表

Table 1. Plasma-substitute solutions and their physicochemical properties. The viscosities at 10 and 1000 s^{-1} are almost identical, indicating that these polymer solutions are Newtonian fluids.

Plasma Substitutes	Mw. (kDa)	Concentration (g/dL) in saline	COP (Torr)	Viscosity (mPa・s)	
				at 10 s^{-1}	at 1000 s^{-1}
DEX	40	10	44	4.5	4.5
MFG	30	4	44	2.2	2.3
HES_{670}	670	6	27	4.5	4.4
HES_{200}	200	6	29	2.5	2.5
HES_{130}	130	6	35	2.3	2.3
HES_{70}	70	6	34	2.0	2.0
rHSA	66.5	5	19	1.3	1.2

DEX, dextran; HES, hydroxyethyl starch; MFG, modified fluid gelatin; rHSA, recombinant human serum albumin; COP, colloid osmotic pressure.

Table 1

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Rheological properties of hemoglobin-vesicles (artificial red cells) suspended in a series of plasma-substitute solutions and their mixtures with blood.

Atsushi Sato[1], Hiromi Sakai[2], Shinji Takeoka[1], and Eishun Tsuchida[2]

1) Graduate School of Science and Engineering, