., Teramura, Y., Takeoka, S., and Tsuchida, E. (2000) Synthesis of multiacyl poly(ethylene glycol) for the conjugation of cytochrome c to phospholipid vesicle. *Bioconjugate Chem. 11*, 815–821.

(90) Sou, K., Goins, B., Takeoka, S., Tsuchida, E., and Phillips, W. T. (2007) Selective uptake of surface-modified phospholipid vesicles by bone marrow macrophages *in vivo*. *Biomaterials 28*, 2655–2666.

(91) Teramura, Y., and Iwata, H. (2008) Islets surface modification prevents blood-mediated inflammatory responses. *Bioconjugate Chem. 19*, 1389–1395.

(92) Obata, Y., Suzuki, D., and Takeoka, S. (2008) Evaluation of cationic assemblies constructed with amino acid based lipids for plasmid DNA delivery. *Bioconjugate Chem. 19*, 1055–1063.

(93) Sou, K., Inenaga, S., Takeoka, S., and Tsuchida, E. (2008) Loading of curcumin into macrophages using lipid-based nanoparticles. *Int. J. Pharm. 352*, 287–293.

(94) Sou, K., Klipper, R., Goins, B., Tsuchida, E., and Phillips, W. T. (2005) Circulation kinetics and organ distribution of Hb vesicles developed as a red blood cell substitute. *J. Pharmacol. Exp. Ther. 312*, 702–709.

(95) Bradley, A. J., Devine, D. V., Ansell, S. M., Janzen, J., and Brooks, D. E. (1998) Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch. Biochem. Biophys. 357*, 185–194.

(96) Hu, Q., and Liu, D. (1996) Co-existence of serum-dependent and serum-independent mechanisms for liposome clearance and involvement of non-Kupffer cells in liposome uptake by mouse liver. *Biochim. Biophys. Acta 1284*, 153–161.

(97) Shibuya-Fujiwara, N., Hirayama, F., Ogata, Y., Ikeda, H., and Ikebuchi, K. (2001) Phagocytosis *in vitro* of polyethylene glycol-modified liposome-encapsulated hemoglobin by human peripheral blood monocytes plus macrophages through scavenger receptors. *Life Sci. 70*, 291–300.

(98) Sakai, H., Horinouchi, H., Tomiyama, K., Ikeda, E., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am. J. Pathol. 159*, 1079–1088.

(99) Sakai, H., Horinouchi, H., Masada, Y., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2004) Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials 25*, 4317–4325.

(100) Sakai, H., Masada, Y., Horinouchi, H., Ikeda, E., Sou, K., Takeoka, S., Suematsu, M., Takaori, M., Kobayashi, K., and Tsuchida, E. (2004) Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J. Pharmacol. Exp. Ther. 311*, 874–884.

(101) Sakai, H., Horinouchi, H., Yamamoto, M., Ikeda, E., Takeoka, S., Takaori, M., Tsuchida, E., and Kobayashi, K. (2006) Acute 40% exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. *Transfusion 46*, 339–347.

(102) Sakai, H., Seishi, Y., Obata, Y., Takeoka, S., Horinouichi, H., Tsuchida, E., and Kobayashi, K. (2009) Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: Profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days, *Shock 31*, 192−200.

(103) Yamaoka, T., Tabata, Y., and Ikada, Y. (1994) Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *J. Pharm. Sci. 83*, 601–606.

(104) Ohki, N., Kimura, T., and Ogata, Y. (1998) The reduction of methemoglobin in Neo Red Cell. *Artif. Cells Blood Substit. Immobil. Biotechnol. 26*, 477–485.

(105) Sakai, H., Tsai, A. G., Rohlfs, R. J., Hara, H., Takeoka, S., Tsuchida, E., and Intaglietta, M. (1999) Microvascular responses to hemodilution with Hb vesicles as red cell substitutes: Influences of O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol. 276*, H553−H562.

(106) Wang, L., Morizawa, K., Tokuyama, S., Satoh, T., and Tsuchida, E. (1992) Modulation of oxygen-carrying capacity of artificial red cells (ARC). *Polym. Adv. Technol. 4*, 8–11.

(107) Cabrales, P., Sakai, H., Tsai, A. G., Takeoka, S., Tsuchida, E., and Intaglietta, M. (2005) Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am. J. Physiol. Heart Circ. Physiol. 288*, H1885−H1892.

(108) Izumi, Y., Sakai, H., Hamada, K., Takeoka, S., Yamahata, T., Kato, R., Nishide, H., Tsuchida, E., and Kobayashi, K. (1996) Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical oxygen tension. *Crit. Care Med. 24*, 1869–1873.

(109) Sakai, H., Takeoka, S., Wettstein, R., Tsai, A. G., Intaglietta, M., and Tsuchida, E. (2002) Systemic and Microvascular responses to the hemorrhagic shock and resuscitation with Hb vesicles. *Am. J. Physiol. Heart Circ. Physiol. 283*, H1191−H1199.

(110) Sakai, H., Masada, Y., Horinouchi, H., Yamamoto, M., Ikeda, E., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2004) Hemoglobin vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit. Care Med. 32*, 539–545.

(111) Yoshizu, A., Izumi, Y., Park, S. I., Sakai, H., Takeoka, S., Horinouchi, H., Ikeda, E., Tsuchida, E., and Kobayashi, K. (2004) Hemorrhagic shock resuscitation with an artificial oxygen carrier hemoglobin vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma necrosis factor alpha (TNFα). *ASAIO J. 50*, 458–463.

(112) Terajima, K., Tsueshita, T., Sakamoto, A., and Ogawa, R. (2006) Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. *Shock 25*, 184–189.

(113) Yamazaki, M., Aeba, R., Yozu, R., and Kobayashi, K. (2006) Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. *Circulation 114* (1 Suppl), I220−I225.

(114) Tsai, A. G., Vandegriff, K. D., Intaglietta, M., and Winslow, R. M. (2003) Targeted O_2 delivery by low-P_{50} hemoglobin: a new basis for O_2 therapeutics. *Am. J. Physiol. Heart Circ. Physiol. 285*, H1411−H1419.

(115) Plock, J. A., Tromp, A. E., Contaldo, C., Spanholtz, T., Sinovcic, D., Sakai, H., Tsuchida, E., Leunig, M., Banic, A., and Erni, D. (2007) Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue. *Crit. Care Med. 35*, 899–905.

(116) Nolte, D., Pickelmann, S., Lang, M., Keipert, P., and Messmer, K. (2000) Compatibility of different colloid plasma expanders with perflubron emulsion: an intravital microscopic study in the hamster. *Anesthesiology 93*, 1261–1270.

(117) Jouan-Hureaux, V., Audonnet-Blaise, S., Lacatusu, D., Krafft, M. P., Dewachter, P., Cauchois, G., Stoltz, J. F., Longrois, D., and Menu, P. (2006) Effects of a new perfluorocarbon emulsion on human plasma and whole-blood viscosity in the presence of albumin, hydroxyethyl starch, or modified fluid gelatin: an in vitro rheologic approach. *Transfusion 46*, 1892–1898.

(118) Vandegriff, K. D., McCarthy, M., Rohlfs, R. J., and Winslow, R. M. (1997) Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated. *Biophys. Chem. 69*, 23–30.

(119) Kobayashi, K. (2006) Summary of recombinant human serum albumin development. *Biologicals 34*, 55–59.

(120) Meyuhas, D., Nir, S., and Lichtenberg, D. (1996) Aggregation of phospholipid vesicles by water-soluble polymers. *Biophys. J. 71*, 2602–2612.

(121) Neu, B., and Meiselman, H. J. (2002) Depletion-mediated red blood cell aggregation in polymer solutions. *Biophys. J. 83*, 2482–2490.

(122) Sakai, H., Sato, A., Takeoka, S., and Tsuchida, E. (2007) Rheological property of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma substitute aqueous solutions. *Langmuir 23*, 8121–8128.

(123) Tsai, A. G., Friesenecker, B., McCarthy, M., Sakai, H., and Intaglietta, M. (1998) Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. *Am. J. Physiol. Heart Circ. Physiol. 275*, H2170−H2180.

(124) Intaglietta, M., Cabrales, P., and Tsai, A. G. (2006) Microvascular perspective of oxygen-carrying and -noncarrying blood substitutes. *Annu. Rev. Biomed. Eng. 8*, 289–321.

(125) Rebel, A., Ulatowski, J. A., Kwansa, H., Bucci, E., and Koehler, R. C. (2003) Cerebrovascular response to decreased hematocrit: effect of cell-free hemoglobin, plasma viscosity, and CO_2. *Am. J. Physiol. Heart Circ. Physiol. 285*, H1600−H1608.

(126) Dimino, M. L., and Palmer, A. F. (2007) High O_2 affinity hemoglobin-based oxygen carriers synthesized via polymerization of hemoglobin with ring-opened 2-chloroethyl-β-D-fructopyranoside and 1-o-octyl-β-D-glucopyranoside. *Biotechnol. Bioeng. 97*, 462–472.

(127) Plock, J. A., Contaldo, C., Sakai, H., Tsuchida, E., Leunig, M., Banic, A., Menger, M. D., and Erni, D. (2005) Is the Hb in Hb vesicles infused for isovolemic hemodilution necessary to improve oxygenation in critically ischemic hamster skin? *Am. J. Physiol. Heart Circ. Physiol. 289*, H2624−H2631.

(128) Contaldo, C., Plock, J., Sakai, H., Takeoka, S., Tsuchida, E., Leunig, M., Banic, A., and Erni, D. (2005) New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue. *Crit. Care Med. 33*, 806–812.

(129) Verdu, E. F., Bercik, P., Huang, X. X., Lu, J., Al-Mutawaly, N., Sakai, H., Tompkins, T. A., Croitoru, K., Tsuchida, E., Perdue, M., and Collins, S. M. (2008) The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection. *Am. J. Physiol. Gastrointest. Liver Physiol. 295*, G664−G670.

(130) Komatsu, H., Furuya, T., Sato, N., Ohta, K., Matsuura, A., Ohmura, T., Takagi, S., Matsuura, M., Yamashita, M., Itoda, M., Itoh, J., Horinouchi, H., and Kobayashi, K. (2007) Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. *Neurosci. Lett. 421*, 121–125.

(131) Yamamoto, M., Izumi, Y., Horinouchi, H., Teramura, Y., Sakai, H., Kohno, M., Watanabe, M., Kawamura, M., Adachi, T., Ikeda, E., Takeoka, S., Tsuchida, E., Kobayashi, K. (2009) Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation. *J. Surg. Res.* doi:10.1016/j.jss. 2007 12 770.

