Conclusion

While more research is required for a full understanding of the biological and pharmacological properties of this rHSA-FeP, the present results obviously indicate that the albumin-based synthetic O_2-carrying hemoprotein excludes unfavorable hemodynamic responses observed in the modified-Hb solutions. Thus, rHSA-FeP can be utilized not only as a safe and effective red blood cell substitute, but also as an O_2-carrying medicine which will be adopted for use in several clinical applications, such as myocardial infraction, tracheal blockade, preservation of organs for transplantation, etc.

Acknowledgement

We thank Prof. Dr. Koichi Kobayashi (Keio University) for his helpful suggestion and discussions in the animal experiments. Dr. Hiromi Sakai and Miss Yasuko Matsukawa (Waseda University) are greatly acknowledged for their skilful animal experiments. This work was partially supported by Health Science Research Grants (Research on Pharmaceutical and Medical Safety).

[1] Biopure Cooperation News Release (4/10/2001).
[2] G. Edwards, L. Levien, C. Benn, Program in The 9th International Symposium on Blood Substitutes, p. 38, Tokyo March 2003.
[3] (a) T. Komatsu, K. Hamamatsu, J. Wu, E. Tsuchida, *Bioconjugate Chem.* **1999**, *10*, 82; (b) E. Tsuchida, T. Komatsu, Y. Matsukawa, K. Hamamatsu, J. Wu, *Bioconjugate Chem.* **1999**, *10*, 797; (c) T. Komatsu, Y. Matsukawa, E. Tsuchida, *Bioconjugate Chem.* **2000**, *11*, 772; (d) T. Komatsu, T. Okada, M. Moritake, E. Tsuchida, *Bull. Chem. Soc. Jpn.* **2001**, *74*, 1695; (e) T. Komatsu, Y. Matsukawa, E. Tsuchida, *Bioconjugate Chem.* **2002**, *13*, 397.
[4] E. Tsuchida, T. Komatsu, K. Hamamatsu, Y. Matsukawa, A. Tajima, A. Yoshizu, Y. Izumi, K. Kobayashi, *Bioconjugate Chem.* **2000**, *11*, 46.
[5] (a) T. Komatsu, Y. Matsukawa, E. Tsuchida, *Chem.Lett.* **2000**, 1060; (b) T. Komatsu, Y. Matsukawa, E. Tsuchida, *Bioconjugate Chem.* **2001**, *12*, 71.
[6] T. Yonetani, H. Yamamoto, J. E. Erman, J. S. Leigh, Jr., G. H. Reed, *J. Biol. Chem.* **1972**, *247*, 2447.
[7] T. Yoshimura, *Bull. Chem. Soc. Jpn.* **1991**, *64*, 2819.
[8] M. Hoshino, M. Kogure, *J. Phys. Chem.* **1989**, *93*, 5478.
[9] E. L. Rose, B. M. Hoffman, *J. Am. Chem. Soc.* **1983**, *105*, 2866.
[10] T. G. Traylor, V. S. Sharma, *Biochemistry* **1992**, *31*, 2847.
[11] E. G. Moore, Q. H. Gibson, *J. Biol. Chem.* **1976**, *251*, 2788.
[12] E. Tsuchida, T. Komatsu, Y. Matsukawa, A. Nakagawa, H. Sakai, K. Kobayashi, M. Suematsu, *J. Biomed. Mater. Res.* **2003**, *64A*, 257
[13] A. C. Guyton, J. M. Ross, O. Carrier, J. R. Walker, *Circ. Res.* **1964**, *14*, 1.

Am J Physiol Heart Circ Physiol 285: H1140–H1147, 2003.
First published May 8, 2003; 10.1152/ajpheart.00285.2003.

Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity

Claudio Contaldo,[1] Sören Schramm,[1] Reto Wettstein,[1] Hiromi Sakai,[2] Shinji Takeoka,[2] Eishun Tsuchida,[2] Michael Leunig,[1] Andrej Banic,[1] and Dominique Erni[1]

[1]*Department of Orthopedic, Plastic and Hand Surgery, Inselspital University Hospital, 3010 Berne, Switzerland; and* [2]*Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan*

Submitted 1 April 2003; accepted in final form 5 May 2003

Contaldo, Claudio, Sören Schramm, Reto Wettstein, Hiromi Sakai, Shinji Takeoka, Eishun Tsuchida, Michael Leunig, Andrej Banic, and Dominique Erni. Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am J Physiol Heart Circ Physiol* 285: H1140–H1147, 2003. First published May 8, 2003; 10.1152/ajpheart.00285.2003.—The aim of this study was to test the influence of oxygen affinity of Hb vesicles (HbVs) and level of blood exchange on the oxygenation in collateralized, ischemic, and hypoxic hamster flap tissue during normovolemic hemodilution. Microhemodynamics were investigated with intravital microscopy. Tissue P_{O_2} was measured with Clark-type microprobes. HbVs with a P_{50} of 15 mmHg (HbV15) and 30 mmHg (HbV30) were suspended in 6% Dextran 70 (Dx70). The Hb concentration of the solutions was 7.5 g/dl. A stepwise replacement of 15%, 30%, and 50% of total blood volume was performed, which resulted in a gradual decrease in total Hb concentration. In the ischemic tissue, hemodilution led to an increase in microvascular blood flow to maximally 141–166% of baseline in all groups (median; $P < 0.01$ vs. baseline, not significant between groups). Oxygen tension was transiently raised to 121 ± 17% after the 30% blood exchange with Dx70 ($P < 0.05$), whereas it was increased after each step of hemodilution with HbV15-Dx70 and HbV30-Dx70, reaching 217 ± 67% ($P < 0.01$) and 164 ± 33% ($P < 0.01$ vs. baseline and other groups), respectively, after the 50% blood exchange. We conclude that despite a decrease in total Hb concentration, the oxygenation in the ischemic, hypoxic tissue could be improved with increasing blood exchange with HbV solutions. Furthermore, better oxygenation was obtained with the left-shifted HbVs.

blood substitutes; artificial red blood cells; microhemodynamics; hypoxia; collateral circulation

MAINTAINING ADEQUATE OXYGENATION is crucial for functional recovery and survival of cerebral, myocardial, mesenteric, or peripheral tissues rendered ischemic due to acute obstruction of their anatomic blood supply. In this scenario, oxygenation is determined by the amount of oxygen that is transported into the infarcted tissue via a collateral vasculature as well as by the potential of the oxygen carrier to deliver oxygen to this tissue.

Oxygenation and survival of ischemic myocardial (7, 19), cerebral (3, 18, 29), and peripheral tissues (2) could successfully be improved after the infusion of solutions containing artificial oxygen carriers, such as perfluorocarbons and chemically modified Hbs. These solutions have initially been developed with the scope of reducing the need of allogeneic blood transfusions, and at least four compounds are currently in advanced clinical trials to evaluate their potential as red blood cell (RBC) substitutes in blood loss (1, 10).

In a recent study (6), we were able to demonstrate that hypoxia in ischemic, collateralized hamster flap tissue was attenuated by a 50% blood exchange with a solution containing Hb vesicles (HbVs) suspended in 6% Dextran 70 (Dx70). The effect was associated with an increased capacity to transport oxygen to the ischemic tissue, which was related to the presence of HbVs and to an improvement of microcirculatory blood flow.

The HbV consists of isolated, purified human Hb that is encapsulated with a double phospholipid membrane coated with polyethylene glycol (22). The encapsulation of Hb prolongs the circulation time in the organism and prevents direct contact of Hb with the endothelial lining, thus suppressing vasoconstriction due to NO scavenging, which has been attributed to chemically modified Hbs (20). Another major advantage of the HbV is that oxygen affinity may easily be adapted to the needs of the tissue by supplementing the appropriate amount of coencapsulated allosteric effector (pyridoxal 5′-phosphate) (24). It was shown that after a 80% blood exchange with HbV solutions, oxygen consumption of the microvasculature was near to normal for a P_{50} of 16 and 30 mmHg, respectively, whereas it was significantly reduced for a P_{50} of 9 mmHg. The development of artificial RBC substitutes has been characterized by the assumption that their oxygen affinity should be similar or lower than that of blood to facilitate the delivery of oxygen to the tissues in need. On the other hand, it has been postulated that shifting the oxygen dissociation curve of RBC-bound Hb to the left (26, 27) or applying artificial oxygen

Address for reprint requests and other correspondence: D. Erni, Div. of Plastic and Reconstructive Surgery, Inselspital Univ. Hospital, CH-3010 Berne, Switzerland (E-mail: dominique.erni@insel.ch).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

carriers with high oxygen affinity (11) may be beneficial for the oxygenation of hypoxic tissues, in which O_2 diffusion from oxygen carrier to tissue is ensured by the high gradient of P_{O_2}.

The aim of this study was to test the effect obtained by increasing the oxygen affinity of HbVs on the oxygenation of the ischemic hamster flap tissue during normovolemic hemodilution with HbVs suspended in 6% Dx70. To this end, HbV with a P_{50} of 15 mmHg (HbV15) was compared with an HbV (P_{50} = 30 mmHg; HbV30) with an oxygen affinity similar to that of hamster blood (P_{50} = 28 mmHg). Furthermore, we wanted to evaluate the influence of the degree of normovolemic blood replacement with the HbV solutions. We chose a protocol that included three steps of hemodilution up to a level of 50% blood exchange, beyond which a clinical use does not appear to be reasonable.

MATERIALS AND METHODS

Experiments were performed according to the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and with the approval of the local Animal Ethics Committee. Thirty-seven male Syrian golden hamsters weighing 65–85 g were used in this study. The animals were randomly assigned to the control group (n = 9) or to one of three groups subjected to stepwise normovolemic hemodilution with 6% Dx70 (n = 10) or HbV15 or HbV30, respectively, suspended in 6% Dx70 (HbV15-Dx70, n = 9; HbV30-Dx70, n = 9).

Animal and flap preparation. The experiments were performed in a hamster skin flap model as described previously (4–6). Anesthesia was induced by pentobarbital injected intraperitoneally (100 mg/kg body wt, Nembutal, Abbott Laboratories; Chicago, IL). The carotid artery and jugular vein were cannulated for blood pressure monitoring and for the blood exchange and laboratory analysis, respectively. Catheterization and flap dissection were performed with the aid of an operating microscope at ×10 magnification (Wild; Heerbrugg, Switzerland). After the animal was shaved and the back skin of the animal was epilated, the vascular anatomy was identified by diaphanoscopy. An island flap measuring 30 × 20 mm was dissected free from the surrounding tissue. The animal was then placed in a lateral postition on a specially designed Plexiglas stage providing a platform for mounting the flap, which was positioned with the skin lying on the platform and kept at its original size by sutures. The panniculus carnosus was meticulously removed except for a single layer of muscle tissue left in place to protect the vascular network. The flap was merely perfused via one artery and vein, which bifurcate into two equal-sized branches within the flap, each of them supplying a separate vascular territory. One of the branches was transected after being secured with microsurgical ligatures. Therefore, one vascular territory was anatomically perfused by the intact branch, whereas the other was indirectly perfused through the collateral vasculature connecting the two vascular networks. The raw surface of the flap was finally covered with a polyvinyl film to isolate the tissue from the environment. During surgery, 4 mg papaverine hydrochloride (Sigma; St. Louis, MO) dissolved in 1 ml physiological saline solution was applied to the pedicle by a soaked cotton tip to prevent vascular spasm.

Laboratory analysis. Blood samples were collected in 40 μl heparin-washed microtubes for measurement of total Hb concentration and arterial blood gases (ABL 625, Radiometer; Copenhagen, Denmark). Hematocrit was determined by centrifugation.

Microhemodynamic measurements. Investigations were performed using an intravital microscope (Axioplan 1, Zeiss; Jena, Germany). Microscopic images were captured by a television camera (intensified charge-coupled device camera, Kappa Messtechnik; Gleichen, Germany), recorded on video (50 Hz, Panasonic; Osaka, Japan), and displayed on a television screen (Trinitron PVM-1454QM, Sony; Tokyo, Japan). The preparation was observed visually with a ×40 objective resulting in a total optical magnification of ×909 on the videomonitor. Microvascular diameter was measured by transillumination with a green filter, which gave a well-defined image of the width of the erythocyte column. For the assessment of centerline velocity, white blood cells (WBCs) were stained in vivo with rhodamine 6G (2 μmol/kg body wt iv, Sigma). Fluorescence was visualized with the aid of an excitation filter (530–560 nm), a dichroic mirror (580 nm), and a barrier filter (580 nm) and through epi-illumination by a mercury lamp (Atto Arc, Zeiss) (13). Velocity was calculated by measuring the distance covered by the WBC during one time frame (20 ms). Microvascular blood flow (Q) was calculated by means of *Eq. 1*

$$Q = \pi \times (\text{WBC velocity}/1.6) \times (\text{diameter}/2)^2 \quad (1)$$

The value 1.6 represents the empirically determined ratio of centerline velocity to whole blood velocity (9).

The microvessels were classified according to physiological and anatomic features into conduit arterioles (connections to each other), end arterioles, and small venules (4, 12). The vessels were chosen for examination according to their optical clarity.

Tissue oxygen tension. Tissue P_{O_2} was assessed with Clark-type microprobes consisting of polarographic electrodes and an oxygen-sensitive microcell (Revoxode CC1, GMS; Kiel, Germany). According to the manufacturer, the Revoxode CC1 provides reproducible values for several consecutive days without the need of recalibration. The length of the cell was 1 mm, and the sampling area was within 1 mm of the cell. The probes were inserted into the subcutaneous tissue in the center of each vascular territory under visual control and microscopic magnification. Care was taken to place the probes in such a way that no arterioles or large venules lay within the sampling area.

Table 1. *Physicochemical characteristics of hamster blood and the diluents*

	Hamster Blood	Dx70	HbV15-Dx70	HbV30-Dx70
P_{50}, mmHg	28		15	30
Hill number	2.8		1.7	2.3
OTE, %	31		19	35
Oxygen capacity, ml/100 ml	24.3		9.1	9.1
[Hb], g/dl	17	0	7.5	7.5
[metHb], %			5.2	2.3
Oncotic pressure, mmHg	18	50	50	50
Viscosity, cP	4.5	2.8	8.7	8.7

HbV15-Dx70 and HbV30-Dx70, hemoglobin vesicles (HbVs) with a P_{50} of 15 mmHg (HbV15) and 30 mmHg (HbV30) suspended in 6% Dextran 70 (Dx70), OTE, oxygen transport efficiency (arteriovenous difference in oxygen saturation of Hb in hamsters); metHb, methemoglobin. Viscosity was measured at 37°C and at 150 s^{-1}.

Table 2. *MAP and laboratory data at baseline and during blood exchange*

	Baseline	Level of Blood Exchange		
		15%	30%	50%
Hematocrit				
Control	0.54 ± 0.05	0.53 ± 0.04	0.51 ± 0.03	0.51 ± 0.05
Dx70	0.51 ± 0.04	0.39 ± 0.05†	0.30 ± 0.06†	0.20 ± 0.05†
HbV15-Dx70	0.51 ± 0.07	0.41 ± 0.08†	0.32 ± 0.07†	0.22 ± 0.07†
HbV30-Dx70	0.56 ± 0.06	0.46 ± 0.05†	0.33 ± 0.06†	0.20 ± 0.03†
Total Hb concentration, g/dl				
Control	16.6 ± 1.0	16.3 ± 0.8	16.1 ± 0.7	16.0 ± 0.8
Dx70	16.7 ± 1.0	12.9 ± 1.7†	9.9 ± 2.0†	6.5 ± 1.5†
HbV15-Dx70	16.7 ± 2.1	14.2 ± 2.6†	11.7 ± 2.5†	8.7 ± 2.2†
HbV30-Dx70	18.1 ± 1.8	14.6 ± 1.3†	11.1 ± 1.2†	8.0 ± 1.0†
MAP, mmHg				
Control	87 ± 11	80 ± 8	83 ± 9	79 ± 5
Dx70	81 ± 7	81 ± 10	81 ± 8	79 ± 8
HbV15-Dx70	78 ± 7	81 ± 9	80 ± 8	78 ± 4
HbV30-Dx70	91 ± 8	90 ± 9	90 ± 9	86 ± 11
P_{O_2}, mmHg				
Control	47 ± 6	48 ± 7	45 ± 7	47 ± 8
Dx70	43 ± 10	49 ± 18	58 ± 19*	71 ± 17†
HbV15-Dx70	45 ± 8	47 ± 13	49 ± 14	53 ± 13
HbV30-Dx70	42 ± 7	47 ± 15	51 ± 14	52 ± 7*
P_{CO_2}, mmHg				
Control	62 ± 8	62 ± 9	62 ± 9	60 ± 8
Dx70	61 ± 5	59 ± 8	53 ± 8†	51 ± 7†
HbV15-Dx70	59 ± 6	56 ± 8	55 ± 8	53 ± 8
HbV30-Dx70	55 ± 4	52 ± 6	51 ± 5	50 ± 5
pH				
Control	7.23 ± 0.05	7.25 ± 0.06	7.25 ± 0.08	7.25 ± 0.09
Dx70	7.24 ± 0.04	7.24 ± 0.05	7.28 ± 0.05	7.30 ± 0.07
HbV15-Dx70	7.23 ± 0.05	7.25 ± 0.07	7.27 ± 0.06	7.30 ± 0.08
HbV30-Dx70	7.26 ± 0.05	7.27 ± 0.06	7.29 ± 0.05	7.29 ± 0.05

Values are means ± SD. MAP, mean arterial blood pressure. *$P < 0.05$ and †$P < 0.01$ vs. baseline.

HbV solutions. The HbVs were prepared as previously reported (22, 24). They consisted of isolated human Hb encapsulated in a phospholipid vesicle coated with polyethylene glycol. The size of the vesicles was 253 ± 63 nm. The oxygen affinity (P_{50}) was regulated by adding the coencapsulated allosteric effector pyridoxal 5′-phosphate, and it was calculated from the O_2 equilibrium curve measured with a Hemox Analyzer (TCS Medical Products) at 37°C (24). The HbVs were suspended in 6% Dx70 (B. Braun Medical; Emmenbrucke, Switzerland). The physical characteristics of the so-

Table 3. *Microhemodynamic data in anatomically perfused and ischemic tissue at baseline*

	Diameter, μm			Centerline Velocity, mm/s		
	Conduit arterioles	End arterioles	Venules	Conduit arterioles	End arterioles	Venules
			Anatomically perfused tissue			
Control	55 ± 18	6.5 ± 2.2	75 ± 18	7.3 ± 2.2	1.6 ± 0.8	2.1 ± 1.2
n	20	15	8	20	15	8
Dx70	52 ± 29	7.2 ± 2.6	88 ± 7	7.8 ± 3.0	3.0 ± 1.6	2.1 ± 1.1
n	19	29	9	19	29	9
HbV15-Dx70	53 ± 23	6.4 ± 1.7	83 ± 9	6.4 ± 2.7	1.9 ± 1.0	1.1 ± 0.4
n	13	23	7	13	23	7
HbV30-Dx70	52 ± 16	7.5 ± 1.8	86 ± 19	7.3 ± 2.6	2.2 ± 0.7	1.8 ± 0.8
n	25	16	17	25	16	17
			Ischemic tissue			
Control	56 ± 29	7.8 ± 3.4	83 ± 13	0.5 ± 0.4	0.3 ± 0.2	0.2 ± 0.0
n	18	21	10	18	21	10
Dx70	63 ± 34	8.3 ± 3.0	90 ± 11	0.8 ± 0.7	0.5 ± 0.3	0.4 ± 0.3
n	21	31	9	21	31	9
HbV15-Dx70	70 ± 31	6.7 ± 2.2	89 ± 13	0.4 ± 0.3	0.2 ± 0.1	0.2 ± 0.1
n	15	25	9	15	25	9
HbV30-Dx70	54 ± 20	8.6 ± 2.3	77 ± 16	0.7 ± 0.3	0.4 ± 0.2	0.2 ± 0.1
n	27	25	12	27	25	12

Values are means ± SD; *n*, sample size. Note that centerline velocity was significantly lower in the ischemic vessels than in the anatomically perfused vessels ($P < 0.01$).

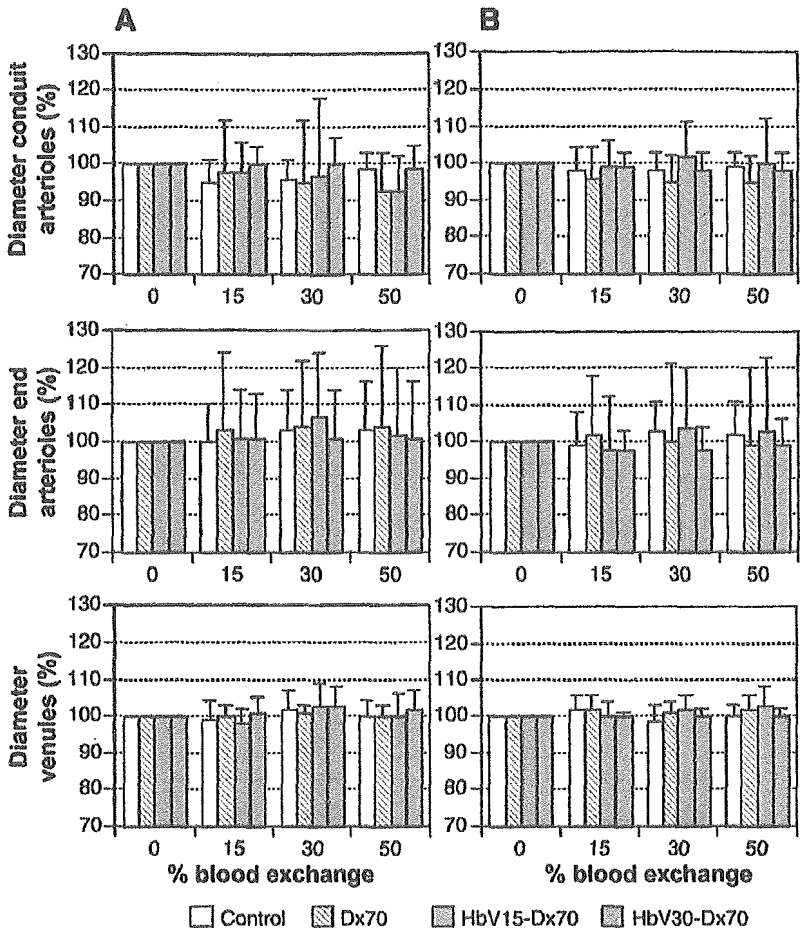


Fig. 1. Microvascular diameters in the anatomically perfused (*A*) and ischemic (*B*) tissues at baseline and after stepwise exchange of 15%, 30%, and 50% total blood volume with 6% Dextran 70 (Dx70) and Hb vesicles (HbVs) with a P_{50} of 15 mmHg (HbV15) and 30 mmHg (HbV30) suspended in Dx70. Data are given as a percentage of baseline and represent means ± SD.

lutions are summarized in Table 1. Oncotic pressure and viscosity were measured with a colloid osmometer (model 4420, Wescor; Logan, UT) and a cone-plate viscometer (PVII+, Brookfield Engineering; Middleboro, MA), respectively (30).

Protocol. The animals were kept under light anesthesia with a continuous infusion of 50 mg/ml pentobarbital given at a rate of ~0.5 mg·min^{-1}·kg body wt^{-1} throughout the experiment. The depth of anesthesia was regulated by tolerance of a noxious reflex due to pinching of the hind paw but no nonaversive reflexes (palpebral, corneal, and jaw reflex). A constant temperature in the animal and flap preparation was maintained by means of a heating pad and by keeping the room temperature at 28°C. Normovolemic hemodilution was achieved by simultanous replacement of withdrawn blood over 10 min. Hemodilution was performed in three steps at an interval of 1 h, thus reaching levels of 15%, 30%, and 50% of blood exchange. Measurements were taken at the end of each interval.

Inclusion criteria were a normal vascular anatomy, sufficient optical clarity of the preparation, and a mean arterial pressure above 60 mmHg.

The animals were euthanized with an overdose of pentobarbital at the end of the experiment.

Statistical analysis. The InStat version 3 program (Graph Pad Software, San Diego, CA) was utilized for statistical analysis. If the assumption of a normal distribution was appropriate, data were presented as means ± SD; otherwise, they represented median and 25th and 75th percentiles. For normally distributed data, the time-related differences between repeat measurements and the differences between the groups were assessed by the paired and unpaired ANOVA, respectively, whereas the nonparametric Friedman and Kruskal-Wallis tests were used for not normally distributed data analysis. All tests were followed by the Bonferroni post test. A value of $P < 0.05$ was taken to represent statistical significance.

RESULTS

Five animals (1 control, 1 Dx70, 1 HbV15-Dx70, and 2 HbV30-Dx70) did not fulfill the inclusion criteria and were excluded from this study.

The systemic data are summarized in Table 2. Similar hematocrits were obtained in all hemodiluted animals. After the 50% blood exchange, hemodilution with Dx70 resulted in a mean total Hb concentration of 6.5 g/dl, whereas the addition of HbV to the diluents enhanced the total Hb concentration to 8.7 and 8.0 g/dl, respectively ($P < 0.05$). Arterial Po_2 was gradually raised after each step of hemodilution, reaching 71 mmHg in the Dx70 group ($P < 0.01$ vs. baseline) and 53 and 52 mmHg for HbV15-Dx70 [not significant (NS)] and HbV30-Dx70 ($P < 0.05$), respectively (both $P < 0.05$ vs. Dx70). Furthermore, the blood exchange was followed by gradual reductions of arterial Pco_2 ($P <$

0.01 for Dx70; NS for the HbV groups) and increases in pH (NS).

At baseline, the microhemodynamic data were similar in all groups. The diameters and centerline velocities for conduit arterioles, end arterioles, and venules in each part of the flap are summarized in Table 3. Mean centerline velocities were significantly reduced in the ischemic vessels compared with the anatomically perfused vessels ($P < 0.01$).

The behavior of the microvascular diameters in both parts of the flap are shown in Fig. 1. A slight vasoconstriction was observed in the conduit arterioles after hemodilution with Dx70 (93 ± 10% in the anatomically perfused tissue and 95 ± 7% in the ischemic tissue, both NS) and HbV15-Dx70 (93 ± 9% anatomically perfused, NS), whereas the diameters remained virtually stable in the other microvessels in all groups.

Microvascular blood flow did not show any relevant changes in the anatomically perfused vessels (Fig. 2), whereas it was significantly increased in all vessels in the ischemic tissue due to hemodilution ($P < 0.05$ for Dx70 and HbV15-Dx70; $P < 0.01$ for HbV30-Dx70). The highest values were obtained with the 30% and 50% blood exchanges, reaching 144% (108–160%) for Dx70, 166% (152–192%) for HbV15-Dx70, and 141% (118–165%) for HbV30-Dx70.

Mean Po_2 ranged between 23.0 ± 4.3 and 26.7 ± 2.2 mmHg in the anatomically perfused tissue. Tissue Po_2 was significantly reduced in the ischemic part, where the values were between 7.7 ± 3.2 and 10.9 ± 3.4 mmHg ($P < 0.01$). In the anatomically perfused tissue, oxygenation was virtually not influenced by hemodilution with or without HbVs (Fig. 3). In the ischemic tissue, a transient improvement was observed during hemodilution with Dx70. The maximum was obtained after the 30% exchange (121 ± 17% of baseline, $P < 0.05$). Each step of blood exchange caused an increase in ischemic tissue oxygenation if the diluents contained HbV. Tissue Po_2 was enhanced up to 217 ± 67% and 164 ± 33% for HbV15-Dx70 and HbV30-Dx70, respectively (both $P < 0.01$ vs. baseline and other groups).

DISCUSSION

The principal findings of this study were that *1*) oxygenation in the ischemic, collateralized, and hypoxic flap tissue was raised with each step of hemodilution with both HbV solutions and that higher values

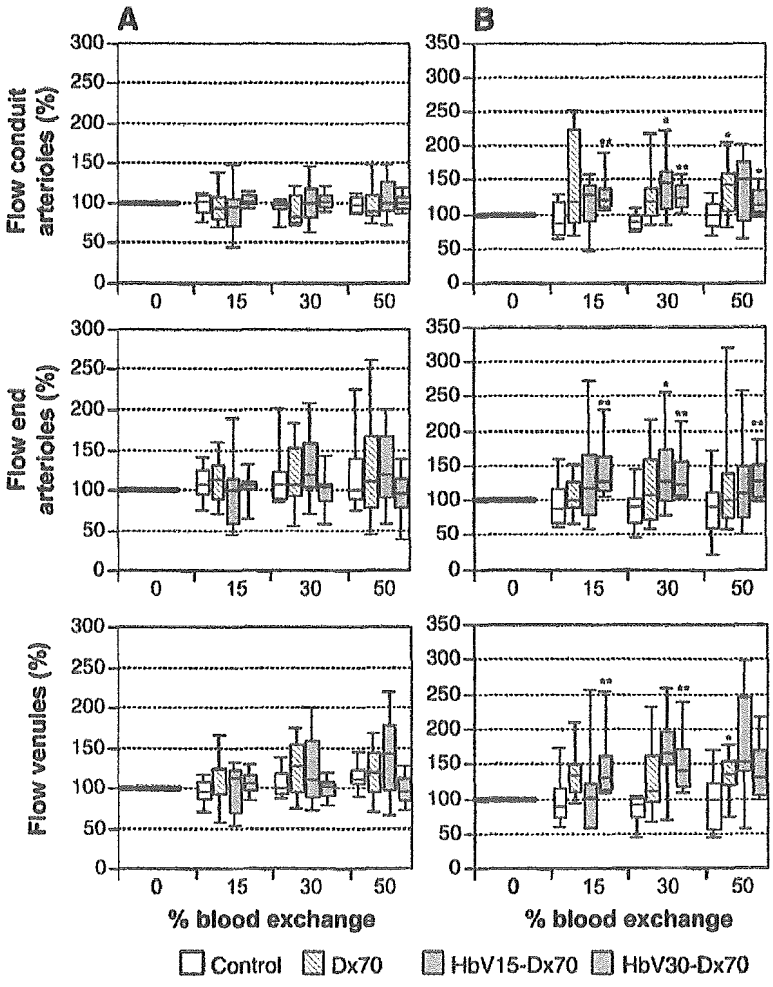


Fig. 2. Microvascular blood flow in the anatomically perfused (*A*) and ischemic (*B*) tissues at baseline and after stepwise exchange of 15%, 30%, and 50% total blood volume with 6% Dx70, HbV15-Dx70, and HbV30-Dx70. Data are given as a percentage of baseline and are presented in box plots reflecting 10th percentile, 25th percentile, median, 75th percentile, and 90th percentile. *$P < 0.05$ and **$P < 0.01$ vs. baseline.

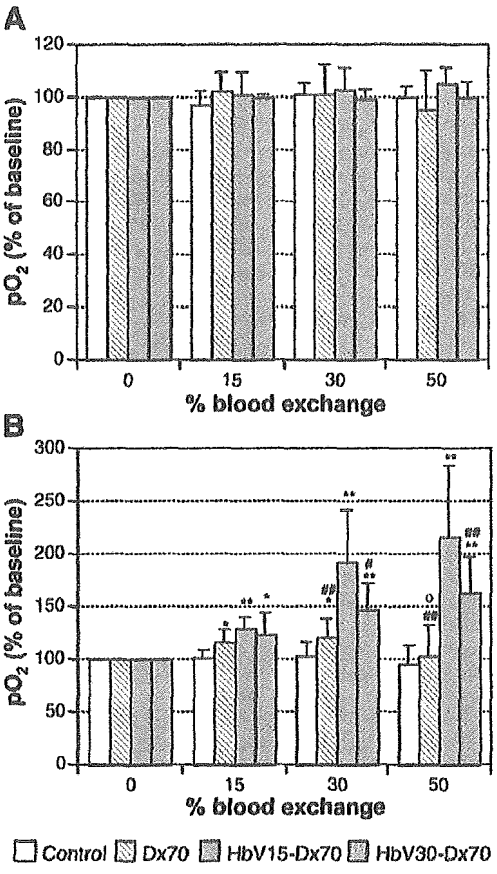


Fig. 3. Po_2 in the anatomically perfused (*A*) and ischemic (*B*) tissues at baseline and after stepwise exchange of 15%, 30%, and 50% total blood volume with 6% Dx70, HbV15-Dx70, and HbV30-Dx70. Data are given as a percentage of baseline and represent means ± SD. $^*P < 0.05$ and $^{**}P < 0.01$ vs. baseline; $\#P < 0.05$ and $\#\# P < 0.01$ vs. HbV15-Dx70; $^oP < 0.01$ vs. HbV30-Dx70.

were obtained 2) by increasing the oxygen affinity of HbV and 3) with HbV-containing solutions compared with Dx70 only.

Normovolemic hemodilution with Dx70 transiently improved the oxygenation in the ischemic tissue, reaching a peak of 121% of baseline at the 30% blood exchange. This level of hemodilution is considered to provide the highest RBC flux at the capillary level (15), thus resulting in maximal oxygen transport capacity both systemically (28) and in locally ischemic tissue (25). Furthermore, arterial Po_2 was increased at this level of hemodilution due to hyperventilation. As we (5) demonstrated in previous experiments in the same model, increased arterial Po_2 values were transferred as far as the collateral arterioles, which is where the blood circulation enters the ischemic tissue. In the present study, however, the improvement of oxygenation in the ischemic tissue was by far higher if HbV was added to the Dx70 solution, although microvascular blood flow, Hb concentration, and arterial Po_2 were similar or lower. Moreover, the oxygenation was enhanced despite a simultaneous decrease in total Hb concentration due to hemodilution with the HbV-Dx70 solutions. These results suggest that under the given conditions, the presence of HbVs increases the capacity of blood to deliver oxygen to the ischemic tissue and that the effect is related to the level of blood exchange with HbV solutions.

The effect may be achieved due to the small size of the HbVs, which might allow them to perfuse capillaries that are no longer accessible to RBCs due to intraluminal obstructions or reduced perfusion pressure that have to be assumed in the ischemic, collateralized tissue in the present model. Indeed, circulating HbVs could be observed in capillaries that were no longer considered functional because of the cessation of RBC flux (23). However, our previous experiments (6) showed that the improvement of oxygenation in the ischemic tissue obtained after hemodilution with HbV solutions was dependent on an increased RBC flux in this tissue, which indicates that mechanisms different from the passage of HbVs through vascular obstructions may be present.

On the basis of previous intravascular oxygen tension measurements, it was estimated that 40–50% of the systemic arterial oxygen content exited the upstream vasculature before reaching the collateralized, ischemic flap tissue (5, 24). It has been shown in both experimental (8, 24) and theoretical (31) studies that oxygen delivery may be delayed in favor of the downstream vasculature if oxygen carriers with increased oxygen affinity are infused. It is conceivable that this effect was responsible for the increased ischemic tissue oxygenation obtained with HbV15-Dx70 in our study. The high oxygen affinity of HbV15 did not seem to hamper the unloading of oxygen to this tissue, which is promoted by the high oxygen tension gradient and the increased residence time of circulating blood (11). Furthermore, it may be assumed that oxygen delivery is facilitated due to metabolic acidosis, thus causing a shift of the oxygen dissociation curve to the right.

The results obtained with HbV30-Dx70 suggest that prevention of oxygen loss in the upstream vasculature may have been accomplished without raising the oxygen affinity of HbV. It has been shown that the diffusion of oxygen through the plasma may substantially be influenced by adding oxygen carriers (14, 16, 17). According to the Stokes-Einstein equation, the diffusivity of oxygen is inversely proportional to the size of the plasma-bound oxygen carrier and the viscosity of the suspension. In mathematical models and in vitro experiments, facilitated oxygen diffusion was ascribed to small-sized Hbs (14, 16, 17, 21), whereas, because of their large size, this effect was abolished if HbVs were used (21). Although not measured in our study, there is sufficient evidence to assume a marked increase in viscosity of the plasma suspension obtained during hemodilution with the HbV-Dx70 solutions, because an increase in plasma viscosity from 1.2 to 1.4 cP has been observed in hamsters after severe hemodilution with Dx70 (30), which has a threefold lower viscosity than HbV-Dx70. Taken together, it may be assumed that hemodilution with the HbV-Dx70 solutions caused a retention of oxygen in the upstream vasculature, which

was related to both the size of the HbV and the increasing composite viscosity of the plasma suspension consisting in original hamster blood plasma, Dx70 molecules, and HbV, and which resulted in a shift of oxygen delivery in favor of the collateralized, ischemic and hypoxic flap tissue.

Additional studies will be necessary to outline the influence of both HbV concentration and viscosity of the solutions, and the long-term benefit of the observed improvement in oxygenation would yet have to be confirmed by evaluating the functionality and survival of the jeopardized tissue. Moreover, our data may not be extrapolated to ischemic conditions in other, vital organs, such as the myocardium or cerebral tissue, in which the oxygen demand is substantially higher than in the present flap tissue.

In summary, we conclude that up to a 50% blood exchange, normovolemic hemodilution with HbV-Dx70 solutions led to a dose- and oxygen affinity-dependent improvement of oxygenation in the ischemic, hypoxic flap tissue, which was not related to the oxygen-carrying capacity of the circulating blood. Thus our study strongly emphasizes the potential function of HbVs as a therapeutic that may be used to improve the delivery of oxygen to ischemic and hypoxic tissues, which exceeds its role as a simple RBC substitute.

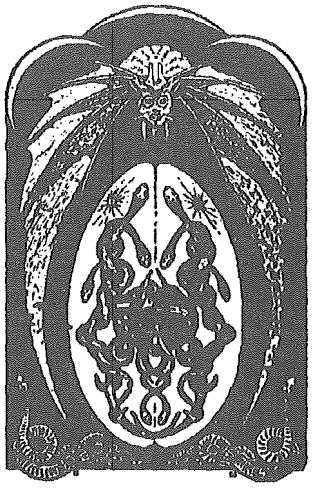
DISCLOSURES

This research was supported by Swiss National Foundation for Scientific Research Grants 32-054092.98 (to D. Erni) and 32-050771.97 (to M. Leunig), by the Department of Clinical Research, University of Berne, Switzerland, and by Health Sciences Research, Research on Advanced Medical Technology, Artificial Blood Project, Grant 12090101 from the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

1. **Chang TM.** Oxygen carriers. *Curr Opin Investig Drugs* 3: 1187–1190, 2002.
2. **Chowdary RP, Berkower AS, Moss ML, and Hugo NE.** Fluorocarbon enhancement of skin flap survival in rats. *Plast Reconstr Surg* 79: 98–101, 1987.
3. **Cole DJ, Drummond JC, Patel PM, Nary JC, and Applegate RL 2nd.** Effect of oncotic pressure of diaspirin cross-linked hemoglobin (DCLHb) on brain injury after temporary focal cerebral ischemia in rats. *Anesth Analg* 83: 342–347, 1996.
4. **Erni D, Sakai H, Banic A, Tschopp HM, and Intaglietta M.** Quantitative assessment of microhemodynamics in ischemic skin flap tissue by intravital microscopy. *Ann Plast Surg* 43: 405–415, 1999.
5. **Erni D, Sakai H, Tsai AG, Banic A, Sigurdsson GH, and Intaglietta M.** Haemodynamics and oxygen tension in the microcirculation of ischaemic skin flaps after neural blockade and haemodilution. *Br J Plast Surg* 52: 565–572, 1999.
6. **Erni D, Wettstein R, Schramm S, Contaldo C, Sakai H, Takeoka S, Tsuchida E, Leunig M, and Banic A.** Normovolemic hemodilution with Hb vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am J Physiol Heart Circ Physiol* 284: H1702–H1709, 2003.
7. **Faithfull NS, Fennema M, and Erdmann W.** Protection against myocardial ischaemia by prior haemodilution with fluorocarbon emulsions. *Br J Anaesth* 60: 773–778, 1988.
8. **Intaglietta M.** Microcirculatory basis for the design of artificial blood. *Microcirculation* 6: 247–258, 1999.
9. **Intaglietta M and Tompkins WR.** Microvascular measurements by video image shearing and splitting. *Microvasc Res* 5: 309–312, 1973.
10. **Jahr JS, Nesargi SB, Lewis K, and Johnson C.** Blood substitutes and oxygen therapeutics: an overview and current status. *Am J Ther* 9: 437–443, 2002.
11. **Kavdia M, Pittman RN, and Popel AS.** Theoretical analysis of effects of blood substitute affinity and cooperativity on organ oxygen transport. *J Appl Physiol* 93: 2122–2188, 2002.
12. **Kerger H, Saltzman DJ, Menger MD, Messmer K, and Intaglietta M.** Systemic and subcutaneous microvascular P_{O_2} dissociation during 4-h hemorrhagic shock in conscious hamsters. *Am J Physiol Heart Circ Physiol* 270: H827–H836, 1996.
13. **Leunig M, Demhartner TJ, Sckell A, Fraitzl CR, Gries N, Schenk RK, and Ganz R.** Quantitative assessment of angiogenesis and osteogenesis after transplantation of bone: comparison of isograft and allograft bone in mice. *Acta Orthop Scand* 70: 374–380, 1999.
14. **McCarthy MR, Vandegriff KD, and Winslow RM.** The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers. *Biophys Chem* 92: 103–117, 2001.
15. **Mirhashemi S, Ertefai S, Messmer K, and Intaglietta M.** Model analysis of the enhancement of tissue oxygenation by hemodilution due to increased microvascular flow velocity. *Microvasc Res* 34: 290–301, 1987.
16. **Nishide H, Chen XS, and Tsuchida E.** Facilitated oxygen transport with modified and encapsulated hemoglobins across non-flowing solution membrane. *Artif Cells Blood Substit Immobil Biotechnol* 25: 335–346, 1997.
17. **Page TC, Light WR, McKay CB, and Hellums JD.** Oxygen transport by erythrocyte/hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance. *Microvasc Res* 55: 54–64, 1998.
18. **Powanda DD and Chang TM.** Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artif Cells Blood Substit Immobil Biotechnol* 30: 23–37, 2002.
19. **Premaratne S, Harada RN, Chun P, Suehiro A, and McNamara JJ.** Effect of perfluorocarbon exchange transfusion on reducing myocardial infarct size in a primate model of ischemia-reperfusion injury: a prospective, randomized study. *Surgery* 117: 670–676, 1995.
20. **Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, and Intaglietta M.** Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* 279: H908–H915, 2000.
21. **Sakai H, Suzuki Y, Kinoshita M, Takeoka S, Maeda N, and Tsuchida E.** Oxygen release from Hb-vesicles: comparison with red blood cells and acellular Hb solution using an artificial oxygen permeable narrow tube with 28 μm inner diameter (Abstract). *Artif Blood* 11: 113, 2003.
22. **Sakai H, Takeoka S, Park SI, Kose T, Hamada K, Izumi Y, Yoshizu A, Nishide H, Kobayashi K, and Tsuchida E.** Surface modification of hemoglobin vesicles with poly(ethyleneglycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjug Chem* 8: 15–22, 1997.
23. **Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, and Tsuchida E.** Systemic and microvascular responses to hemorrhagic shock and resuscitation with Hb vesicles. *Am J Physiol Heart Circ Physiol* 283: H1191–H1199, 2002.
24. **Sakai H, Tsai AG, Rohlfs RJ, Hara H, Takeoka S, Tsuchida E, and Intaglietta M.** Microvascular response to hemodilution with Hb vesicles as red blood cell substitutes: influence of O_2 affinity. *Am J Physiol Heart Circ Physiol* 276: H552–H562, 1999.
25. **Schramm S, Wettstein R, Wessendorf R, Jakob SM, Banic A, and Erni D.** Acute normovolemic hemodilution improves oxygenation in ischemic flap tissue. *Anesthesiology* 96: 1478–1484, 2002.

26. **Schumacker PT, Suggett AJ, Wagner PD, and West JB.** Role of hemoglobin P_{50} in O_2 transport during normoxic and hypoxic exercise in the dog. *J Appl Physiol* 59: 749–757, 1985.
27. **Stein CJ and Ellsworth ML.** Capillary oxygen transport during severe hypoxia: role of hemoglobin oxygen affinity. *J Appl Physiol* 75: 1601–1607, 1993.
28. **Sunder-Plassmann L, Klövekorn WP, Holper K, Hase U, and Messmer K.** The physiological significance of acutely induced hemodilution. In: *Proc 6th Eur Conf Microcirculation*, edited by Ditzel J and Lewis DH. Basel, Switzerland: Karger, 1971, p. 23–28.
29. **Sutherland GR, Farrar JK, and Peerless SJ.** The effect of Fluosol on oxygen availability in focal cerebral ischemia. *Stroke* 15: 829–835, 1984.
30. **Tsai AG, Friesenecker B, McCarthy M, Sakai H, and Intaglietta M.** Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. *Am J Physiol Heart Circ Physiol* 275: H2170–H2180, 1998.
31. **Vadapalli A, Goldman D, and Popel AS.** Calculations of oxygen transport by red blood cells and hemoglobin solutions in capillaries. *Artif Cells Blood Substit Immobil Biotechnol* 30: 157–188, 2002.



Am J Physiol Heart Circ Physiol 285: H2543–H2551, 2003.
First published July 24, 2003; 10.1152/ajpheart.00537.2003.

O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs

Hiromi Sakai,[1] Yoji Suzuki,[2] Megumi Kinoshita,[2] Shinji Takeoka,[1] Nobuji Maeda,[2] and Eishun Tsuchida[1]
[1]*Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555; and*
[2]*Department of Physiology, School of Medicine, Ehime University, Shigenobu, Ehime 791-0295, Japan*

Submitted 11 June 2003; accepted in final form 21 July 2003

Sakai, Hiromi, Yoji Suzuki, Megumi Kinoshita, Shinji Takeoka, Nobuji Maeda, and Eishun Tsuchida. 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–H2551, 2003. First published July 24, 2003; 10.1152/ajpheart.00537.2003.—A phospholipid vesicle that encapsulates a concentrated hemoglobin (Hb) solution and pyridoxal 5′-phosphate as an allosteric effector [Hb vesicle (HbV) diameter, 250 nm] has been developed to provide an O_2 carrying ability to plasma expanders. The O_2 release from flowing HbVs was examined using an O_2-permeable, fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm) exposed to a deoxygenated environment. Measurement of O_2 release was performed using an apparatus that consisted of an inverted microscope and a scanning-grating spectrophotometer with a photon-count detector, and the rate of O_2 release was determined based on the visible absorption spectrum in the Q band of Hb. HbVs and fresh human red blood cells (RBCs) were mixed in various volume ratios at a Hb concentration of 10 g/dl in isotonic saline that contained 5 g/dl albumin, and the suspension was perfused at the centerline flow velocity of 1 mm/s through the narrow tube. The mixtures of acellular Hb solution and RBCs were also tested. Because HbVs were homogeneously dispersed in the albumin solution, increasing the volume of the HbV suspension resulted in a thicker marginal RBC-free layer. Irrespective of the mixing ratio, the rate of O_2 release from the HbV/RBC mixtures was similar to that of RBCs alone. On the other hand, the addition of 50 vol% of acellular Hb solution to RBCs significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 7 nm) that affects their diffusion for the facilitated O_2 transport.

blood substitutes; red blood cells; hemoglobin; microcirculation; oxygenation; liposome

VARIOUS KINDS OF HEMOGLOBIN (Hb)-based O_2 carriers (HBOCs) have been developed for the substitution of the function of red blood cells (RBCs) including intramolecularly cross-linked, polymerized, polymer-conjugated, and liposome-encapsulated Hbs (LEHs; Refs. 6, 42). Historically, it has been regarded that the O_2 affinity (expressed as P_{50}; the O_2 tension at which Hb is half-saturated with O_2) should be regulated similar to that of RBCs, namely, ~25–30 Torr, using an allosteric effector or by a direct chemical modification of the Hb molecules. Theoretically, this allows sufficient O_2 unloading during blood microcirculation as can be evaluated by the arteriovenous difference in O_2 saturation (So_2) in accordance with an O_2 equilibrium curve. It has been expected that decreasing O_2 affinity (increasing P_{50}) results in an increase in O_2 unloading. This is supported by the result in a mouse model (31) that RBCs with a high P_{50} value show enhanced O_2 release for improved exercise capacity.

The size of HBOCs is much smaller than that of RBCs. Thus even if the O_2 affinity of both O_2 carriers is similar in the equilibrated condition, the O_2 releasing and binding rates should be different depending on the flow conditions of the carriers and the diffusion of O_2. In a stopped-flow analyses, significantly faster O_2 release rates for an unmodified Hb solution or LEHs rather than RBCs were confirmed (e.g., O_2 dissociation rate constant, 84 s^{-1} for unmodified Hb solution vs. 4.4 s^{-1} for RBCs; Refs. 8, 30, 45). However, these observations were performed under homogeneous conditions and at dilute (<10 μM) Hb concentrations ([Hb]), and it was not clear whether this significant difference would actually be observed in the in vivo condition and how the microcirculation would respond to the difference.

O_2 transfer from blood to tissues in the microcirculatory network is the result of a complex process whereby a substantial fraction of O_2 is exchanged in the arterioles and venules (10, 11, 14) where RBCs are not homogeneously distributed. RBCs tend to move to the centerline in laminar flow, and there is a plasma layer in the marginal zone as is clearly demonstrated in microvessels (17). For this reason, studies of O_2 release in microvessels as well as in capillaries are physiologically important (14, 34, 38, 39, 43). Therefore, the O_2 release rates under more physiological conditions, at a higher [Hb], and in flow conditions in

Address for reprint requests and other correspondence: E. Tsuchida, Advanced Research Institute for Science and Engineering, Waseda Univ., Tokyo 169-8555, Japan (E-mail: eishun@waseda.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

microvessels have been required for discussion of the dynamics of the O_2 releasing that couples with tissue oxygenation. The measurement can be performed using an artificial, narrow, O_2-permeable tube (35). In the case of blood, a cell-free plasma layer between a RBC-flow column and a vessel wall and a highly viscous Hb solution inside RBCs could be barriers to the O_2 diffusion. On the other hand, HBOCs are so small that they are homogeneously dispersed in the plasma phase. Page et al. (20) demonstrated using an artificial flow channel excavated in an O_2-permeable silicone rubber film that a polymerized bovine Hb showed facilitated O_2 release when the Hb was mixed with RBCs.

Phospholipid vesicles that encapsulate concentrated Hb have been developed [Hb vesicles (HbVs) or LEHs; diameter, 250 nm] as another type of HBOC that possesses a cell structure similar to RBCs (21, 24–30, 42). Both RBCs and HbVs have a lipid-bilayer membrane that prevents direct contact of Hb with blood components and endothelial lining. Furthermore, Hb encapsulation in the vesicle suppresses hypertension induced by vasoconstriction; this mechanism is presumably due to the effects of free Hb, which scavenges the endogenous vasorelaxation factors, nitric oxide, and carbon monoxide (13, 24) due to their high affinity with Hb. In this report, we evaluate for the fist time (based on microscopic observations) the O_2 unloading profile of our HbVs compared with RBCs and an acellular unmodified Hb solution using an artificial, narrow, O_2-permeable tube with a 28-μm inner diameter. This methodology has been well established by Tateishi et al. (35, 36).

MATERIALS AND METHODS

Preparation of HbVs, Hb solutions, and an RBC suspension. The HbVs were prepared under sterile conditions as previously reported (24, 27, 32, 33). Hb was purified from outdated donated blood obtained from the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo). The encapsulated purified Hb (38 g/dl) contained 17.6 mM of pyridoxal 5′-phosphate (PLP; Sigma; St. Louis, MO) as an allosteric effector at a molar PLP/Hb ratio of 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate in a 5:5:1 molar ratio (Nippon Fine Chemical; Osaka, Japan). The surface of the HbVs was modified with polyethylene glycol (PEG; 5,000 mol wt) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-polyethylene glycol (NOF; Tokyo; 0.3 mol% of total lipids). HbV diameter was 252 ± 53 nm (determined by the light-scattering method). The P_{50} value was 28 Torr at 37°C, which was measured with a Hemox analyzer (TCS Medical Products; Huntingdon Valley, PA). HbVs were resuspended in either 5 g/dl recombinant human serum albumin (HSA; 69,000 mol wt; Nipro; Osaka; HbV_{HSA}) or in 6 g/dl hydroxylethyl starch (HES; 70,000 mol wt; Kyorin Chemistry Lab; Tokyo; HbV_{HES}), and the final [Hb] was adjusted to 10 g/dl.

The purified Hb solution (38 g/dl) was diluted with a phosphate-buffered saline to 10 g/dl, and PLP was added at a molar Hb/PLP ratio of 1:3 or 1:1. Under these conditions, the P_{50} values were 29 and 15 Torr, respectively, and these Hb solutions were termed Hb29 and Hb15, respectively. Fresh human RBCs were obtained from a healthy donor (Y. Suzuki). RBCs were washed twice with isotonic saline and suspended in the saline that contained 5 g/dl HSA, and the [Hb] was adjusted to 10 g/dl. The RBC suspension was used within a day of the blood collection. The physicochemical properties of all O_2 carriers are summarized in Table 1. The viscosities of the suspensions were measured with either a capillary rheometer (oscillatory capillary rheometer OCR-D; Anton Paar; Graz, Austria) or a cone-plate viscometer (model E; Tokyo Keiki; Tokyo) at 37°C and at the shear rates of 300 and 71 s^{-1}. The latter shear rate is approximately identical to the wall shear rate of the narrow tube (inner diameter, 28 μm) as performed in the present study when the centerline velocity is 1 mm/s. The HbV or Hb solutions were mixed with the RBC suspension at volume ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. For example, the mixture of 10 vol% HbV_{HSA}s and 90 vol% RBCs is abbreviated as $10HbV_{HSA}$/90RBC for convenience.

Perfusion of HbV/RBC or Hb/RBC mixtures through narrow tubes. Narrow, O_2-permeable tubes (inner diameter, 28.1 or 28.6 μm; wall thickness, 37.5 μm; length, 150 mm) were produced from a fluorinated ethylenepropylene copolymer at Hirakawa Hewtech (Ibaraki, Japan) as described by Kubota et al. (16). One end of the narrow tube was connected to a reservoir of the HbV/RBC or Hb/RBC suspension. The narrow tube was placed horizontally between two wide acrylic plates with a short gap (~2 mm) on the stage of an inverted microscope (IMT-2; Olympus Optics; Tokyo). The suspension in the reservoir was gently and continuously mixed with a magnetic stirrer. The gap between the two acrylic plates was filled with nitrogen-bubbled saline that contained 10 mM sodium hydrosulfite ($Na_2S_2O_4$; Wako Pure and Fine Chemical; Tokyo). The narrow tube was not permeable to sodium hydrosulfite. The HbVs distributed homogeneously in the narrow tube so that it was difficult to determine the marginal HbV-free layer. Therefore, the thickness of the marginal RBC-free layer, the distance between the tube inner wall and a nearest RBC, was measured at 50 points using an image-processing technique (35), and the values were averaged. The perfusion pressure was monitored with a sensor (P-231D; Nihon Kohden; Tokyo) equipped with an amplifier (AP-601G; Nihon Kohden). The entire perfusion experiment was performed at 25°C.

Table 1. *Physicochemical properties of Hb solutions and HbV and RBC suspensions*

	Hb15	Hb29	HbV_{HSA}	HbV_{HES}	RBC
[Hb], g/dl	10	10	10	10	10
Hb/PLP	1:1	1:3	1:2.5	1:2.5	
P_{50}, Torr	15	29	28	28	28
Diameter, nm	7	7	252 ± 53	252 ± 53	~8,000
Suspending medium	Saline	Saline	5 g/dl HSA	6 g/dl HES	5 g/dl HSA
Viscosity					
cP at 300 s^{-1}	1	1	3.5	5.1	3.0
cP at 71 s^{-1}	1	1	3.9	5.7	3.9

Values are means ± SD for diameter measurements of hemoglobin (Hb) vesicles (HbV). Hb and HbV viscosity was measured with a capillary viscometer; red blood cell (RBC) viscosity was measured with a cone-plate viscometer. Hb15 and Hb29, purified Hb with P_{50} values of 15 and 29 Torr, respectively; HbV_{HSA} and HbV_{HES}, HbVs suspended with human serum albumin (HSA) or hydroxylethyl starch (HES), respectively; [Hb], Hb concentration; PLP, pyridoxal 5′-phosphate.

Measurement of So_2 in narrow tubes. The apparatus (35) consisted of an inverted microscope with an objective lens of ×40 magnification (ULWD CDPlan 40PL; Olympus Optics), a scanning-grating spectrophotometer with a sensitive photon-counting detector that counted 1×10^3 to 3×10^6 photons/s (USP-410; Unisoku; Osaka), and a computer (PC-386V; Epson; Tokyo). The light intensity of a halogen lamp in the microscope was controlled by a current stabilizer (NLO 18-10; Takasago; Tokyo). The spectrophotometer was connected to the microscope eyepiece through a thin light guide (diameter, 0.4 mm) made of quartz and was operated by the computer. The grating was scanned in 508 steps over the wavelength range of 499.2–600.8 nm with a gate time of 20 ms/step for photon counting; data were obtained every 0.2 nm. One visible spectrum from a 5-μm-diameter spot over the centerline of the narrow tube was recorded within 20 s. A measuring spot on the narrow tube within the visual field of the microscope was fixed on a monitor (PVM-1371; Sony; Tokyo) through a video camera attached to the vertical eyepiece (DXC-930; Sony) by sliding the stage of the microscope and/or by rotating the cylindrical mirror. The centerline flow velocity was determined using the cross-correlation technique (2, 15), and the velocity was adjusted to 1.0 mm/s by changing the pressure applied to the reservoir of HbV/RBC or Hb/RBC mixtures. Under these conditions, the Reynolds number was 0.025 and the laminar flow was theoretically established.

The spectroscopic measurements were performed at traveling distances of 15, 30, 50, 80, and 100 mm. After a steady flow of RBCs and a steady oxygenation state of the flowing RBCs and HbVs (or Hbs) were attained a few minutes later, 10 scans were accumulated. Numerical filtering was then applied with a moving average of 5 successive values (e.g., absorbances at 499.6, 499.8, 500.0, 500.2, and 500.4 nm) in the scanning step to obtain a smoothed absorption spectrum (used to improve the signal-to-noise ratio). One scan of RBCs flowing in the narrow tube showed a remarkable deviation in absorbance values (Fig. 1*A*). On the other hand, HbVs showed less deviation due to the homogeneous dispersion of the vesicles. The accumulation of 10 scans followed by the moving averaging of 5 successive values in the scanning step of every 0.2 nm provided a smooth spectrum even for the RBC suspension (Fig. 1*B*). However, owing to the light scatter by fine particles, the absorbance of the HbVs at a shorter wavelength was slightly higher than that at a longer wavelength (29).

In the spectra of the 100%-deoxygenated and 100%-oxygenated HbVs, two isosbestic points (522 and 586 nm; Ref. 44) in the Q band of Hb were connected by a straight line as the baseline (Fig. 1*C*). The absorbances at 555 nm (ΔA_{555}, λ_{max} of deoxyHb) and 576 nm (ΔA_{576}, λ_{max} of oxyHb) from the baseline were obtained to make a calibration line that shows the relationship between the So_2 (in %) and the ratio of the two absorbances ($R = \Delta A_{555}/\Delta A_{576}$) as $So_2 = (35 - 15R)/(0.25R + 0.21)$. The So_2 values of each sample were plotted versus the traveling distance.

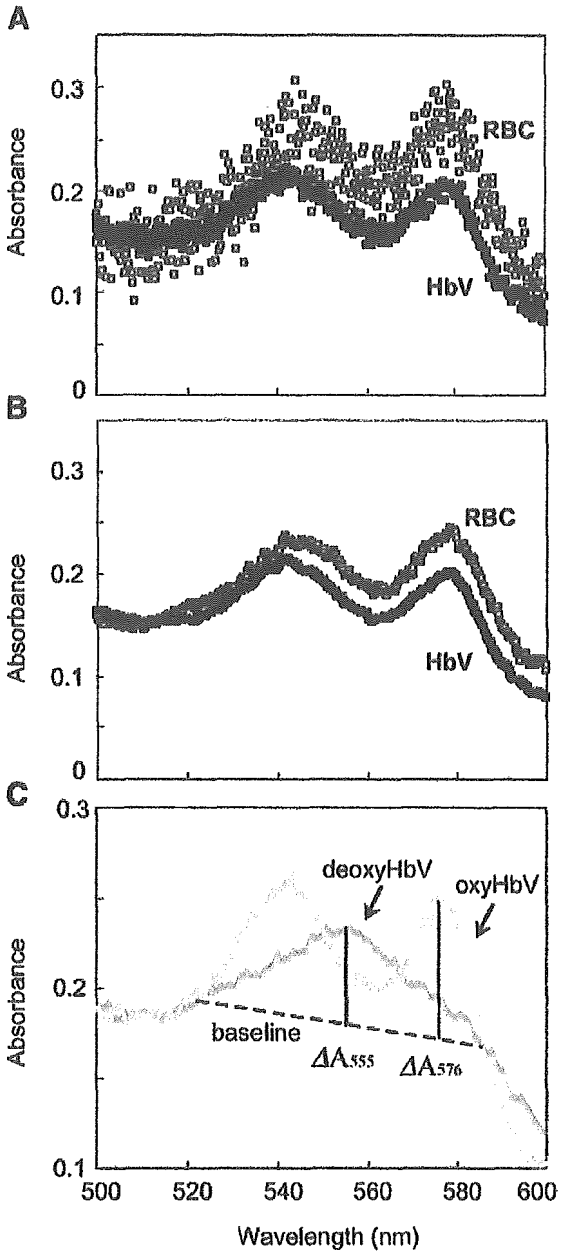


Fig. 1. *A*: one scan of the red blood cell (RBC) suspension showed a significantly scattered spectrum, whereas hemoglobin (Hb) vesicles (HbVs) showed a smooth spectrum. *B*: accumulation of 10 scans followed by the moving average of 5 successive values (using a 0.2-nm scanning step) provided enough resolution to calculate the O_2 saturation (So_2). *C*: for So_2 determination, two isobestic points of the spectra of deoxygenated (deoxy-) and oxygenated (oxy)HbVs (at 522 and 586 nm) were connected by a straight line (baseline). On the basis of the absorbances at 555 (ΔA_{555}, λ_{max} of deoxyHb) and 576 (ΔA_{576}, λ_{max} of oxyHb) nm from the baseline, a relationship between So_2 and the ratio of the two absorbances ($R = \Delta A_{555}/\Delta A_{576}$) was expressed as So_2 (in %) = $(35 - 15R)/(0.25R + 0.21)$.

RESULTS

Flow patterns of HbV/RBC mixtures in narrow tube. Figure 2 shows a microscopic view of the mixtures of RBCs and HbV_{HSA}s flowing in the narrow tubes. The thickness of the RBC-free marginal layer increased with increasing mixing ratio of HbV_{HSA}, and the layer seemed to be slightly turbid and dark colored due to the presence of HbV particles with a 250-nm diameter (29). The thickness of the RBC-free layer was 2.7 ± 1.7 μm for RBCs alone (100RBC), 3.5 ± 1.8 μm for $10HbV_{HSA}$/90RBC, 4.8 ± 2.2 μm for $50HbV_{HSA}$/50RBC, and 7.0 ± 1.6 μm for $90HbV_{HSA}$/10RBC. On the other hand, the mixture of RBCs and the Hb solution produced a transparent layer, but the distribution of RBCs was not

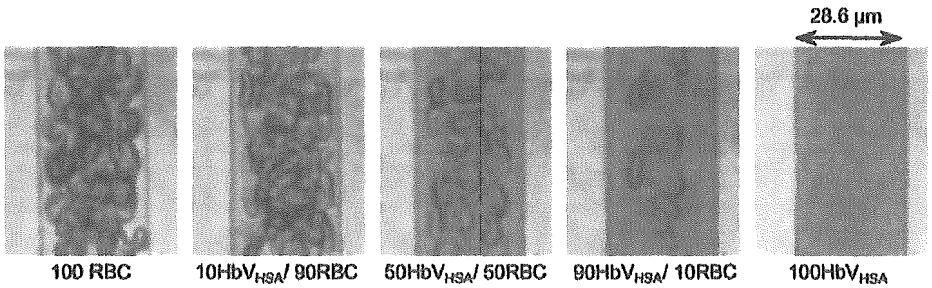


Fig. 2. Flow patterns of RBCs mixed with HbVs suspended in recombinant human serum albumin (HbV$_{HSA}$/RBC) in a narrow tube. HbV particles were homogeneously dispersed in a suspension medium. They tended to distribute in the marginal zone of the flow. Thickness of the RBC-free layer increased with increasing amount of HbV$_{HSA}$. RBC-free phase became darker and more semitransparent, which indicates the presence of HbVs. Tube diameter, 28 μm; Hb concentration ([Hb]), 10 g/dl; centerline flow velocity, 1 mm/s.

changed significantly compared with the HbV/RBC mixtures (data not shown).

Perfusion pressure of narrow tube. The perfusion pressure of the RBC suspension mixed with HbV$_{HSA}$, HbV$_{HES}$, or Hb29 in various ratios was examined at the constant centerline flow velocity of 1 mm/s (Fig. 3). The addition of the Hb29 solution to the RBC suspension in HSA (3.9 cP; Table 1) decreased the perfusion pressure from 10 kPa (100RBC) to 6 kPa (90Hb29/10RBC) due to the lower viscosity of the Hb solution (1.3 cP at 71 s^{-1}). On the other hand, the addition of HbVs increased the perfusion pressure in proportion to the HbV/RBC mixing ratio. Especially, the perfusion pressure (41 kPa) of the mixture of 90% HbV$_{HES}$s (5.7 cP; Table 1) and 10% RBC (3.9 cP) was more than four times higher than that of RBCs alone (10 kPa).

Spectroscopic changes of HbV/RBC and Hb/RBC mixtures. Figure 4 shows the representative spectroscopic changes of the mixtures of RBCs with HbVs or Hbs at various traveling distances in the narrow tubes. The most significant change was observed for the mixture of 90 vol% Hb29 solution and 10 vol% RBCs (90Hb29/10RBC). Two characteristic peaks of oxyhemoglobin in the Q band significantly decreased with traveling distance, and at 100 mm, the two peaks were flattened. On the other hand, the 90HbV$_{HSA}$/10RBC mixture showed slight changes, but the changes were almost identical to those observed for the 100RBC suspension. The addition of 10 vol% Hb29s to 90 vol% RBCs (to make 10Hb29/90RBC) did not seem to significantly change the spectrum.

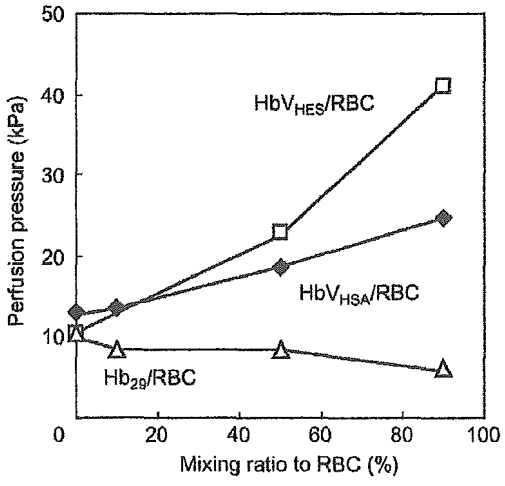


Fig. 3. Perfusion pressure measurements of RBCs mixed with HbV$_{HSA}$ (HbV$_{HSA}$/RBC), hydroxylethyl starch (HbV$_{HES}$/RBC), or purified Hb and added pyridoxal 5′-phosphate (PLP), which has a P_{50} value of 29 Torr (Hb29/RBC). [Hb] flowing in the narrow tube, 10 g/dl; centerline flow velocity, 1 mm/s.

O_2 release from HbV/RBC mixtures. According to the calibration line for the So_2, the So_2 values (in %) were plotted vs. the traveling distances. Figure 5 summarizes the O_2 release from the HbV/RBC mixtures in various mixing ratios. The rates of O_2 release from all the mixtures were similar to that of 100RBC, and ~40% of the O_2 was released at the traveling distance of 100 mm.

The influence of the suspending medium of HbV on O_2 release was compared using HbV$_{HSA}$ and HbV$_{HES}$ in the mixture of 90 vol% HbVs and 10 vol% RBCs (Fig. 6). Irrespective of the suspending medium (HSA and HES), the rates of O_2 release were almost identical for the two mixtures.

O_2 release from Hb/RBC mixtures. Contrary to the results with the HbV/RBC mixtures, the Hb/RBC mixtures showed faster O_2 release rates than RBCs alone at the mixing ratio of 50 vol% for Hb29 and 90 vol% for both Hb29 and Hb15 (Fig. 7). The Hb29 (P_{50} = 29 Torr) possessed a similar P_{50} value as HbV (P_{50} = 30 Torr; Table 1). The 10Hb29/90RBC mixture did not show any apparent change in the O_2 release compared with RBCs alone. However, the addition of 50Hb29/50RBC clearly showed a faster O_2 release. The 90Hb29/10RBC mixture showed a much faster release rate, and 55% of the O_2 was released at the 100-mm traveling distance. Addition of Hb15, which has a higher O_2 affinity (P_{50} = 15 Torr), also enhanced the O_2 release, but the enhancement was less than that of Hb29 as evaluated for the 50Hb15/50RBC mixture. However, at the mixing ratios of 10 and 90 vol%, there were no apparent differences in enhancement of O_2 unloading between Hb15 and Hb29.

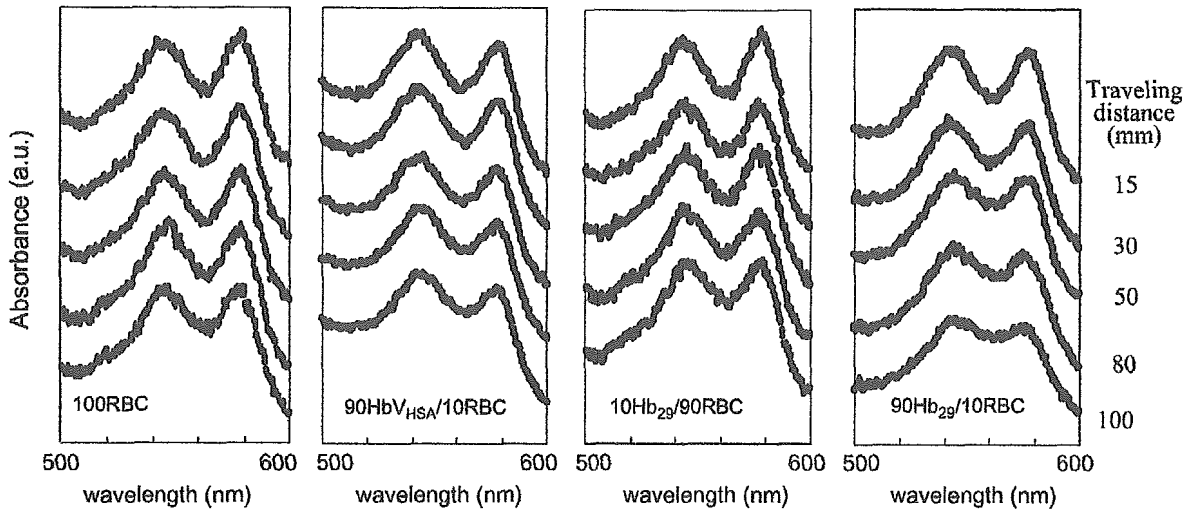


Fig. 4. Representative spectroscopic changes in Q bands of 100% RBCs and various HbV_{HSA}/RBC and Hb29/RBC mixtures compared with traveling distance in the narrow tube. Mixture of 90% Hb29 with 10% RBCs (90Hb29/10RBC) showed significant reduction in the two characteristic peaks attributed to oxyHb. Mixtures shown: 90HbV_{HSA}/10RBC, 90% HbV_{HSA} mixed with 10% RBCs; 10Hb29/90RBC, 10% Hb29 mixed with 90% RBCs.

DISCUSSION

The principal finding of this study using the narrow O_2-permeable tube is that the rate of O_2 release from HbVs is similar to that from RBCs at a constant [Hb] of 10 g/dl. On the other hand, acellular, unmodified Hb showed faster O_2 release rates. This property of HbVs is outstanding compared with previous findings for O_2 release rates measured by the stopped-flow method. The stopped-flow analysis confirmed that HbVs released O_2 significantly faster than RBCs and slower than acellular, unmodified Hbs (30). Vandegriff and Olson (45) also examined the O_2 release of RBCs of different sizes (diameter, 4.32–50 μm) from various species and of LEHs (diameter, 200 nm, which is similar in size to HbVs). They clarified that the O_2 release rate depended primarily on the cell surface area-to-volume ratio and the intracellular [Hb] and that LEHs had the fastest release rate. These discrepancies of the results between the perfusion study using the O_2-permeable tube and the stopped-flow methods should be mainly due to the differences in the experimental conditions. The stopped-flow studies were performed with homogeneous solutions at the low [Hb] of 10 μM (0.065 g/dl) in a mixing cuvette in which the dissociated O_2 immediately diminished with sodium hydrosulfite and the O_2 gradient between intra- and extracellular compartments was extremely high. In the present study using the O_2-permeable tube, the [Hb] was as high as 1.6 mM (10 g/dl) with a high viscosity, and a certain kind of O_2 gradient between the center of the tube and the tube wall would be formed. Therefore, the present experimental conditions were much closer to the in vivo physiological conditions, although the wall of the narrow tube was unavoidably thick.

We confirmed under our experimental conditions that acellular, unmodified Hb shows a much faster O_2 unloading rate than RBCs as reported by other researchers (18, 20). The differences in the rates between

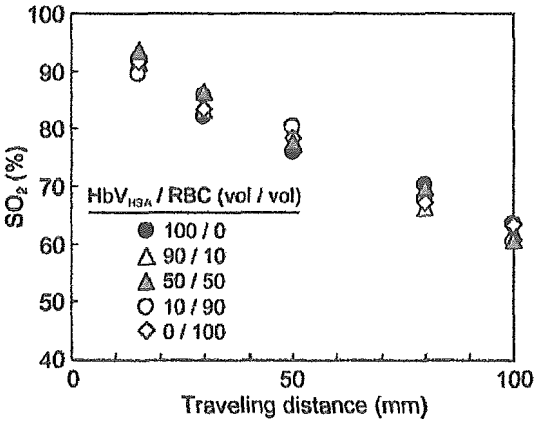


Fig. 5. O_2 release from the HbV_{HSA}/RBC mixture for different mixing ratios determined at various traveling distances.

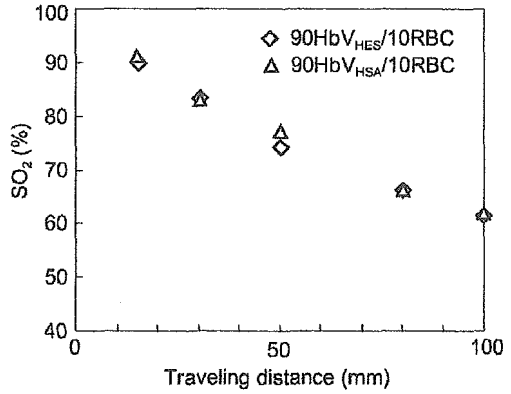


Fig. 6. Effects of suspending media on O_2 release from HbV/RBC mixture: comparison of 90HbV_{HSA}/10RBC and 90% HbV_{HES} with 10% RBCs (90HbV_{HES}/10RBC) determined at various traveling distances.

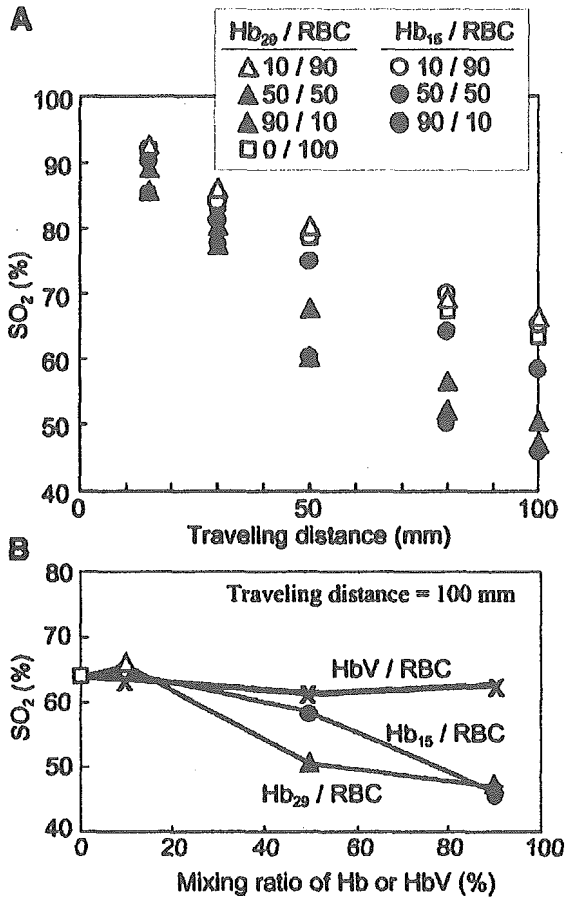


Fig. 7. *A*: effects of O_2 affinity on O_2 release from Hb/RBC mixtures at various traveling distances: comparison of Hb29/RBC and Hb15/RBC mixtures of various ratios. *B*: effects of Hb/RBC or HbV/RBC mixing ratios on S_{O_2}: data from *A* for Hb/RBC and from Fig. 5 for HbV/RBC at the traveling distance of 100 mm. Hb15, purified Hb with pyridoxal 5′-phosphate (PLP) added, which has a P_{50} value of 15 Torr.

the acellular Hb and HbVs could be explained by the theory of facilitated O_2 transport by the diffusion of O_2-bound Hb molecules (1, 5, 7). The total O_2 flux is the sum of the fluxes of free O_2 molecules and O_2 bound as HbO_2. The diffusion of HbO_2 contributes to the facilitated O_2 transport. According to the Stokes-Einstein equation, the diffusion constant (D_{HbO_2}) is defined as

$$D_{HbO_2} = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r} \qquad (1)$$

where k is the Boltzman constant and T is absolute temperature. This equation demonstrates that the diffusion of a macromolecule, i.e., the contribution of facilitated O_2 diffusion, is inversely proportional to the viscosity of the solution (η) and the radius of the macromolecule (r); therefore, the contribution of facilitated O_2 diffusion is inversely proportional to the size of the carriers and the viscosity (18).

Nishide et al. (19) studied O_2 diffusion across a nonflowing solution membrane, and they found that the presence of Hb molecules in a solution (<30 g/dl) significantly facilitated O_2 transport. When comparing HbVs, unmodified human Hb (HbAo), and polymerized Hbs, the "apparent" D_{HbO_2} values are in the following order: HbAo (9×10^{-7} cm^2/s) > polyHbs (2×10^{-7} cm^2/s) > HbVs (3.5×10^{-8} cm^2/s) at [Hb] = 10 g/dl, with solution viscosity values ($1.0 < 1.8 < 3.2$ cP, respectively) and molecular sizes ($7 < \sim 15 < 200$ nm, respectively) that are in inverse order in accordance with *Eq. 1*. As a result, facilitated O_2 transport is significantly reduced for the suspension of HbVs.

According to the study of McCarthy et al. (18), a PEG-conjugated Hb (PEG-Hb) shows a similar O_2 release rate with RBCs in an artificial microflow channel, whereas the acellular cross-linked Hbs and HbAo show faster rates. The PEG chains should not be a barrier layer to the O_2 release. The main difference in the O_2 release between PEG-Hb and other acellular Hbs should be due to the diffusion of Hb and the viscosity: PEG-Hb has a larger molecular size (28 vs. 7 nm) and a higher viscosity (3.2 vs. 0.9 cP) than the Hb molecule. These properties of PEG-Hb may reduce the O_2 unloading, because the diffusion constant of PEG-Hb should be lower than that of an unmodified Hb. The higher O_2 affinity of PEG/Hb (P_{50} = 10 Torr) should also contribute to the slower O_2 unloading, because we confirmed that the Hb15/RBC mixture showed a slower O_2 unloading than did the Hb29/RBC mixture at a 50:50 mixing ratio. Attention should be paid to the theory that facilitated O_2 transport ([O_2] facilitated) decreases with increasing P_{50} according to (1, 19)

$$[O_2]\ \text{facilitated} \propto \frac{[Hb]_0 \cdot D_{Hb}}{1/K + Po_2} \qquad (2)$$

where $[Hb]_0$ is the total [Hb], Po_2 is the O_2 partial pressure, D_{Hb} is the diffusion coefficient of Hb, and K is the equilibrium constant of the O_2 binding reaction (its reciprocal is proportional to the P_{50} value). Accordingly, the faster O_2 release rate for Hb29 than for Hb15 is not due to the higher level of facilitated O_2 transport but is simply a result of the lower O_2 affinity.

When a large amount of HbV is exchange transfused, the addition of a plasma expander is required, because the oncotic pressure of the HbV suspension is close to zero (26). HbV_{HES} shows a higher viscosity (5.7 cP at 71 s^{-1}) compared with HbV_{HSA} (3.9 cP), because the interaction of HbV particles with extended HES chains is possibly stronger than that with globular HSA molecules. Using the optical microscope, however, we could not observe the HbV aggregates in the suspension; this was possibly due to the low molecular weight of HES (70,000). The marginal RBC-free layer formed by axial accumulation of RBCs is important for the maintenance of blood flow as a lubricating layer (3). The addition of HbV_{HSA} to RBCs increased the thickness of the RBC-free layer from 2.7 ± 1.7 (100RBC) to 7.0 ± 1.6 μm (90HbV_{HSA}/10RBC). This increase was due to preferential flow of smaller particles, HbVs, near the peripheral region of the flow channel (4) and reduction of RBCs (i.e., reduction of hematocrit) (31). Furthermore, the perfusion pressure was increased at a con-

stant flow rate, because the addition of the viscous HbV_{HES} and HbV_{HSA} suspensions increased the viscosity of the RBC-free lubricating layer. The increase in the perfusion pressure was more evident for the more viscous HbV_{HES} than for HbV_{HSA} possibly owing to the differences in the molecular conformations of HES and HSA; also, differences in their concentrations also partly contributed to the perfusion pressure values (37). Despite the viscosity difference between the two suspensions, O_2 release rates from the RBC mixtures were not significantly altered in the present experimental conditions. It has been confirmed (35) that the diffusion constant of the O_2 molecule is not significantly influenced by medium viscosity. The medium viscosity may influence the flow behavior of HbVs in a narrow tube. However, a homogeneous distribution of HbV_{HES} and HbV_{HSA} in the narrow tube was observed by cross-sectional scanning of the narrow tube during flow, and the vesicle-free layer was hardly observed in the marginal region. Therefore, homogeneous distribution of HbVs in the narrow tube and the identical O_2 affinity of the vesicles provided identical O_2 release rates from HbV_{HES} and HbV_{HSA} under a constant flow velocity. This phenomenon also supports the fact that the contribution of HbVs to facilitated O_2 transport by diffusion of HbVs is significantly lower than acellular Hbs; thus viscosity does not influence O_2 unloading. We conclude that under the present experimental conditions, differences in medium viscosity affect the perfusion pressure but not the O_2 release from HbVs and/or RBCs.