(132) Sakai, H., Horinouchi, H., Tsuchida, E., Kobayashi, K. (2008) Hemoglobin-vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model. *Shock*. doi:10 1097/SHK.0b013e318189017a.

(133) Peters, T. (1996) *All about Albumin*: Biochemistry, Genetics and Medical Applications, Academic Press, San Diego.

(134) He, X. M., and Carter, D. C. (1992) Atomic structure and chemistry of human serum albumin. *Nature 358*, 209–215.

(135) Carter, D. C., and Ho, J. X. (1994) Structure of serum albumin. *Adv. Protein Chem. 45*, 153–203.

(136) Curry, S., Madelkow, H., Brick, P., and Franks, N. P. (1998) Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Biol. 5*, 827–835.

(137) Bhattacharya, A. A., Grune, T., and Curry, S. (2000) Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J. Mol. Biol. 303*, 721–732.

(138) Ghuman, J., Zunszain, P. A., Petitpas, I., Bhattacharya, A. A., Otagiri, M., and Curry, S. (2007) Structural basis of the drug-binding specificity of humans serum albumin. *J. Mol. Biol. 353*, 38–52.

(139) Tsuchida, E., Komatsu, T., Matsukawa, Y., Hamamatsu, K., and Wu, J. (1999) Human serum albumin incorporating tetrakis(o-pivalamido)phenyl porphinatoiron(II) derivative as a totally synthetic O_2-carrying hemoprotein. *Bioconjugate Chem. 10*, 797–802.

(140) Komatsu, T., Hamamatsu, K., Wu, J., and Tsuchida, E. (1999) Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis-(o-pivalamido) phenylporphyrinatoiron(II) derivatives. *Bioconjugate Chem. 10*, 82–86.

(141) Tsuchida, E., Komatsu, T., Yanagimoto, T., and Sakai, H. (2003) Preservation stability and *in vivo* administration of albumin-heme hybrid solution as an entirely synthetic O_2-carrier. *Polym. Adv. Technol. 13*, 848–850.

(142) Huang, Y., Komatsu, T., Nakagawa, A., Tsuchida, E., and Kobayashi, S. (2003) Compatibility *in vitro* of albumin-heme (O_2 carrier) with blood cell components. *J. Biomed. Mater. Res. 66A*, 292–297.

(143) Komatsu, T., Huang, Y., Wakamoto, S., Abe, H., Fujihara, M., Azuma, H., Ikeda, H., Yamamoto, H., Horinouchi, H., and Kobayashi, K. (2007) Influence of O_2-carrying plasma hemoprotein "albumin-heme" on complement system and platelet activation *in vitro* and physiological responses to exchange transfusion. *J. Biomed. Mater. Res. 81A*, 821–826.

(144) Tsuchida, E., Nakagawa, A., and Komatsu, T. (2003) Coordination structure of active site in synthetic hemoprotein (albumin-heme) with dioxygen and carbon monoxide. *Macromol. Symp. 195*, 275–280.

(145) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2000) Nitrosyl iron(II) complex of *meso*-tetrakis(α,α,α,α-o-pivalamidophenyl)porphyrin with a covalently linked 2-methylimidazolylalkyl group. *Chem. Lett.* 1060–1061.

(146) Komatsu, T., Matsukawa, Y., and Tsuchida, E. (2001) Reaction of nitric oxide with synthetic hemoprotein, human serum albumin incorporating tetraphenylporphinatoiron(II) derivatives. *Bioconjugate Chem. 12*, 71–75.

(147) 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.

(148) Traylor, T. G., Tsuchiya, S., Campbell, D., Mitchell, M., Stynes, D., and Koga, N. (1985) Anthracene-heme cyclophanes. Sterice in CO, O_2 and RNC binding. *J. Am. Chem. Soc. 107*, 604–614.

(149) 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.

(150) 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.

(151) Nakagawa, A., Komatsu, T., Iizuka, M., and Tsuchida, E. (2008) O_2 binding to human serum albumin incorporating iron porphyrin with a covalently linked methyl-L-histidine isomer. *Bioconjugate Chem. 19*, 581–584.

(152) Nakagawa, A., Komatsu, T., and Tsuchida, E. (2007) meso-Tetrakis(α,α,α,α-o-amidophenyl) porphinatoiron(II) bearing a proximal histidyl group at the β-pyrrolic position via an acyl bond: synthesis and O_2 coordination in aqueous media. *Chem. Lett. 36*, 640–641.

(153) Komatsu, T., Okada, T., Moritake, M., and Tsuchida, E. (2001) O_2-binding properties of double-sided porphinatoiron(II)s with polar substituents and their human serum albumin hybrids. *Bull. Chem. Soc. Jpn. 74*, 1695–1702.

(154) Nakagawa, A., Komatsu, T., Iizuka, M., and Tsuchida, E. (2006) Human serum albumin hybrid incorporating tailed porphyrinatoiron(II) in the $\alpha,\alpha,\alpha,\beta$-conformer as an O_2-binding site. *Bioconjugate Chem. 17*, 146–151.

(155) Nakagawa, A., Komatsu, T., Ohmichi, N., and Tsuchida, E. (2003) Synthetic dioxygen-carrying hemoprotein. human serum albumin including iron(II) complex of protoporphyrin IX with an axially coordinated histidylglycyl-propionate. *Chem. Lett. 32*, 504–505.

(156) Nakagawa, A., Komatsu, T., Ohmichi, N., and Tsuchida, E. (2004) Synthesis of protoheme IX derivatives with a covalently linked proximal base and their human serum albumin hybrids as artificial hemoprotein. *Org. Biomol. Chem. 2*, 3108–3112.

(157) Wang, R.-M., Komatsu, T., Nakagawa, A., and Tsuchida, E. (2005) Human serum albumin bearing covalently attached iron(II) porphyrins as O_2-coordination sites. *Bioconjugate Chem. 16*, 23–26.

(158) Harris, J. M. (1992) *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications* (Harris, J. M., Ed.) Plenum Press, New York.

(159) Veronese, F. M., and Harris, J. M. (2002) Introduction and overview of peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54*, 453–456.

(160) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54*, 459–476.

(161) Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405–417.

(162) Nucci, M. L., Shorr, R., and Abuchowski, A. (1991) The therapeutic value of poly(ethylene glycol) modified proteins. *Adv. Drug Delivery Rev. 6*, 133–151.

(163) Kawahara, N. Y., and Ohno, H. (1997) Induced thermostability of poly(ethylene oxide)-modified hemoglobin in glycols. *Bioconjugate Chem. 8*, 643–648.

(164) Huang, Y., Komatsu, T., Wang, R.-M., Nakagawa, A., and Tsuchida, E. (2006) Poly(ethylene glycol)-conjugated human serum albumin including iron porphyrins: surface modification improves the O_2-transporting ability. *Bioconjugate Chem. 17*, 393–398.

(165) Cabrales P., Tsai, A. G., Intaglietta, M. (2004) Microvascular pressure and functional capillary density in extreme hemodilution with low- and high-viscosity dextran and a low-viscosity Hb-based O_2 carrier. *Am. J. Physiol. Heart Circ. Physiol. 287*, H363−H373.

(166) Martini, J., Cabrales, P., K, A., Acharya, S. A., Intaglietta, M., Tsai, A. G. (2008) Survival time in severe hemorrhagic shock after perioperative hemodilution is longer with PEG-conjugated human serum albumin than HES 130/0.4: a microvasucular perspective. *Crit. Care 12*, R54.

(167) Sugawara, Y., and Shikama, K. (1980) Autoxidation of native oxymyoglobin. *Eur. J. Biochem. 110*, 241–246.

(168) Tsuchida, E., Komatsu, T., Hamamatsu, K., Matsukawa, Y., Tajima, A., Yoshizu, A., Izumi, Y., and Kobayashi, K. (2000) Exchange transfusion with 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.

(169) Huang, Y., Komatsu, T., Yamamoto, H., Horinouchi, H., Kobayashi, K., and Tsuchida, E. (2006) PEGylated albumin-heme as an oxygen-carrying plasma expander: exchange transfusion into acute anemia rat model. *Biomaterials 27*, 4477–4483.

(170) 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.

(171) Nakagawa, A., Komatsu, T., Huang, H., Lu, G., and Tsuchida, E. (2007) O_2-binding albumin thin films: solid membranes of poly(ethylene glycol)-conjugated human serum albumin incorporating iron porphyrin. *Bioconjugate Chem. 18*, 1673–1677.

(172) Goa, K. L., and Benfield, P. (1994) Hyaluronic-acid - a review of its pharmacology and use as a surgical and in ophthalmology, and its therapeutic potential in joint disease and wound-healing. *Drugs 47*, 536–566.

(173) Adams, P. A., and Berman, M. C. (1980) Kinetics and mechanism of the interaction between human serum albumin and monomeric haemin. *Biochem. J. 191*, 95–102.

(174) Zunszain, P. A., Ghuman, J., Komatsu, T., Tsuchida, E., and Curry, S. (2003) Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. *BMC Struct. Biol. 3*, 6.

(175) Wardell, M., Wang, Z., Ho, J. X., Robert, J., Ruker, F., Ruble, J., and Carter, D. C. (2002) The atomic structure of human methemalbumin at 1.9 Å. *Biochem. Biophys. Res. Commun. 291*, 813–819.

(176) Komatsu, T., Ohmichi, N., Zunszain, P. A., Curry, S., and Tsuchida, E. (2004) Dioxygenation of human serum albumin having a prosthetic-heme group in a tailor-made heme pocket. *J. Am. Chem. Soc. 126*, 14304–14305.

(177) Komatsu, T., Ohmichi, N., Nakagawa, A., Zunszain, P. A., Curry, S., and Tsuchida, E. (2005) O_2 and CO binding properties of artificial hemoproteins formed by complexing iron protoporphyrin IX with human serum albumin mutants. *J. Am. Chem. Soc. 127*, 15933–15942.

(178) Vickery, L., Nozawa, T., and Sauer, K. (1976) Magnetic circular dichroism studies of myoglobin complexes. Correlation with-heme spin state and axial ligation. *J. Am. Chem. Soc. 98*, 343–350.

(179) Antonini, E., and Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*, p 18, North-Holland Publishers, Amsterdam.

(180) Rohlfs, R., Mathews, A. J., Carver, T. E., Olson, J. S., Springer, B. A., Egeberg, K. D., and Sligar, S. G. (1990) The effects of amino acid substitution at position E7 (residue 64) on the kinetics of ligand binding to sperm whale myoglobin. *J. Biol. Chem. 265*, 3168–3176.

(181) Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970) Studies of the function of abnormal hemoglobins I. An improved method for automatic measurement of the oxygen equilibrium curve of hemoglobin. *Biochim. Biphys. Acta 200*, 189–197.

(182) Vandegriff, K. D., Young, M. A., Lohman, J., Bellelli, A., Samaja, M., Malavalli, A., and Winslow, R. M. (2008) CO-MP4, a polyethylene glycol-conjugated haemoglobin derivative and carbon monoxide carrier that reduces myocardial infarct size in rats. *Br. J. Pharmacol. 154*, 1649–1561.

(183) Creighton, T. E. (1992) *Proteins: Structures and Molecular Properties*, p 242, W. H. Freeman and Co., New York.

(184) Chu, M. M. L., Castro, C. E., and Hathaway, G. M. (1978) Oxidation of low-spin iron(II) porphyrins by molecular oxygen. An outer sphere mechanism. *Biochemistry 17*, 481–486.

(185) Tsuchida, E., Nishide, H., Sato, Y., and Kaneda, M. (1982) The preparation of protoheme mono-N-[5-(2-methyl-1-imidazolyl)pentyl]amide and its oxygenation. *Bull. Chem. Soc. Jpn. 55*, 1890–1895.

(186) Uno, T., Sakamoto, R., and Tomisugi, Y. (2003) Inversion of axial coordination in myoglobin to create a "proximal" ligand binding pocket. *Biochemistry 42*, 10191–10199.

(187) Springer, B. A., Sligar, S. G., Olson, J. S., and Philips, G. N. (1994) Mechanisms of a ligand recognition in myoglobin. *Chem. Rev. 94*, 699–714.

(188) Komatsu, T., Nakagawa, A., Zunszain, P. A., Curry, S., and Tsuchida, E. (2007) Genetic engineering of the heme pocket in human serum albumin: modulation of O_2 binding of iron protoporphyrin IX by variation of distal amino acids. *J. Am. Chem. Soc. 129*, 11286–11295.

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Artificial Organs

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Review of Hemoglobin-Vesicles as Artificial Oxygen Carriers

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Abstract: Blood transfusion systems have greatly benefited human health and welfare. Nevertheless, some problems remain: infection, blood type mismatching, immunological response, short shelf life, and screening test costs. Blood substitutes have been under development for decades to overcome such problems. Plasma component substitutes have already been established: plasma expanders, electrolytes, and recombinant coagulant factors. Herein, we focus on the development of red blood cell (RBC) substitutes. Side effects hindered early development of cell-free hemoglobin (Hb)-based oxygen carriers (HBOCs) and underscored the physiological importance of the cellular structure of RBCs. Well-designed artificial oxygen carriers that meet requisite criteria are expected to be realized eventually. Encapsulation of Hb is one idea to shield the toxicities of molecular Hbs. However, intrinsic issues of encapsulated Hbs must be resolved: difficulties related to regulating the molecular assembly, and management of its physicochemical and biochemical properties. Hb-vesicles (HbV) are a cellular type of HBOC that overcome these issues. The in vivo safety and efficacy of HbV have been studied extensively. The results illustrate the potential of HbV as a transfusion alternative and promise its use for other clinical applications that remain unattainable using RBC transfusion. **Key Words:** Blood substitutes—Hemoglobin—Liposome—Transfusion—Perfusion.

PROBLEMS OF TRANSFUSION SYSTEM AND EXPECTATIONS FOR ARTIFICIAL O_2 CARRIERS

Allogeneic blood transfusion was developed early in the last century as a routine clinical practice; it has contributed immensely to human health and welfare. Infectious diseases such as hepatitis and HIV are social problems associated with transfusion, but strict virus testing using the nucleic acid amplification test (NAT) is effective to reduce the risk of infection. Even so, NAT poses problems such as detection limits during the window period and limited species of viruses for testing. The continuing emergence of new viruses and a new type of pathogen, the prion, both threaten humans. In Japan, the storage period of donated red blood cells (RBCs) is limited to 3 weeks. Platelets can be preserved for only a few days. Immunological responses (such as anaphylaxis and graft vs. host disease) and contingencies of blood type incompatibility further limit the usefulness of blood products. To obviate or minimize homologous transfusion, the transfusion trigger has been reconsidered, and roughly reduced to 6 g/dL. Bloodless surgery and preoperational enhancement of erythropoiesis for storing autologous blood have become common. However, these treatments are not always practical for all patients. Some developed countries with aging populations must confront a decreasing number of young donors and an increasing number of aged recipients. On the other hand, in some developing countries, establishment of a safe blood donation system is difficult. Under such circumstances, research to develop blood substitutes has gathered great attention and has continued to develop

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worldwide. In Japan, for example, the government has given strong support to a spectrum of projects for development of blood substitutes in the wake of two tragedies: the infection, with AIDS, of hemophiliac patients who had received nonpasteurized plasma products; and the Great Hanshin Earthquake disaster.

Among blood components, some substitutes for plasma fractions are established, such as electrolyte solutions, plasma expanders, and recombinant coagulation factors. Especially, the commercialization of recombinant human serum albumin (rHSA) was finally approved in Japan in 2008. On the other hand, substitutes for cellular components—platelets and RBCs—are challenging. This review of the literature specifically examines hemoglobin (Hb)-based oxygen carriers (HBOCs). The optimal molecular structures and the physicochemical properties of HBOCs are selected based on the sciences of molecular assemblies of biopolymers, bio-conjugation, metal complexes, and nanotechnology, along with important results of physiological, and pharmacological studies (1,2).

THE CONCEPT OF CELLULAR HB-BASED O_2 CARRIERS AND THEIR DEVELOPMENT

Historically, the first attempt to use an Hb-based O_2 carrier was made in the 1930s. Stroma-free Hb was used because Hb in RBCs binds and releases O_2. However, several problems became apparent: impurity of stroma-free Hb, dissociation into dimers, a short circulation time, renal toxicity, high oncotic pressure, and high O_2 affinity. Since the 1970s, various approaches have been developed to overcome these problems, especially in the USA. In some cases, chemically modified Hbs (intramolecularly crosslinked Hb, recombinant Hb, polymerized Hb that were contaminated with nonpolymerized Hb) caused side effects such as vasoconstriction and abnormal esophageal function (3). Those effects are presumably attributable to the specific affinity of Hb to endogenous gas molecules, NO and CO, which are important messenger molecules for vasorelaxation (4). Although many companies have developed chemically modified Hb solutions as transfusion alternatives for elective surgery and trauma, some have suspended clinical trials because of vasoactive properties. The fact that myocardial lesions are caused by intramolecular crosslinked and polymerized Hbs has deterred further development of these HBOCs (5–7). The side effects of molecular Hbs described above imply the importance of the cellular structure and the larger particle dimension of HBOCs.

For those reasons, the optimal structure of HBOCs might be to mimic the RBC cellular structure. Pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed by Chang in 1957, who prepared microcapsules (5 μm) made of nylon, collodion, and other materials. In Japan, Toyoda and the Kambara-Kimoto group investigated encapsulation of Hbs with gelatin, gum arabic, silicone, etc. in the 1960s. Nevertheless, results underscored the extreme difficulty in regulating the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility simultaneously. After discovery of liposomes in 1964, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. Hunt et al. prepared neohemocytes (7). The US Naval Research Laboratories showed remarkable progress in the use of LEH (8). Terumo Corp. (Tokyo) was supported by the New Energy and Industrial Technology Development Organization (2003–2005) for industrialization in its development of different LEH, so-called Neo Red Cells or TRM-645 (9,10) (Table 1).

In fact, Hb encapsulation is expected to shield toxic effects of molecular Hbs. However, the intrinsic issues of encapsulated Hbs have emerged that are related to the nature of molecular assembly and particle dispersion, such as particle size distribution, protein purity, high Hb content, blood compatibility, and stability for long-term storage, and prompt degradation (2,9). To overcome such difficulties, the Waseda–Keio group has extensively studied the physicochemical properties, safety, and efficacy of Hb-vesicles (HbV) that mimic the cellular structure of RBC with the support of Japan's Ministry of Health, Labour and Welfare (1997–2008) (2) (Fig. 1, Tables 1 and 2). The HbV characteristics are as follows: (i) human Hb is purified via pasteurization at 60°C and ultrafiltration to eliminate pathogens; (ii) a concentrated Hb solution, nearly 35–40 g/dL, is encapsulated within a thin bilayer membrane; the Hb concentration of the resulting HbV suspension is 10 g/dL; (iii) a new synthetic lipid is used to prevent platelet activation and complement activation (11). (iv) Subsequently, polyethylene glycol-modification guarantees long-term storage of more than 2 years at room temperature (12), with blood compatibility, and extended circulation half-life. (v) The resultant cellular structure shields all side effects of molecular Hb (13). (vi) The particle size (250 nm) is appropriate for homogeneous distribution in the plasma phase to flow through narrower vessels, where large RBCs

TABLE 1. *Representative liposome-encapsulated Hbs (LEH) studied extensively for future commercialization*

Product Name	Group	Characteristics	References	Current status
Hb-vesicles (HbV)	Waseda Univ. & Keio Univ.	1. Pasteurization of HBOC at 60°C for virus inactivation, and high purity and concentration of encapsulated Hb (35–40 g/dL) 2. Lipid composition to improve blood compatibility 3. PEG modification and deoxygenation for 2 years storage 4. [Hb] = 10 g/dL (and others, see Table 2)	(2, 4, 11, 12)	Preclinical
Neo Red Cells (NRC) TRM-645	Terumo Corp.	1. Inositol hexaphosphate to regulate P_{50} (= 51.4 torr) 2. Lipids: soybean hydrogenated phosphatidylcholine / cholesterol / stearic acid / PEG-DSPE 3. Storage in a refrigerator for 6 months 4. Preservation of enzymes for metHb reduction 5. [Hb] = 6.2 g/dL	(9, 10)	Preclinical
Artificial Red Cells (ARC)	NOF Corp. & Waseda Univ.	1. Polymerized lipids (DODPC) for stabilization 2. Storage in powdered or frozen state 3. Difficulty in degradation in RES	See Refs. in (1, 2)	Suspended
Liposome-encapsulated Hb (LEH)	US Naval Research Laboratory	1. Freeze-dried powder with trehalose 2. Low Hb encapsulation efficiency 3. Thrombocytopenia, complement activation	(8)	Suspended
Neohemocyte (NHC)	Univ. of California, San Francisco	1. Wide particle distribution (0.1–1.5 μm) 2. Lipids: egg yolk phosphatidylcholine / dipalmitoyl-phosphatidic acid / cholesterol / alpha tocopherol 3. [Hb] = 15.1 g/dL 4. P_{50} = 24 torr	(7)	Suspended
Synthetic Erythrocytes (SE)	Rush–Presbyterian–St. Luke's Medical Center, Univ. Illinois	1. First attempt of LEH	See Refs. in (1, 2)	Suspended

cannot flow. (vii) Finally, HbV are captured by the reticuloendothelial system and are degraded promptly without decomposition (hemolysis) during blood circulation. Phospholipid vesicles for encapsulation of Hb are expected to be beneficial for heme detoxification through their preferential delivery to the reticuloendothelial system, a physiological compartment for degradation of senescent RBCs, even at

FIG. 1. (Left) Schematic representation of HbV. One particle contains about 30 000 Hb molecules. The surface of one particle is modified using polyethylene glycol (PEG) chains, which ensures the dispersion stability of HbV during storage and during circulation in the bloodstream. The average particle diameter is about 250 nm. (Middle) The packed HbV suspension appears turbid, like a mixture of milk and red wine, because of light-scattering of the particle suspension. (Right) Reoxygenation of HbV by a membrane oxygenator (artificial lung), clearly displaying the change of color from purple to red. HbV would be useful as a priming fluid for extracorporeal membrane oxygenators and for perfusion of organs for transplantation.