The low contribution of HbVs to facilitated O_2 transport could explain the similar O_2 release rates for HbVs (250 nm) and RBCs (8 μm). Under conditions with little facilitated O_2 transport for both HbV and RBC suspensions, only the O_2 affinity (P_{50} value) would be the determining factor for the O_2 release rate, because the P_{50} values for HbVs and RBCs were the same (28 Torr) in the present study. This speculation has to be confirmed in a future study of HbVs and RBCs with different P_{50} values.

Experiments of Page et al. (*experiment A;* Ref. 20) and ours (*experiment B*) showed the enhancement of O_2 unloading by addition of acellular Hb solutions. However, there are some discrepancies between the two groups. *Experiment A* showed that the addition of only 10 vol% of Hb solution to the RBC suspension enhanced O_2 release, whereas in *experiment B*, significant enhancement was observed with addition of 50 vol% of Hb solution, and enhancement was hardly observed for addition of 10 vol% of Hb solution. Furthermore, the reduction of So_2 in *experiment A* was much faster than in *experiment B* despite the identical Hb concentration (10 g/dl) and similar tube diameter (25 μm). For example, the 60% So_2 level of RBCs was attained after 100 s and 100 mm of traveling in *experiment B*, whereas the same So_2 level was attained after <0.2 s and only 4 mm of traveling distance in *experiment A*; the flow velocity was much faster in *experiment A* (~30 mm/s) than in *experiment B* (1.0 mm/s).

These differences between *experiments A* and *B* may be explained as follows. *1*) O_2 permeability of microflow channels: the silicone tube has a higher O_2 permeability [~1,000–6,000 × $cm^3(STP)\cdot cm^{-2}\cdot cm^{-1}\cdot s^{-1}\cdot mmHg^{-1} \times 10^{10}$] compared with the fluorinated ethyleneepropylene copolymer tube [59 × $cm^3(STP)\cdot cm^{-2}\cdot cm^{-1}\cdot s^{-1}\cdot mmHg^{-1} \times 10^{10}$] used in *experiment B*. Thus the silicone tube extracts O_2 from the RBC suspension very rapidly by exposure to the deoxygenated environment. *2*) Temperature: *experiment A* was conducted at 37°C, whereas *experiment B* was performed at 25°C. The higher temperature results not only in a higher P_{50} value (22) but also in more facilitation of the process of O_2 unloading in *experiment A*. *3*) Flow velocity: measurements in *experiment B* were carried out when the RBC flow and oxygenation attained steady state at a flow velocity of 1 mm/s. However, the measured So_2 of the flowing RBCs at an observation point showed the RBCs to be in the process of O_2 unloading. In this condition, the measured So_2 would be higher than in the RBC-O_2 equilibrium state. The O_2 distribution in the tube has a parabolic shape; therefore, O_2 is transferred from the centerline toward the wall of the tube. Such an O_2 gradient in the tube may be more prominent at a higher flow velocity and with higher O_2 permeability of the tube as in *experiment A*, as there would be less time for O_2 unloading. Conversely, the thicker marginal RBC-free layer formed at the faster flow velocity may make the Hb-induced facilitation of O_2 release more evident. *4*) Measurement of So_2: in *experiment B*, So_2 was measured on a 5-μm-diameter spot over the centerline of the narrow tube in which O_2 was retained for a longer traveling distance, whereas in *experiment A*, the So_2 was measured for the entire section of the flow channel. However, we confirmed that the So_2 at the center was not significantly different from that at a point a half-radius distance from the center after 100 mm of travel. In relation to the wavelength for the spectroscopy, the measurement in the Soret band (400–450 nm) in *experiment A* was remarkably influenced by the light scattering of the RBCs. On the other hand, the measurement in the Q band with a longer wavelength in *experiment B* was less influenced by the light scattering. These differences in spectroscopy do not seem to provide any quantitative differences in the O_2 release rate. In conclusion, the quantitative difference between O_2 release in *experiments A* and *B* is mainly due to the difference in the O_2 permeability of the flow channels and partly due to the differences in temperature and flow velocity.

It has been suggested that faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation (20). However, this concept is controversial with regard to the recent findings, because an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders (1, 23, 41). We confirmed that HbVs do not induce vasoconstriction and hypertension. This is not only owing to the reduced inactivation of nitric oxide as an endothelium-derived vasorelaxation factor (24) but also possibly due to the moderate O_2 release rate that is similar to RBCs as confirmed in this

study. Very recently, Erni et al. (9, 12) demonstrated that HbVs suspended in dextran effectively oxygenated ischemic collaterized tissue in skin flaps. This phenomenon should be explained by the functional characteristics of HbVs; namely, HbV suspension continues to retain O_2 in upstream vessels and reaches the ischemic tissue to release the O_2 (9, 12). In this regard, it is expected that HbVs with a lower P_{50} value would show much slower O_2 unloading, which would be advantageous for ischemic tissue oxygenation. Moreover, the higher viscosity and the resulting higher perfusion pressure (as shown in Fig. 3) would be beneficial to increase the shear stress on the vascular wall for vasorelaxation and to homogeneously transmit the pressure to the microvascular network and thus supply blood to whole capillaries (39). Our experimental results contribute importantly to the design and optimization of O_2 carriers and suggest the possible utilization of HbVs for new clinical indications other than blood substitution.

The authors are grateful to Dr. N. Tateishi (Ehime University) for setting up the microscope for the measurements and for the computer programming and to Dr. M. Intaglietta (University of California, San Diego) for promoting this study.

DISCLOSURE

This work was supported by Health Sciences Research Grants (Research on Pharmaceutical and Medical Safety, Artificial Blood Project); the Ministry of Health, Labor, and Welfare, Japan, and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B12480268); and 21 COE, Practical Nano-Chemistry, from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

REFERENCES

1. **Baines AD, Adamson G, Wojciechowski P, Pliura D, Ho P, and Kluger R.** Effect of modifying O_2 diffusivity and delivery on glomerular and tubular function in hypoxic perfused kidney. *Am J Physiol Renal Physiol* 274: F744–F752, 1998.
2. **Baker M and Wayland H.** On-line volume flow rate and velocity profile measurement for blood in microvessels. *Microvasc Res* 7: 131–143, 1974.
3. **Bayliss LE.** The flow of RBCs in capillary tubes. Changes in the cell-free marginal sheath with changes in the shearing stress. *J Physiol (Lond)* 175: 1–25, 1965.
4. **Beck MR Jr and Eckstein EC.** Preliminary report on platelet concentration in capillary tube flows of whole blood. *Biorheology* 17: 455–464, 1980.
5. **Bouwer ST, Hoofd L, and Kreuzer F.** Diffusion coefficients of oxygen and hemoglobin measured by facilitated oxygen diffusion through hemoglobin solutions. *Biochim Biophys Acta* 1338: 127–136, 1997.
6. **Chang TMS.** *Blood Substitutes: Principles, Methods, Products, and Clinical Trials.* Basel: Karger, 1997.
7. **Chen N, Nishide H, and Tsuchida E.** Analysis of facilitated oxygen transport in a liquid membrane of hemoglobin. *Bull Chem Soc Jpn* 69: 255–259, 1996.
8. **Coin JT and Olson JS.** The rate of oxygen uptake by human red blood cells. *J Biol Chem* 254: 1178–1190, 1979.
9. **Contaldo C, Schramm S, Wettstein R, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A, and Erni D.** Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am J Physiol Heart Circ Physiol* 285: H1140–H1147, 2003.
10. **Duling BR and Berne RM.** Longitudinal gradients in periarteriolar oxygen tension: a possible mechanism for the participation of oxygen in local regulation of blood flow. *Circ Res* 27: 669–678, 1970.
11. **Ellsworth ML, Ellis CG, Popel AS, and Pittman RN.** Roles of microvessels in oxygen supply to tissue. *News Physiol Sci* 9: 119–123, 1994.
12. **Erni D, Wettstein R, Schramm S, Sakai H, Takeoka S, Tsuchida E, Leunig M, and Banic A.** Normovolemic hemodilution with hemoglobin-vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am J Physiol Heart Circ Physiol* 284: H1702–H1709, 2003.
13. **Goda N, Suzuki K, Naito S, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, and Suematsu M.** Distribution of heme oxygenase isoform in rat liver: topographic basis for carbon monoxide-mediated microvascular relaxation. *J Clin Invest* 101: 604–612, 1998.
14. **Intaglietta M, Johnson PC, and Winslow RM.** Microvascular and tissue oxygen distribution. *Cardiovasc Res* 32: 632–643, 1996.
15. **Intaglietta M, Silverman NR, and Tompkins WR.** Capillary flow velocity measurements in vivo and in situ by television method. *Microvasc Res* 10: 165–179, 1975.
16. **Kubota K, Tamura J, Shirakura T, Kimura M, Yamanaka K, Isozaki T, and Nishio I.** The behaviour of red cells in narrow tubes in vitro as a model of the microcirculation. *Br J Haematol* 94: 266–272, 1996.
17. **Maeda N.** Erythrocyte rheology in microcirculation. *Jpn J Physiol* 46: 1–14, 1996.
18. **McCarthy MR, Vandegriff KD, and Winslow RM.** The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers. *Biophys Chem* 92: 103–117, 2001.
19. **Nishide H, Chen XS, and Tsuchida E.** Facilitated oxygen transport with modified and encapsulated hemoglobin across non-flowing solution membrane. *Artif Cells Blood Substit Immobil Biotechnol* 25: 335–346, 1997.
20. **Page TC, Light WR, McKay CB, and Hellums JD.** Oxygen transport by erythrocyte/hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance. *Microvasc Res* 55: 54–66, 1998.
21. **Phillips WT, Lemen L, Goins B, Rudolph AS, Klipper R, Fresne D, Jerabek PA, Emch ME, Martin C, Fox PT, and McMahan CA.** Use of oxygen-15 to measure oxygen-carrying capacity of blood substitutes in vivo. *Am J Physiol Heart Circ Physiol* 272: H2492–H2499, 1997.
22. **Reeves RB.** The effect of temperature on the oxygen equilibrium curve of human blood. *Respir Physiol* 42: 317–328, 1980.
23. **Rohlfs RJ, Bruner E, Chiu A, Gonzales A, Gonzales ML, Magde D, Magde MD Jr, Vandegriff KD, and Winslow RM.** Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. *J Biol Chem* 273: 12128–12134, 1998.
24. **Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, and Intaglietta M.** Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension in conscious hamster model. *Am J Physiol Heart Circ Physiol* 279: H908–H915, 2000.
25. **Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E.** Hemoglobin vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* 159: 1079–1088, 2001.
26. **Sakai H, Masada Y, Horinouchi H, Takeoka S, Kobayashi K, and Tsuchida E.** Hemoglobin vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med.* In press.
27. **Sakai H, Masada Y, Takeoka S, and Tsuchida E.** Characteristics of bovine hemoglobin for the potential source of hemoglobin-vesicles as an artificial oxygen carrier. *J* Biochem 131: 611–617, 2002.
28. **Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, and Tsuchida E.** Systemic and microvascular responses to hemorrhagic shock and resuscitation with Hb vesicles. *Am J Physiol Heart Circ Physiol* 283: H1191–H1199, 2002.
29. **Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, and Tsuchida E.** Pretreatment of serum

containing Hb vesicles (oxygen carriers) to avoid their interference in laboratory tests. *Clin Chem Lab Med* 41: 222–231, 2003.
30. **Sakai H, Tsai AG, Rohlfs RJ, Hara H, Takeoka S, Tsuchida E, and Intaglietta M.** Microvascular responses to hemodilution with Hb vesicles as red cell substitutes: influence of O_2 affinity. *Am J Physiol Heart Circ Physiol* 276: H553–H562, 1999.
31. **Shirasawa T, Izumizaki M, Suzuki YI, Ishihara A, Shimizu T, Tamaki M, Huang F, Koizumi KI, Iwase M, Sakai H, Tsuchida E, Ueshima U, Inoue H, Koseki H, Senda H, Kuriyama T, and Homma I.** Oxygen affinity of hemoglobin regulates O_2 consumption, metabolism, and physical activity. *J Biol Chem* 278: 5035–5043, 2003.
32. **Sou K, Naito Y, Endo T, Takeoka S, and Tsuchida E.** Effective encapsulation of proteins into size-controlled phospholipid vesicles using the freeze-thawing and extrusion. *Biotechnol Progr.* First published 25 July 2003; 10.1021/bp0201004.
33. **Takeoka S, Ohgushi T, Terase K, Ohmori T, and Tsuchida E.** Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir* 12: 1755–1759, 1996.
34. **Tateishi N, Maeda N, and Shiga T.** A method for measuring the rate of oxygen release from single microvessels. *Circ Res* 70: 812–819, 1992.
35. **Tateishi N, Suzuki Y, Cicha I, and Maeda N.** O_2 release from erythrocytes flowing in a narrow O_2-permeable tube: effects of erythrocyte aggregation. *Am J Physiol Heart Circ Physiol* 281: H448–H456, 2001.
36. **Tateishi N, Suzuki Y, Shirai M, Cicha I, and Maeda N.** Reduced oxygen release from erythrocytes by the acceleration-induced flow shift, observed in an oxygen-permeable narrow tube. *J Biomech* 35: 1241–1251, 2002.
37. **Tateishi N, Suzuki Y, Soutani M, and Maeda N.** Flow dynamics of erythrocytes in microvessels of isolated rabbit mesentery: cell-free layer and flow resistance. *J Biomech* 27: 1119–1125, 1994.
38. **Tateishi N, Suzuki Y, Tanaka J, and Maeda N.** Imaging of oxygen saturation and distribution of erythrocytes in microvessels. *Microcirculation* 4: 403–412, 1997.
39. **Tsai AG and Intaglietta M.** High viscosity plasma expanders: volume restitution fluid for lowering the perfusion trigger. *Biorheology* 38: 229–237, 2001.
40. **Tsai AG, Johnson PC, and Intaglietta M.** Oxygen gradients in the microcirculation. *Physiol Rev* 83: 933–963, 2003.
41. **Tsai AG, Kerger H, and Intaglietta M.** Microcirculatory consequences of blood substitution with αα-hemoglobin. In: *Blood Substitutes: Physiological Basis of Efficacy*, edited by Winslow RM, Vandegriff K, and Intaglietta M. Boston, MA: Birkhauser, 1995, p. 155–174.
42. **Tsuchida E, ed.** *Blood Substitutes: Present and Future Perspectives*. Amsterdam: Elsevier, 1998.
43. **Vadapalli A, Goldman D, and Popel AS.** Calculations of oxygen transport by red blood cells and hemoglobin solutions in capillaries. *Artif Cells Blood Substitutes Immobilization Biotechnol* 30: 157–188, 2002.
44. **van Assendelft OR.** *Spectrophotometry of Haemoglobin Derivatives*. Assen, The Netherlands: Royal Vangorcum, 1970.
45. **Vandegriff KD and Olson JS.** The kinetics of O_2 release by human red blood cells in the presence of external sodium dithionite. *J Biol Chem* 259: 12609–12618, 1984.