TABLE 2. *Characteristics of Hb-vesicles (HbV) developed at Waseda University*

Parameter	
Particle diameter	250–280 nm
P_{50}*	25–28 torr
Hb concentration	10 g/dL
Suspending medium	Physiologic saline solution (0.9% NaCl)
MetHb	<3%
Rate of MetHb formation† (at 37°C, Po_2 = 40 torr)	4%/h
Colloid osmotic pressure	0 torr
Intracellular Hb concentration	ca. 35–40 g/dL
Lipid composition‡	DPPC / cholesterol / DHSG / DSPE-PEG_{5000}
Weight ratio of Hb to lipids	1.6–1.9 (w/w)
Stability for storage at room temperature	2 years
Circulation half-life	32 h (rats)

* P_{50} is regulated with co-encapsulated pyridoxal 5′-phosphate.
† The rate of autoxidation is dependent on Po_2, and accelerated at a reduced Po_2. Because of the pasteurization of Hb solution (60°C) to obtain purified Hb and guarantee the utmost safety from infection, the resulting HbV contains no enzymes. Therefore, autoxidation of Hb is unavoidable. Recently, it was clarified that co-encapsulation of L-tyrosine significantly retards autoxidation because of the catalase-like activity provided by metHb and L-tyrosine, which eliminates H_2O_2, a by-product of Hb autoxidation.
‡ DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexhadecyl-*N*-succinyl-L-glutamate.

doses greater than putative clinical doses (2). Results of animal tests have clarified the efficacy of HbV as a transfusion alternative and their possible use for other clinical applications. The results of ongoing HbV research make us confident in advancing further development of HbV, with the expectation of its eventual clinical application.

HB-VESICLES AS A TRANSFUSION ALTERNATIVE

Advantages of the HBOCs are the absence of blood-type antigens and infectious viruses, and their stability for long-term storage: those advantages overwhelm those of present RBC transfusion. The shorter half lives of HBOCs in the bloodstream (2–3 days) limit their use, but they are applicable in shorter periods for use as (i) a resuscitative fluid for hemorrhagic shock in emergency situations for temporary benefits or bridging until packed RBCs are available, or (ii) as a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion, or (iii) as a priming solution for the circuit of an extacorporeal membrane oxygenator.

One particle of HbV (~250 nm diameter) contains about 30 000 Hb molecules. Because HbV acts as a particle in the blood, not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It necessitates the addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates for use as plasma expanders are HSA, hydroxyethyl starch, dextran, and gelatin, depending on the clinical setting, cost, country, and clinician. rHSA is becoming an alternative in Japan. The absence of any infectious disease from humans is the greatest advantage of rHSA. Moreover, it presents none of the immunological and hematological abnormalities that are often associated with using dextran. Aiming at application of HbV suspended in a plasma expander to the above indications, HbV was tested in rodent models for resuscitation from hemorrhagic shock and extreme hemodilution. Physiological and pharmacological studies of HbV have clarified that HbV can maintain the fundamental parameters of respiration, hemodynamics, and tissue oxygenation that are comparable to results of RBC transfusion. Realization of an HBOC will contribute considerably to the renovation of present clinical practices, especially in emergency medicine and surgery, where the HBOC can be injected instantaneously without concern of blood type mismatch, infection, or other blood-borne disease.

Hb-vesicles used as a perioperative infusion fluid or as a priming fluid for extracorporeal circuit

Extensive in vivo studies of HbV suspended in plasma-derived HSA or rHSA revealed sufficient O_2 transporting efficiency that is apparently comparable to RBCs in extreme blood exchange experiments with up to 90% blood exchange (14). All systemic parameters and tissue oxygen tensions were well preserved, although the blood exchange with HSA showed considerable hypotension, acidosis, and death within 30 min after completion of blood exchange. As confirmed in rat models after 40% blood exchange with HbV in clinically relevant conditions, hematopoietic activity was preserved and the decreased hematocrit reverted to its original level within 1–2 weeks, whereas HbV captured in RES disappeared completely (15), indicating that HbV is useful for preoperative blood exchange or perioperative infusion in the event of hemorrhage to prevent or minimize homologous blood transfusion.

Although miniaturization of the cardiopulmonary bypass (CPB) circuit has reduced the priming volume, it remains insufficiently low to achieve an acceptable level of hemodilution in small patients.

Homologous blood use is considered the gold standard for CPB priming in infants despite exposure of patients to potential cellular and humoral antigens. A recent experimental study of HbV suspended in rHSA as a priming solution for CPB in a rat model demonstrated that HbV protects neurocognitive function by transporting O_2 to brain tissues, even when the hematocrit is markedly reduced (16). The results indicate that the use of HbV for CPB priming might prevent neurocognitive decline in infants caused by considerable hemodilution.

Hb-vesicles as a resuscitative fluid for hemorrhagic shock

The first attempt using an HbV suspension to restore systemic conditions after hemorrhagic shock was conducted with anesthetized Wistar rats (17). Shock was induced by 50% blood withdrawal. The rats exhibited hypotension and considerable metabolic acidosis and hyperventilation. After 15 min, they received isovolemic HbV suspended in rHSA (HbV/rHSA, [Hb] = 8.6 g/dL), shed autologous blood (SAB), or rHSA alone. The HbV/rHSA group restored mean arterial pressure, similarly to the SAB group, which was significantly higher than the rHSA group. No remarkable difference was observed in the blood gas variables between the resuscitated groups. However, two of eight rats in the rHSA group died before 6 h.

After removing the catheters and awakening, the rats were housed in cages for up to 14 days. The HbV/rHSA group gained body weight; the reduced hematocrit reverted to the original level in 7 days, indicating elevated hematopoiesis. Both groups showed AST and ALT elevation at 1 day because of systemic ischemia reperfusion injury. Splenomegaly was significant in the HbV/rHSA group at 3 days because of HbV accumulation, but it had subsided by 14 days. Histopathological observation revealed that a substantial amount of HbV accumulated in the spleen macrophages, although it had completely disappeared by 14 days. In conclusion, HbV showed a sufficient resuscitative effect that was comparable to that achieved with transfusion. The injected HbV were phagocytized in the reticuloendothelial system by 14 days. The elevated hematopoietic activity caused the complete recovery of hematocrit by 7 days.

These results reflect that HbV is useful as a resuscitative fluid for hemorrhagic shock. Its performance is comparable to that of SAB. We have progressed to similar experiments using beagles. Those results confirm the long-term survival of more than one year after resuscitation with HbV.

OTHER POTENTIAL CLINICAL APPLICATIONS OF HB-VESICLES

One advantage of HbV is that the physicochemical properties of HbV are adjustable, such as oxygen affinity (P_{50}, oxygen partial pressure at which Hb is half saturated with oxygen) and rheological properties. Historically, it has been widely believed that the O_2 affinity should be regulated similarly to RBC, at about 25–30 torr, using an allosteric effector or by a direct chemical modification of the Hb molecules. Theoretically, this enables sufficient O_2 unloading during blood microcirculation, as can be inferred from the arterio-venous difference in the levels of O_2 saturation in accordance to an O_2 equilibrium curve. It has been expected that decreasing the O_2 affinity (increasing P_{50}) increases O_2 unloading. Regarding the blood viscosity, lowered viscosity is believed to increase cardiac output and facilitate peripheral blood flow. However, these beliefs are being reviewed and revised in the field of blood substitute research. The suspension of HbV can provide unique opportunities to modify these physicochemical properties easily and to observe their physiological impacts.

Oxygenation of ischemic tissues using HbV

In ischemic tissues, blood flow is extremely reduced. As a result, O_2 tension is very low, for example, 5 torr. Normal RBCs are expected to have already released O_2 before they reach the ischemic tissue. The left-shifted curve (lower P_{50}) indicates that Hb does not release O_2, even in the venous side in a normal condition. However, RBC or an HBOC with a P_{50} lower than usual can carry O_2 to an ischemic tissue (18). Dr. Erni and colleagues at Inselspital Hospital of the University of Berne developed a hamster skin flap model in which the blood flow of one branch is blocked completely; the tissue becomes completely ischemic. Exchange transfusion was performed using low and high P_{50}-HbV, which revealed improved oxygenation of the ischemic part, especially with the low P_{50}-HbV (19,20). Collateral blood flow is expected to occur even to the ischemic part and the HbV conveys O_2 to the ischemic part via the collateral arteries. This is the first example demonstrating the effectiveness of HbV for an ischemic tissue, implying its applicability for other ischemic diseases. In addition to the lower P_{50}, the viscosity of the HbV suspension is expected to contribute to improvement of microcirculation. The combination of HbV and dextran solution or hydroxyethyl starch solution induces flocculation of HbV, thereby rendering the suspension non-Newtonian and viscous (21).

The higher viscosity of the circulation fluid would increase shear stress on the vascular wall, thereby inducing vasorelaxation. A viscous fluid also homogeneously pressurizes the capillaries to improve the functional capillary density. Recent studies have demonstrated the effectiveness of HBOCs with a lower P_{50} (higher O_2 affinity) as a means of implementing O_2 delivery targeted to ischemic tissues (22). Our experimental data support those earlier observations and ensure the possible utilization of HBOCs for remedying ischemic conditions (23).

Oxygenation of organs for transplantation and regenerated tissues in the future

Even though the organ transplant law went into effect in 1997 in Japan, organ transplants are expected to occur far less frequently than in the USA or Europe, probably because of the ethical and religious controversy related to the criteria for diagnosis of brain death. However, it is expected to be accepted eventually in Japan with the prevailing view of the need for transplantation and carrying an organ donor card. In a clinical setting of transplantation, its success is dependent in part on the prevention of ischemia-reperfusion injury after transplantation by an improved preservation condition. The representative organ preservation fluid is the University of Wisconsin (UW) solution, which comprises not only crystalloids, but also a plasma expander. The two-layer method is to dip the dissected organ at the interface of the UW solution and a perfluorocarbon (PFC) solution. Oxygen diffuses from PFC to the organ, and the transport of nutrients and metabolites takes place through contact with the UW solution. It is speculated that there should be a limitation of the distance for diffusion of oxygen, carbon dioxide, and small molecules to keep the tissues alive. One idea is to use HbV as an intra-arterial perfusion fluid to carry oxygen, nutrients, and metabolites. Actually, we tested perfusion of the liver, heart, and intestine with HbV (24,25). We confirmed the preservation of organ functions for a few hours. Our next step will be to prolong the perfusion period to the greatest extent possible. In fact, HbV can be reoxygenated easily by perfusion through an oxygenating "artificial lung" (Fig. 1). We must design the composition of HbV suspension to provide not only oxygen but also nutrients and homogeneous fluid distribution to all capillaries, which would presumably require a certain level of viscosity.

Tissue reconstruction and tissue regeneration have become popular since Vacanti et al. reported the formation of an auricular-shaped cartilage on the back of a mouse by cell culturing onto a polymer scaffold. In Japan, an innovation occurred with the discovery of thermoresponsive polymer gel, poly-(isopropylacrylamide), for cell culturing by Okano et al. This material is hydrophobic at 37°C during cell culturing in a Petri dish; it becomes hydrophilic at 32°C. Moreover, it can be removed easily from the cells to provide a cell sheet. The resultant two-dimensional cell sheet is applicable to many clinical indications. Cell culture requires not only a supply of oxygen and nutrition but also the removal of metabolites, which can be achieved by replacing the culturing media periodically in the case of a two-dimensional cell culturing. However, in the case of constructing a three-dimensional bulky tissue, it would require perfusion with a fluid that can serve the functions of blood in addition to angiogenesis in a regenerating tissue on a scaffold. Such functionality would necessitate the design of the composition of HbV described above. Consequently, HbV can provide unique opportunities to manipulate physicochemical properties that cannot be provided by RBCs.

CONCLUSION

The circulation half-life of HBOCs including HbV after intravenous administration is much shorter than that of RBC. The total amount of the membrane component of HbV is 2–3 times more than that of RBCs. HbV degraded promptly and completely in the reticuloendothelial system. However, a massive dosage of HbV transiently induces splenohepatomagaly, reduces phagocytosis, and modifies immunological responses (2). On the other hand, HBOCs are superior to conventional transfusion in terms of their absence of blood type and pathogens, and their long storage period. The outstanding difference between HbV and other HBOCs such as chemically modified Hb solutions is that the physicochemical properties of HbV, not only P_{50}, but also rheological properties, are adjustable. Accordingly, the use of HbV provides unique opportunities for versatile therapeutic approaches that cannot be attained using a conventional transfusion system.

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REFERENCES

1. Sakai H, Tsuchida E. Blood substitutes. In: Akay M, ed. *Wiley Encyclopedia of Biomedical Engineering*. Hoboken, NJ: John Wiley & Sons, Inc, 2006;613–21.
2. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. *J Intern Med* 2008;263:4–15.
3. de Figueiredo LF, Mathru M, Solanki D, Macdonald VW, Hess J, Kramer GC. Pulmonary hypertension and systemic vasoconstriction may offset the benefits of acellular hemoglobin blood substitutes. *J Trauma* 1997;42:847–54.
4. Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E. Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J Biol Chem* 2008;283:1508–17.
5. Burhop K, Gordon D, Estep T. Review of hemoglobin-induced myocardial lesions. *Artif Cells Blood Substit Immobil Biotechnol* 2004;32:353–74.
6. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA* 2008;299:2304–12.
7. Hunt CA, Burnette RR, MacGregor RD, et al. Synthesis and evaluation of a prototypal artificial red cell. *Science* 1985; 230:1165–8.
8. Rudolph AS, Klipper RW, Goins B, Phillips WT. In vivo biodistribution of a radiolabeled blood substitute: 99mTc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. *Proc Natl Acad Sci USA* 1991;88:10976–80.
9. Pape A, Kertscho H, Meier J, et al. Improved short-term survival with polyethylene glycol modified hemoglobin liposomes in critical normovolemic anemia. *Intensive Care Med* 2008;34: 1534–43.
10. Kawaguchi AT, Fukumoto D, Haida M, Ogata Y, Yamano M, Tsukada H. Liposome-encapsulated hemoglobin reduces the size of cerebral infarction in the rat: evaluation with photochemically induced thrombosis of the middle cerebral artery. *Stroke* 2007;38:1626–32.
11. Abe H, Azuma H, Yamaguchi M, et al. Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. *Artif Cells Blood Substit Immobil Biotechnol* 2007;35:157–72.
12. Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. Poly-(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug Chem* 2000;11:425–32.
13. Sakai H, Hara H, Yuasa M, et al. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* 2000;279:H908–15.
14. Izumi Y, Sakai H, Kose T, et al. Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J* 1997;43:289–97.
15. Sakai H, Horinouchi H, Yamamoto M, et al. Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. *Transfusion* 2006;46:339–47.
16. Yamazaki M, Aeba R, Yozu R, Kobayashi K. Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. *Circulation* 2006;114:1220–5.
17. Sakai H, Horinouchi H, Masada Y, et al. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 2004;32:539–45.
18. Linberg R, Conover CD, Shum KL, Shorr RG. Increased tissue oxygenation and enhanced radiation sensitivity of solid tumors in rodents following polyethylene glycol conjugated bovine hemoglobin administration. *In Vivo* 1998;12:167–73.
19. Contaldo C, Plock J, Sakai H, et al. New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue. *Crit Care Med* 2005; 33:806–12.
20. Plock JA, Tromp AE, Contaldo C, et al. Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue. *Crit Care Med* 2007;35:899–905.
21. Sakai H, Sato A, Takeoka S, Tsuchida E. Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. *Langmuir* 2007;23:8121–8.
22. Tsai AG, Vandegriff KD, Intaglietta M, Winslow RM. Targeted O_2 delivery by low-P_{50} hemoglobin: a new basis for O_2 therapeutics. *Am J Physiol Heart Circ Physiol* 2003;285: H1411–9.
23. Komatsu H, Furuya T, Sato N, et al. Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. *Neurosci Lett* 2007;421:121–5.
24. Verdu EF, Bercik P, Huang XX, et al. The role of luminal factors in the recovery of gastric function and behavioral changes after chronic Helicobacter pylori infection. *Am J Physiol Gastrointest Liver Physiol* 2008;295:G664–70.
25. Yamagishi T, Bessho M, Hama M, et al. Hemoglobin-vesicle aids recovery of cardiac function during ischemia-reperfusion in Langendorff perfused rat hearts. *Artif Blood* 2006;14:92–7.

海外文献紹介

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ヘモグロビン内包リポソームによる脳への酸素供給：出血性ショックラットモデルでのPETイメージングによる評価

Cerebral Oxygen Delivery by Liposome-encapsulated Hemoglobin: A Positron-emission Tomographic Evaluation in a Rat Model of Hemorrhagic Shock

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訳者のコメント

ヘモグロビン（Hb）をリン脂質小胞体に内包した酸素運搬体は，赤血球と同様の細胞型構造を有している．主に米国で開発が進められている非細胞型の修飾Hbの副作用が明らかになるにつれ，細胞型構造の重要性が強調されるようになってきた．Hbをリン脂質小胞体に内包する試みは，synthetic erythrocytesとして1977年に報告される[1]．1980～90年代にかけて米国海軍研究所（Naval Research Laboratory）が赤血球代替物として利用するための開発研究を進めた．Naval Research Laboratoryで調製されていた製剤はLiposome-encapsulated hemoglobin（LEH）と呼ばれ，米国のいくつかの研究グループで安全性や有効性が評価されている．ここで紹介する文献の著者，W.T. Phillips（Dept. of Radiology, University of Texas Health Science Center, San Antonio）は核医学を専門とする臨床医であり，1988年頃よりNaval Research LaboratoryでLEHの開発を進めていたA.S. Rudolphとの共同研究を開始している．著者らが担当したのは放射化標識したLEH投与後の血中滞留時間と体内分布の計測であり，メトHbの還元剤としてHbと共に内包しているグルタチオンをテクネチウム-99mと複合化させることで，LEHを安定に放射化標識できることを見出している[2]．LEHの膜成分はdistearoyl phosphatidylcholine, cholesterol, dimyristoyl phosphatidylglycerol, α-tocopherol（50:38:10:2, モル比）の4種で構成されており，粒子径300～700nm程度の小胞体にbovine Hbや架橋Hbを内包したものが報告されている．当時のLEHの血中半減期は20時間以内（25% top-load in rabbits）であり[2]，また投与後の補体活性化などの安全性の問題が指摘されていた[3,4]．実際，投与中の心停止が頻発するため様子を見ながら低速での投与が必要であったと聞いている．この問題の原因として，負電荷成分としてdimyristoyl phosphatidylglycerolを使用していたことが挙げられる[5]．

著者らは，補体活性化の回避と体内動態の最適化を目的としてLEHの脂質組成や粒子径の改良を行い，多量のポリエチレングリコール（PEG）（10 mol%）で表面修飾した小粒径の中性小胞体（<200 nm）にHbを内包したLEHを調製し，血中半減期を65時間（25% top-load in rabbits）まで延長することに成功している[6]．しかし，酸素運搬体として利用するにはHb含量が低すぎる点が課題として挙げられている．Hb内包効率は，小胞体の粒子径，PEG修飾量，脂質膜の表面電荷に影響され，粒子径の減少，PEG修飾量の増大，中性小胞体はいずれもHbの内包効率低下の原因となる．血中滞留性の向上を目的に改良されたLEHの粒子径，PEG修飾量，表面電荷はいずれもHbの内包において極めて不利な条件であることがわかる．実際，報告されているスペックから換算すると単位総脂質量あたり内包できるHb量（Hb/Lipids比）は0.1以下であり，Hb含量は1.2g/dLである[6]．一般的に輸血のトリガーとされるHb値が7～8 g/dLであることを考えれば，このLEHを輸血代替物として利用するのが困難であったことは容易に想定できる．現在，Naval Research LaboratoryはLEHの開発を行っていないが，著者らは放射性同元素利用のイメージング技術による酸素運搬体の評価法確立のため，LEHを利用しながら研究を継続している．試料は一貫してLEHと呼ばれているが，酸素運搬体としての機能評価にはHb内包効率を上げる必要があり改良が続けら