Prolonged Oxygen-Carrying Ability of Hemoglobin Vesicles by Coencapsulation of Catalase in Vivo

Yuji Teramura, Hideo Kanazawa, Hiromi Sakai, Shinji Takeoka, and Eishun Tsuchida*

Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan
Received April 23, 2003; Revised Manuscript Received August 21, 2003

Hemoglobin (Hb) vesicles (particle diameter, ca. 250 nm) have been developed as Hb-based oxygen carriers in which a purified Hb solution is encapsulated with a phospholipid bilayer membrane. The oxidation of Hb to nonfunctional ferric Hb (metHb) was caused by reactive oxygen species, especially hydrogen peroxide (H_2O_2), in vivo in addition to autoxidation. We focused on the enzymatic elimination of H_2O_2 to suppress the metHb formation in the Hb vesicles. In this study, we coencapsulated catalase with Hb within vesicles and studied the rate of metHb formation in vivo. The Hb vesicles containing 5.6×10^4 unit mL^{-1} catalase decreased the rate of metHb formation by half in comparison with Hb vesicles without catalase. We succeeded in prolonging the oxygen-carrying ability of the Hb vesicle in vivo by the coencapsulation of catalase.

INTRODUCTION

Ferrous hemoglobin (Hb) reversibly binds oxygen molecules to carry oxygen to terminal tissues and is gradually autoxidized to nonfunctional ferric Hb (metHb). In red blood cells, reduction systems such as NADH–cytochrome b_5, NADPH–flavin, glutathione, and ascorbic acid reduce metHb to ferrous Hb. Superoxide dismutase (SOD) and catalase exist in the cell to eliminate the superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2), respectively, and the percentage of metHb in red blood cells is normally maintained at less than 1.0% (*1*, *2*).

At present, several hemoglobin (Hb)-based oxygen carriers, which have been developed as red blood cell substitutes (*3*–*5*), are generally classified into two types: one is the acellular-type modified Hb molecules such as intramolecularly cross-linked Hb (*6*), recombinant cross-linked Hb (*7*), intermolecularly polymerized Hb (*8*), and poly(ethylene glycol) (PEG)-conjugated Hb (*9*). The other is a cellular-type Hb such as Hb vesicles (*10*) or liposome-encapsulated Hb (*11*), in which Hb molecules are encapsulated with a phospholipid bilayer membrane. Some of the acellular-type Hb modifications have advanced to phase III clinical trials (*12*, *13*). Though the Hb vesicles have not yet been clinically studied, their excellent oxygen-carrying ability and high safety have been confirmed in vivo (*10*, *14*–*19*).

When using Hb-based oxygen carriers, the oxidation of ferrous Hb to metHb is an important issue (*20*). Enzymes such as SOD and catalase, and enzymes in metHb reduction systems have been used to limit the metHb formation (*21*, *22*). In the case of acellular Hb, the rate of metHb formation in blood circulation was suppressed compared with that in vitro, because metHb was reduced by reductants contained in the plasma such as ascorbic acid and glutathione. For example, about 40% glutaraldehyde-polymerized bovine Hb was reported to be oxidized to metHb at 72 h after a 90% exchange transfusion in ovines, and no further increase in the metHb percentage was observed (*23*). In the dextran-conjugated Hb, the metHb percentage was maintained at 35%, 12 h after the 50% exchange transfusion in guinea pigs (*24*). On the other hand, in the Hb vesicles, the reductants in plasma are not available because of their low membrane permeability. Among the reactive oxygen species (ROS) such as nitric oxide (NO), $O_2^{-\bullet}$, and H_2O_2, which are known to promote metHb formation, H_2O_2 can permeate through the bilayer membrane, and a relatively large amount of H_2O_2 such as 4–5 μM is constantly generated in normal human plasma (*25*). Therefore, we considered that such exogenous H_2O_2 should be a cause of the metHb formation in addition to the autoxidation of Hb and endogenous H_2O_2 generated by the autoxidation of Hb in the Hb vesicles. Therefore, the metHb formation of Hb vesicles was expected to be suppressed by H_2O_2 elimination.

Although the rate of metHb formation was actually suppressed in vitro by the coencapsulation of catalase, the rate was dramatically increased in vivo. This was because catalase, which was purchased for laboratory use, was contaminated with LPS, and the resulting catalase-coencapsulated Hb vesicles were also highly contaminated with LPS (>10 EU mL^{-1}). We considered that the LPS would promote the rate of metHb formation in vivo because the inflammatory reaction would produce reactive oxygen species. In this study, we prepared the Hb vesicles coencapsulating LPS-free catalase (below 0.1 EU mL^{-1}) and administered the Hb vesicle dispersion to Wistar rats (20 mL kg^{-1}) to study the suppression of the rate of metHb formation by the coencapsulation of catalase.

MATERIALS AND METHODS

1. Removal of Lipopolysaccharide (LPS) from Catalase. A catalase solution (from bovine liver, Sigma, St Louis, MO) was mixed with a 10% Triton X-114 solution (Pierce Chemical, Rockford, IL) and incubated at 4 °C for 30 min. After the mixed solution was incubated at 37 °C for 40 min to separate into two phases, the aqueous phase containing catalase was centrifuged at 5000 rpm for 20 min, and the catalase solution was dialyzed against the water for injection at 4 °C for 24 h.

* To whom correspondence should be addressed. E-mail: eishun@waseda.jp.

10.1021/bc0340619 CCC: $25.00
Published on Web 10/29/2003

The determination of LPS was carried out with limulus assay (Limulus ES−II, Wako Pure Chemical, Osaka). The catalase solution was mixed with the LAL reagent, and the concentration of LPS was determined from the gelation time (Toxinometer ET-201, Wako Pure Chemical). The catalase activity was measured from the decreasing rate of the absorbance at 240 nm when the 0.06% (v/v) H_2O_2 solution (2.9 mL) was mixed with the catalase solution (0.1 mL) at 25 °C.

2. Preparation of Hb Vesicles Coencapsulating Catalase (*26*−*28*). Hb was purified from outdated human red blood cells donated from Japanese Red Cross Blood Center (*27*). After the hemolyzed solution was separated from stromata, the ligand exchange of Hb from O_2 to carbon monoxide (CO) was carried out by CO gas flowing. The proteins other than carbonylHb (HbCO) were denatured by heat treatment (60 °C for 12 h) and removed as precipitates. Pyridoxal 5′-phosphate (PLP, Sigma) as an allosteric effector was added to the HbCO solution (36 g dL^{-1}) at a 2.5:1 molar ratio of PLP to Hb, and the catalase solution was added to it. Mixed lipid [1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylchorine (DPPC, Nippon Fine Chemical, Osaka)/cholesterol (Nippon Fine Chemical)/1,5-dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA, Nippon Fine Chemical)/1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG (PEG molecular weight was 5000, Sunbright DSPE−50H, H-form, NOF Co., Tokyo), 5/5/1/0.033 by molar ratio] powder was dispersed with the HbCO solution, and the dispersion was stirred at 10 °C for 12 h. The resulting dispersion of multilamellar vesicles was extruded through the membrane filters (FM series, pore size; 3.00, 0.80, 0.65, 0.45, 0.30, 0.22 μm, Fuji Film Co., Tokyo) with a Remolino (Millipore Co., Ltd., Bedford, MA). The Hb vesicles with an average diameter of 250 nm were prepared after extrusion through the membrane filter with 0.22 μm pore size. After the separation of unencapsulated Hb by ultracentrifugation (10000*g*, 60 min), the precipitate of the Hb vesicles was redispersed into saline. Finally, the Hb concentration of the dispersion was adjusted to 10 g dL^{-1}. After Hb vesicles were solubilized with Triton X, the concentration of Hb was determined by a cyanometHb method. In this study, $[catalase]_{fed}$ was defined as the catalase concentration at the mixed solution of Hb and catalase used in the preparation of the Hb vesicles.

The particle diameter of the Hb vesicles was measured by a dynamic light scattering method (N4 PLUS, Beckman Coulter, Fullerton, CA).

3. Measurement of the Rate of metHb Formation of the Hb Vesicle in Vivo. Wistar rats (195−210 g) were used in the experiments. They were anesthetized with diethyl ether, and the Hb vesicle dispersion was administered to the tail vein (20 mL kg^{-1}). The sample was the Hb vesicles coencapsulating catalase (0−5.6 × 10^4 unit mL^{-1}). The blood withdrawn from the tail vein was centrifuged (12000*g*, 5 min) to collect the Hb vesicles in the upper phase of the precipitate. The metHb percentage was calculated based on the intensity ratio at 405 and 430 nm, which were identified as metHb and deoxyHb, respectively, under the nitrogen condition.

RESULTS AND DISCUSSION

The catalase from bovine liver purchased for laboratory use was contaminated with LPS and was not acceptable for in vivo administration. We tried to remove LPS from the catalase solution using a nonionic detergent, Triton X-114. A solution of Triton X-114, which has a cloud point at 20 °C, is homogeneous below this point and is separated into an aqueous phase and a detergent phase above that point (*29*). LPS is highly lipophilic and is transferred from an aqueous phase containing catalase into the organic phase of Triton X-114. We mixed a catalase solution (2.5 × 10^3 unit mL^{-1}) with a Triton X-114 solution (10%, v/v) at 4 °C and separated the mixed solution into two phases at 37 °C to collect the aqueous phase containing catalase. The LPS concentration of the resulting catalase solution was reduced to 0.0038 EU mL^{-1} from > 10 EU mL^{-1} without significant dilution. We coencapsulated this purified catalase with Hb in the vesicles.

We then examined the stability of the catalase activity in a solution state at 37 °C. The catalase activities after incubation for 24, 48, and 56 h were 82, 82, and 81%, respectively. The constant activity of around 80% at 37 °C for more than 56 h indicate that the catalase should be tolerable during the in vivo study because the circulation half-life of the Hb vesicles was around 24 h in rats (20 mL kg^{-1}).

The particle diameter of the Hb vesicles was 251 ± 80 nm, and the oxygen affinity (P_{50}) was 30 Torr. For all the Hb vesicles used in this study, oxyHb and metHb were more than 97% and less than 3%, respectively. Neither HbCO nor ferrylHb was detected. After the catalase-coencapsulated Hb vesicles were dissolved by the addition of Triton X, the resulting solution was analyzed by electrophoresis (IEF−PAGE). From the band of catalase, it was confirmed that the catalase was exactly coencapsulated in the Hb vesicles. We tried to measure the catalase activity of the Hb vesicles, however, due to the catalase-like activity of Hb, this activity could not be determined. Therefore, $[catalase]_{fed}$ was defined as the catalase concentration at the mixed solution of Hb and catalase in the preparation process.

The LPS concentration in the resulting Hb vesicle dispersion ([Hb] = 10 g dL^{-1}) was below 0.1 EU mL^{-1}, which was measured by limulus assay after the solubilization of the Hb vesicles with poly(ethylene glycol) 10-lauryl ether (*30*).

We studied the reactivity of the Hb vesicles against ROS ($O_2^{-\bullet}$ and H_2O_2). The rate of $O_2^{-\bullet}$ produced in a 106 μM hypoxanthine/0.32 unit mL^{-1} xanthine oxidase system was 0.9 μM s^{-1}, which was determined by cytochrome *c* (*31*). Catalase was also added to this system to eliminate H_2O_2 from the immediate dismutation of $O_2^{-\bullet}$ ($k = 8.5 \times 10^5$ M^{-1} s^{-1}). As shown in Figure 1a, for the Hb solution, the peaks at 542 and 577 nm, identified as oxyHb, were gradually converted to the peak at 630 nm, identified as metHb, indicating that Hb underwent a one-electron oxidation to metHb by $O_2^{-\bullet}$. On the other hand, in the Hb vesicle dispersion, the UV−vis spectrum, where a large decline in the baseline from the lower wavelength typically shows the turbidity of the vesicles, did not change at all as shown in Figure 1b. This indicated that $O_2^{-\bullet}$ could not permeate through the bilayer membrane due to the electrostatic repulsion from the negatively charged membrane (Figure 1c). Therefore, if the exogenous $O_2^{-\bullet}$ would attack the Hb vesicles, it should have little influence on the metHb formation.

Next, we analyzed the reaction of H_2O_2 with the Hb vesicles as shown in Figure 2a ([heme] = 12 μM, $[H_2O_2]$ = 120 μM); oxyHb was converted to metHb via ferrylHb as an intermediate. In our previous report (*19*), H_2O_2 permeated through the bilayer membrane, and Hb in the vesicles reacted with H_2O_2 in a catalase-like reaction to produce metHb or ferrylHb (Fe^{IV}=O) in the presence of excess H_2O_2. In the case of the Hb vesicles coencapsulating catalase ([catalase] = 4.2 × 10^4 unit mL^{-1}, Figure