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れている．

国内では献血由来の期限切れ赤血球の有効利用という側面もあり，期限切れ赤血球から単離精製したHbをリン脂質小胞体に内包したHb小胞体の開発研究が進められてきた[7]．一酸化炭素の利用によりHbの加熱処理を可能にするなどの技術進展により，超高純度・高濃度のHb溶液（>35g/dL）を得る技術が完成している[8]．著者らが血中滞留性の向上を目的にLEHの最適化を試みたのに対し，Hb小胞体はいかにして多量のHbをリン脂質小胞体に内包するかという理工学の立場から脂質成分や調製条件の詳細検討が重ねられ，併せて医側からフィードバックされる投与試験の結果を反映させながら最適化が図られてきた．このため，Hb小胞体のHb/Lipids比は1.7～1.9まで向上しており[9]，先に示したLEHの17倍以上の効率でHbを内包している．このようにHb小胞体とLEHは，日本と米国で各々の専門性を生かして研究開発が進められてきたが，細胞型酸素運搬体に関する基礎科学の進展は共通の課題であり，2001年より著者らのグループと共同でHb小胞体の血中滞留時間や体内分布の評価を行っている．この頃までには脱酸素化によるHb小胞体の長期保存（2年以上）の技術が完成していたため[10]，調製したHb小胞体を海外に運搬輸送しての評価が効果的に行えるようになっていた．この共同研究により，赤血球代替物として大量に投与した場合，Hb小胞体の血中滞留時間は，血中滞留性の向上を目的として改良されたLEHと同等であることが明らかになっている[11]．この点は投与量の効果もあり，低用量投与ではHb小胞体の血中滞留時間が短縮されるのに対し，LEHは投与量によらず比較的長時間滞留する特徴を有している．

著者らがLEHの酸素輸送機能の評価に酸素の放射性同位体（^{15}O，半減期2分）を利用する研究を最初に報告したのは1997年であり，PET（positron-emission tomography）イメージングへの発展的研究と位置付けていた[12]．本論文でそれが実現した格好となり，PETイメージングによる脳酸素代謝率の定量解析から，酸素運搬体の機能評価の結果が報告される．脳酸素消費量は，成人安静時で全身酸素消費量の約20～25%に相当する．脳酸素代謝率（$CMRO_2$）＝脳血流量（CBF）×動静脈酸素含量較差で求められ，PETでは動静脈酸素含量較差は脳酸素摂取率（OEF）×動脈酸素含量（CaO_2）に相当する．CBFが減少すると，OEFを増大させて$CMRO_2$を維持する．PETでは非襲侵で三次元的な脳血流代謝が定量的に測定され画像化でき臨床応用されているが，実験小動物は観測対象が小さいため，臨床用PETに比べ高い空間解像度と装置感度が技術課題とされていた．最近では実験小動物に対応できるまでに空間解像度と装置感度が向上しているので，酸素運搬体による治療法の研究開発，臨床評価に重要な手法になると考えられる．

この報告では，40%の出血性ショックの蘇生液としてヘモグロビン内包リポソーム，脱血液，5%アルブミン，生理食塩水が比較される．PETによる$CMRO_2$の観測結果を比較すると，脳酸素代謝率の改善効果を認めるのは酸素輸送能のあるヘモグロビン内包リポソームと脱血液である．今回試験されたヘモグロビン内包リポソーム（Hb含量; 7.5g/dL）では，ラット血液（Hb含量; 15 g/dL程度）と比較して脳への酸素供給能は劣るものの，Hb含量が血液の半分程度であることを考慮すれば妥当な結果といえる．この結果は，脳への酸素供給において，酸素運搬体のHb含量が重要な意味を持つことを示唆する．LEHにはPEG修飾の方法などに改良が加えられており，論文中に記載のスペックから換算するとHb/Lipids比は0.9程度まで向上していると見積もられるが，血液と対等に評価するにはさらにHb含量を向上させる必要がある．また，酸素親和度（P_{50}）と脳への酸素供給能の関連も極めて興味深い点である．最近の研究から，組織の酸素化にHb小胞体のP_{50}が影響することは示されているが，赤血球より低いP_{50}が効果的な組織もあれば，必ずしもそうでない組織もあるようである[13-15]．多量の酸素を消費する脳への酸素供給におけるP_{50}の効果は，酸素運搬体の開発において重要な指針の一つになると考えられる．細胞型酸素運搬体ではアロステリック因子の内包量によりP_{50}を任意に制御できる利点を有しているため，今後の研究の進展が期待される．

また，コントロールとして設けた5%アルブミン投与群では血液希釈により$CMRO_2$が低下する結果が得られている．従来，血漿増量剤による循環動態の改善は酸素供給に有利に作用する結果も報告されている．一見矛盾するようにも見えるが，この相違は出血性ショックにおける循環動態の臓器特異性を反映するのかもしれない．ショックにおいてもある程度循環動態が維持され，酸素消費量の多い臓器を観測対象とした場合，酸素輸送能を持たない血漿増量剤の投与は血液希釈による酸素供給の低下に作用し，他方，酸素消費量の少ない末端臓器を観測対象とした場合には末梢循環を回復させることが酸素供給に重要なのかもしれない．本論文では，ショック時における脳血流のデータがないため解釈が困難な部分もある．現状，全身的な酸素供給には酸素運搬能と膠質浸透圧が重要であることは確かなようである．詳細なデータは原文を一読されたい．

概　要

Liposome-encapsulated hemoglobin（LEH）はヘモグロビン（Hb）を人工脂質膜で被覆した低毒性のHb由来酸素運搬体（HBOC）として開発されている．酸素運搬体の一般的な評価法は，ショック動物モデルでの生理的代用指標に基づき，HBOCによる実質的な酸素運搬による組織レベルでの酸素代謝改善の評価は技術的に困難であった．本報では，40%の循環血液量減少性ショックのラットモデルでのLEHの^{15}O-陽電子放射断層撮影（^{15}O-PET）評価による所見を報告する．*In vitro*試験において，7.5%Hbと中性脂質（distearoylphosphatidylcholine: cholesterol:α-tocopherol, 51.4:46.4:2.2, モル比）で構成されるPEG修飾LEH製剤が効率的に^{15}O標識酸素ガスを結合することを確認した．最終的にLEHを5%ヒト血清アルブミン（HSA）に分散させて膠質浸透圧を調節した．^{15}O標識酸素ガスを吸入した麻酔ラット脳のPET画像は，LEH製剤の効率的な酸素輸送と供給を示した．PET画像から，ラット脳でLEHの酸素運搬能の直接的な指標として酸素輸送と代謝と同様に脳酸素代謝率（$CMRO_2$）を測定した．対照輸液群（生理食塩水と5%のHSA）

と比較して，LEH投与群ではベースラインの80%程度まで有意に$CMRO_2$を改善した．一方，生理食塩水とHSAによる蘇生では，循環血液量減少による$CMRO_2$低下の改善を認めなかった．脱血液による蘇生は酸素代謝の回復に最も効率的だった．これらの結果は，酸素運搬体と血液代替物の評価に^{15}O-PET技術を適応でき，LEHによる蘇生が出血時の脳組織への酸素供給量を増大して，脳での酸素代謝を改善することを示唆する．

緒　言

出血性ショック蘇生の治療目的は，組織への灌流および酸素供給の維持である．晶質液や膠質液を投与する通常の治療法は出血性ショックでの電解質異常と循環量を補正するが，いずれも血流中の酸素運搬能増大には寄与しない．これらの輸液療法では，心負荷の増加により灌流圧を維持することで組織酸素供給を増大させている．一方，ヘモグロビン由来製剤と赤血球は灌流圧の維持に加え酸素運搬能も増大できる輸液である．

酸素運搬体を用いる蘇生術の重要な問題は，循環中の酸素運搬能と灌流組織での酸素利用の関係である．血液の酸素運搬能の増大が低酸素器官での酸素供給と消費の改善に反映されるかどうか明確にはわかっていない．小児の敗血症性ショック患者での臨床事例として，輸血によりヘマトクリットとヘモグロビン濃度を増大させたが酸素消費量の増加を認めなかったとの報告がある．代謝性酸素需要量が酸素供給を上回る状態としてショックを定義するなら，酸素供給と酸素代謝をモニターする意義はきわめて大きい．全身イメージングと従来の血流モニタリングとを組合せることで，出血性ショックでの酸素供給と酸素代謝のモニターができるかもしれない．

本研究では，脳酸素代謝を定量するために，最高水準の小動物用陽電子放射断層撮影（PET）と生理的トレーサーとして酸素の陽電子放出同位元素である酸素-15（^{15}O）を利用した．酸素運搬体としてLiposome-encapsulated Hemoglobin（LEH）を使用し，40%循環血液量減少性ショックモデルラットに投与して，脳酸素消費量の改善能を評価した．また，コントロールの蘇生液として生理食塩水，脱血液，5%ヒト血清アルブミン（HSA）を使用し，LEHの性能と比較した．結果として，^{15}O-PET技術が酸素輸送体や血液代替物の評価に利用でき，この評価法によりLEHによる蘇生が脳組織に酸素を供給して脳酸素代謝を改善することを実証した．

方　法

蘇生液

次の4種類の蘇生液を使用した．(1) Liposome-encapsulated Hemoglobin（LEH）:[Hb]=7.5 g/dL in 5%ヒト血清アルブミン溶液（HSA），(2) 生理食塩水，(3) 5%ヒト血清アルブミン（Albutein, Grifols Biological）（HSA），(4) 脱血液

^{15}O-micro-PET技術によるLEH酸素輸送評価系

先ず，外科的に無処置のラットでLEHの酸素輸送能と脳酸素供給のイメージングに対する^{15}O-micro-PET技術の適応を試験した．サイクロトロン（Scanditronix model MC17F）で^{15}N(p,n)^{15}O核反応により生成した^{15}O標識体を使用した．少量のLEHをトノメータ内で^{15}O標識酸素ガスに5分間暴露して^{15}O標識LEHを得た．Ketamine/xylazine混合液（50 and 10 mg/kg）筋注麻酔下のラットを，Rodent R4 Micro-PET検出器（Concorde Microsystems, Knoxville, TN）に固定されたラットに対し，^{15}O標識LEH LEH（0.5mL）を尾静脈から急速静注投与して4分間のイメージングを実施した．対照として，^{15}O標識ラット赤血球を同様に投与し評価した．

出血性ショック動物モデルとPETイメージング

雄性Sprague-Dawleyラット（体重225-450 g）の左大腿静脈にカテーテルを挿入した．外科的処置によりカテーテルを挿入固定した後2日間の回復を待った．実験当日，ketamine/xylazine混合液（50 and 10 mg/kg）筋注による麻酔を行い，直腸温と動脈圧をデジタルモニターする装置類（iWorx, Dover, NH）を装着した．Micro-PETイメージングのために，麻酔下の動物を保温ジャケットに包み，Micro-PET探知部に固定した．動物を安定化させた後，^{15}O-PETイメージングを開始した（ベースラインスキャン）．特製のnose coneを使用し，ラットに^{15}O標識酸素ガス（5 mL，1.2mCi）を吸入させた．ヘマトクリットと動脈酸素含量を測定するため，血液（0.2mL）を採取した．ベースライン測定終了後，カテーテルより循環血液の40%を0.5mL/分の速度で脱血した．血液量は体重の5.7%として換算された．脱血後30分間安定させた後，二回目の^{15}O-PETと採血を行った（脱血後スキャン）．脱血後スキャンの直後，脱血液と等容量の蘇生液を0.5mL/分で動物に輸注して蘇生させた．動物を安定させてから，^{15}O-PETイメージングと採血を行った（蘇生スキャン）．

Co-Oximeter（Avox Systems, TX）により，採血液から動脈酸素分圧を見積もった．LEHがCo-Oximeterの機能に干渉するので，LEH製剤の循環動態を考慮してLEH投与群のHb含有量を見積もり，実験条件において95%Hb飽和を標準とした（式1）．LEHの血中半減期は30時間であり，投与後30～60分では99%の投与LEHが循環している．LEH投与群では，動脈血の酸素含量の算出にLEHのHb含量（7.5 g/dL）を考慮した．LEHと脱血液のHb含量は一致していない．

$$\text{酸素含量（mL/dL）} = \frac{\text{Hb 飽和度} \times [\text{Hb}] \times 1.34 + 0.31}{100} \quad \text{（式1）}$$

ここで，0.31は大気中での酸素分圧（100mmHg）における血漿への酸素溶解度である．

データ分析

脳酸素代謝率（$CMRO_2$）はOhtaらのコンパートメントモデルによる数学的アルゴリズムで算出された．PETデータの解析は原文の付録AおよびBに詳細に記述されているので参照されたい．

結　果

LEHによる脳組織への^{15}O標識酸素運搬

^{15}O標識LEHを投与したラット脳の^{15}O-micro-PET画像は，脳に比較的強い^{15}O活性を示し，これは^{15}O標識赤血球を投与した時の画像と類似していた．LEHが赤血球と同様に生体内で酸素を運搬し，脳組織に送出していることは，これらの画像から明らかである．酸素ガスを^{15}O標識一酸化炭素（C^{15}O）に置換した場合，赤血球とLEHは強力にC^{15}Oを結合した．静注されたC^{15}O標識赤血球は，一酸化炭素化赤血球の典型的分布を示した．すなわち，赤血球からごくわずかなC^{15}Oしか放出されないため，C^{15}O標識赤血球画像は脳組織に僅かな^{15}O活性しか示さないことを特徴とし，大部分の活性は血管プールの中に残留した．C^{15}O標識赤血球の画像中では，頸動脈が明瞭に可視化された．

LEHによる脳酸素代謝の改善

ラット40%出血モデルで脳酸素代謝の改善におけるLEHの性能をモニターするため，^{15}O-micro-PET によるイメージングを実施した．この間，動物を麻酔下に保ち，バイタルサインを周期的に確認．温水ジャケットで中心体温を37±1°Cに維持した．脱血後のヘマトクリットは脱血量に相当するレベルまで低下し，ベースラインと脱血後のヘマトクリット値において，群間での有意差はなかった．HSAとLEHによる蘇生後，ヘマトクリットは更に低下し，HSAの血漿増量による血液希釈効果を反映しているものと考えられる．同様の効果は生理食塩水で蘇生した群では観察されなかった．生理食塩水が組織液と急速に平衡化することにより，血管体積が減少したことが示唆される．脱血液による蘇生では，ヘマトクリットがベースラインまで回復した．HSA含有輸液（5%HSAとLEH）群と生理食塩水群間のヘマトクリットには，統計学的に有意差を認めた（$P<0.05$）．LEHで蘇生した動物の採血液を遠心分離した後，ヘマトクリット管の血漿層にLEHの存在が確認された．

出血ショックと蘇生における平均動脈圧（MAP）の変化では，脱血開始から失血量に相関してMAPが減少した．生理食塩水による蘇生では，わずかにMAPが改善されたが，5%HSAではベースライン値の80%まで部分的に血圧を回復することが可能だった．一方，5%HSAに分散させたLEHは，安定してベースライン血圧の90%までMAPを改善した．この結果は，血漿増量剤としてのこれらの輸液の性能を反映し，HSAとLEH群間の差はLEHの酸素運搬能に起因すると考えられる．脱血液が試験した輸液の中で最も優れた輸液であることは明らかであった．これらの結果は，急性出血性ショック処置のための血漿増量蘇生液に酸素運搬体を含有することの有用性を示す．酸素輸送能が急性失血に対する生理的フィードバックを緩和するためであろう．

1mCi ^{15}O標識酸素ガス5mLを吸引させたラットのmicro-PET画像では心臓腔と脳組織が観測でき，脳と心臓心室領域について三次元画像解析を行った．心臓での時間分解^{15}O活性は，速やかに上昇して急速な^{15}O活性の分配へと続く特徴を示し，これは典型的なinput曲線である．一方，脳での^{15}O活性は比較的緩やかに短時間の平坦域に達し，その活性は^{15}O標識酸素が血管領域と平衡化される^{15}O標識水に代謝されるにつれて減少した．これらの曲線では，150秒を越えた部分は相当なノイズを認め，心臓でより顕著であった．これは，特に1s/フレームでの再構成では，拍動する心臓では単一の動脈領域の選択が困難なためである．もう一つの理由は，^{15}O活性（物理的崩壊半減期2分）の急速な分配と崩壊である．

Ohtaのモデル（*J Cereb Blood Flow Metab* 12, 559,1994）を基にしたMATLABプログラムにより，時間分解曲線のデータから脳酸素消費量（$CMRO_2$）算出した．生理食塩水と5%HSAで蘇生後の$CMRO_2$は脱血後の$CMRO_2$と同等ないしむしろ低下し，$CMRO_2$の回復を認めなかった（$p<0.05$）．一方，LEHで蘇生後の$CMRO_2$は脱血後の$CMRO_2$に比して有意に高値を示し（$p<0.05$），ベースラインの80%まで$CMRO_2$を回復できた．脱血液で蘇生した場合，$CMRO_2$はベースライン以上に回復した．これらの結果からLEHが組織に酸素を輸送できることは明らかであり，酸素供給により酸素代謝が改善される．しかしながら，酸素代謝の回復において，新鮮な脱血液の有効性には及ばない．本研究は，出血性ショックモデルでの脳酸素代謝の改善において，人工酸素運搬体の生体内での有効性を示す初めての研究である．また，本研究で使用した^{15}O-PETイメージング技術は，他の代用血液または酸素運搬体の酸素供給能の評価に利用できる．

考　察

健常成人での安静時酸素運搬量（DO_2）は～1 L/分である．運搬された酸素の～25%が組織で抽出され，残りの75%は静脈血に留まる．ある点までDO_2が減少する場合には，酸素抽出量（OER）が増大するため，酸素消費量はほぼ一定に保たれる．しかしながら，この関係の範囲は組織間で異なり，低酸素の程度に依存する．例えば，高度に低酸素の脳では，OER増大は運搬量の不足を補償することができず，酸素消費量が減少する．出血が生理的適応可能な機構を超える時，適切な酸素輸送を維持するために酸素運搬体による蘇生術が必要になる．

DO_2は心拍出量と動脈血酸素含量（CaO_2）の積として算出される．従って，心拍出量かCaO_2を増加させることによって組織酸素輸送を増大できる．しかしながら，ある点を越えると，心拍出量の増大は実質的にショック状態を悪化させる．酸素運搬能の改善によるCaO_2の増大に注意が注がれるのはこのためであり，人工酸素運搬体の開発ではCaO_2の増大を目標とする．

他のヘモグロビンを主成分とする酸素運搬体（HBOCs）と同様，LEHは出血ショックの動物モデルで広範囲に前臨床評価されている．前臨床から臨床段階への移行に成功した酸素運搬体の評価は，蘇生術の後の代用指標に基づいていた．いくつかのHBOCsが臨床治験に進んでいるが，投与後の脳の酸素輸送と代謝の実質的な改善は実証されていない．効果的な酸素運搬体が必ずしも効果的な酸素供給体であるというわけではなく，更に，効果的供給が必ずしも酸素代謝の改善に帰着するわけでない．このため，酸素供給を補うことが蘇生術の主要な目的で

あるなら，酸素供給，酸素消費量と局所組織アシドーシスのモニターが最も重要である．組織アシドーシスは乳酸と塩基欠乏が血清指標となるが，組織酸素化を直接測定するための処置は非常に侵襲が大きい．従来，組織酸素のモニターには，近赤外分光法，ポーラログラフの電極挿入，あるいは他の侵襲技術などが用いられてきた．近赤外分光法は容易で非侵襲性であるが，頸静脈酸素飽和度の標準技術との相互関係の欠如が指摘されている．また，近赤外分光法はヘモグロビン，オキシ・ヘモグロビン，およびチトクローム酸化酵素の吸収スペクトルの相違に基づくため，この技術が酸素代謝やHBOCsの評価にどのように役立つか明らかでない．これらの技術で計測される信号の変化は，組織の特有の機能状態には関連しないことがわかっている．

Hb小胞体を開発しているグループは，Hb小胞体が末梢組織の酸素欠乏を回復させることを間接的測定法により示している．本研究では，HBOCsによる蘇生術の機能評価のためにPETを利用すれば，視覚的，定量的，非侵襲的に生存動物での酸素供給と代謝を評価できることを実証する．$CMRO_2$は，脳血流量（CBF），OERとCaO_2より求められる．膜中の酸素輸送は受動的な拡散に従うので，細胞内酸素分圧（$PicO_2$）はCaO_2に直接関連する脳酸素分圧（$PbrO_2$）によって変動する．$PbrO_2$は35mmHgの正常レベルから，出血性ショックの後に15 - 20mmHgの臨界水準以下まで減少する可能性がある．軽度の失血において，$PbrO_2$は脳の自己調節によって臨界水準以上に維持される．酸素をミトコンドリアに届ける拡散力が重篤な出血で大幅に低下するため，自己調節はMAPが 60-150mmHgの範囲内だけで役立つ．微小血管系からの酸素拡散距離も出血による組織浮腫と損傷のため増加する．拡散係数と脳自己調節の変化により，CaO_2または$PbrO_2$のどちらかを除外した評価は十分でないかもしれず，脳酸素代謝とエネルギー代謝に関する付加的な情報が必要である．CaO_2の増大が$PbrO_2$の増加に必ずしも帰着するわけでなく，より高い$PbrO_2$がより高い$PicO_2$を意味するわけでない．$CMRO_2$が可逆性そして不可逆性脳障害の信頼性の高い予測因子である．基礎レベルの61-69%以下への酸素代謝の低下は危機的とみなされる．このように，$CMRO_2$を変数とするPETイメージから得られる情報は，脳障害の可逆性の予測に有用かもしれない．

著者らは，LEHの酸素供給能を調査するために^{15}O-PETイメージングを適用し，脳血流量と容量を測定しなくても$CMRO_2$を正確に評価できるOhtaの単純化された二相モデルでデータを解析した．この手順は動脈入力関数（AIF）を導くために心動脈スペースを使ってさらに簡便になった（詳細は原文付録A参照）．$CMRO_2$の改善において，LEHは酸素運搬能力を持たない輸液に比較して有意に良好な成績を示した．しかしながら，全血は$CMRO_2$をbaselineレベルまで回復させ，最も効果的な輸液であった．ただし，LEHのHb含量が返血した脱血液の半分に相当する点に留意する必要がある．LEHの大きさ（193.5 nm）は分子状修飾Hbに比較して大きいが，赤血球と比較すれば非常に小さい．小粒径のLEHは赤血球と比較して血管内で均一に分布するため，LEHのHb含有量と関連する酸素供給を助長することが考えられる．等価性の立証には，LEHと血液のHb濃度を合せて更なる検討が必要である．

心血管レベルの容量損失の補正が細胞酸素代謝の回復を必ずしも意味するわけでない．HSAとLEHはそれらの膠質浸透圧によって有意にMAPを改善したが，$CMRO_2$を有意に増大させたのはLEHだけであった．HSAには酸素運搬能がなく，さらに血管内で静水圧の平衡を保つために間隙水を引込むことでヘマトクリットを低下させる傾向がある．一方，生理食塩水にはそのような血液希釈の傾向がない．急性失血において，生理食塩水の輸注は浮腫を誘発する．これは，膠質浸透圧のない輸液が静水圧に従って細胞外間隙に漏れることに起因する．今回のLEHは5%HSAによって等張で，組織への酸素の受動拡散のための推進力である動脈PO_2を増大できる．HSAとLEHで蘇生術に対する循環予備能の適切な評価には，脳血流量と脳血液量の評価を含むより詳細な検討が必要である．

要約すると，LEHによる蘇生術が効率的に酸素を低酸素の脳組織に運搬することで$CMRO_2$を改善できることを実証した．LEHと血液のHb含量を合せてLEHの性能を全血蘇生と比較すれば，より興味深い結果となるであろう．重要臓器での生理的な代謝速度を定量化する^{15}O-PET特有の能力を人工酸素運搬体の評価に初めて適応した．この新しいPET技術は，臨床治験のさまざまなステージにある各種Hb由来酸素運搬体の評価法として広く活用できる．PETの有用性は，酸素代謝の評価に限られるものでない．PETによる酸素代謝の研究は，HBOCsがショック状態を改善する機構の区別を可能にするかもしれない．例えば，Oxyglobinのような分子状修飾Hbは，酸素を輸送できるだけでなく血管内の膠質浸透圧を増加させる．現時点では，HBOCsの酸素運搬あるいは膠質浸透圧がショック状態の改善に関与するかどうかわかっていない．遊離Hbの急速な酸化はHBOCsの酸素運搬能を低下させるが，その膠質浸透圧の大部分はまだ保持されている．分子状修飾Hbの投与後初期の効果には酸素運搬と膠質浸透圧の両方による可能性があり，時間の経過により膠質浸透圧の効果が優勢となるであろう．循環量，酸素運搬能，および$CMRO_2$の改善を同時に測定すれば，HBOCsの作用機序の詳細が明らかになる可能性がある．

参考文献

1. Djordjevich L, Miller IF. Lipid encapsulated hemoglobin as a synthetic erythrocyte. Fed Proc 1977;36:567-571.
2. Rudolph AS, Klipper RW, Goins B, Phillips WT. *In vivo* biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. Proc Natl Acad Sci USA 1991;88:10976-10980.
3. Szebeni J, Wassef NM, Spielberg H, Rudolph AS, Alving CR. Complement activation in rats by liposomes and liposome-encapsulated hemoglobin: evidence for anti-lipid antibodies and alternative pathway activation. Biochem

Biophys Res Commun 1994;205:255-263.
4. Goins B, Phillips WT, Klipper R, Rudolph AS. Role of complement in rats injected with liposome-encapsulated hemoglobin. J Surg Res 1997;68:99-105.
5. Loughrey HC, Bally MB, Reinish LM, Cullis PR. The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. Thromb Haemost 1990;64:172-176.
6. Phillips WT, Klipper RW, Awasthi VD, Rudolph AS, Cliff R, Kwasiborski V, Goins BA. Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. J Pharmacol Exp Ther 1999;288:665-670.
7. 土田英俊, 酒井宏水, 武岡真司, 宗 慶太郎, 小林紘一. 酸素輸液（人工赤血球). 医学のあゆみ 2003;205:558-566.
8. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E. Purification of concentrated Hb using organic solvent and heat treatment. Protein Expression Purif 1993;4:563-569.
9. 武岡真司. 分子集合科学を利用した人工血液の創製. 人工血液 2005;13:136-147.
10. Sakai H, Tomiyama K, Sou K, Takeoka S, Tsuchida E. Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjugate Chem 2000;11:425-32.
11. Sou K, Klipper R, Goins B, Tsuchida E, Phillips WT. Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. J Pharmacol Exp Ther 2005;312:702-709.
12. Phillips WT, Lemen L, Goins B, Rudolph AS, Klipper R, Fresne D, Jerabek PA, Emch ME, Martin C, Fox PT, McMahan CA. Use of oxygen-15 to measure oxygen-carrying capacity of blood substitutes in vivo. Am J Physiol 1997;272:H2492-H2499.
13. Cabrales P, Sakai H, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. Am J Physiol Heart Circ Physiol 2005;288:H1885-92.
14. Plock JA, Tromp AE, Contaldo C, Spanholtz T, Sinovcic D, Sakai H, Tsuchida E, Leunig M, Banic A, Erni D. Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue. Crit Care Med 2007;35:899-905.
15. Yamamoto M, Izumi Y, Horinouchi H, Teramura Y, Sakai H, Kohno M, Watanabe M, Kawamura M, Adachi T, Ikeda E, Takeoka S, Tsuchida E, Kobayashi K. Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation. J Surg Res 2008 (in press).

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Original article

Frequency distribution function of red blood cell velocities in single capillaries of the rat cerebral cortex using intravital laser-scanning confocal microscopy with high-speed camera

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Background: Brain metabolism depends largely on oxygen and therefore constant delivery of oxygen to the brain is more important than to any other organ. Previously we reported a newly developed method to automatically calculate red blood cell (RBC) flow and its temporal modulations at the layer 1 of the rat cerebral cortex.

Objective: To examine a general tendency of frame-rate dependency of RBC velocities and heterogeneity of RBC movements in single capillaries identified by fluorescein isothiocyanate (FITC)-dextran staining.

Methods: In urethane-anesthetized rats with a closed cranial window, intravenously administered FITC-labeled RBCs were traced at 125, 250 or 500 frames/s (fps) with a laser-scanning confocal fluorescence microscope and their velocities were automatically calculated with home-made software KEIO-IS2.

Results: RBC velocities in single capillaries were not constant but variable, and were dependent on frame rate, with average values of 0.85 ± 0.43 mm/s at 125 fps, 1.34 ± 0.73 mm/s at 250 fps, and 2.05 1.59 mm/s at 500 fps. When all capillary RBCs were plotted against their velocities (frequency distribution function of RBC velocities), RBC velocities were clustered at around 1.0 mm/s, smearing at higher velocities up to 9.4 mm/s. High RBC velocity was only detected from frame analysis with high frame rates since such high-flow RBCs were uncounted at low frame rates. RBC velocities higher than 6.5 mm/s were statistically significantly outlined from the main population ($p<0.01$). Such a group of capillaries was considered to belong to thoroughfare channels, although their diameter was almost the same as that of ordinary ones.

Conclusion: Extra-high flow capillaries are confirmed in the cerebral cortex and these may be thoroughfare channels or non-nutritional capillaries carrying 42 % of the blood in reserve.

Keywords: High-speed camera, intraparenchymal single capillary, KEIO-IS2, laser-scanning confocal fluorescence-microscopy, RBC velocity, thoroughfare channel.

Flow adjustment of red blood cells (RBCs), a carrier of oxygen, in capillaries has a critical role to the activity of neurons that require continuous supplies of oxygen. Examination of RBC behavior in capillaries reveals RBC flow to be heterogeneous and somewhat random, although RBC flow must be functionally connected with neuronal activity to reflect the local energy demands of active neurons [1]. However, the details of the relationship remain unknown.

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Various methods have been employed to measure RBC velocity in microvessels of various organs, using video images with an intravital microscope with a two-slit photometric method [2], a flying spot technique based on a cross-correlation method [3], a two-stage prism-grating system [4], on-line measurement techniques with a bidirectional optional three-stage prism grating system [5], and a line-scan CCD image sensor [6]. The various techniques that have been used to measure RBC velocity in microvessels of the brain of rats and cats are summarized in **Table 1**. Notably, the reported mean values of RBC velocity, the most fundamental parameter in capillaries, were quite diverse, ranging from 0.39 to 2.08 mm/s. Such diversity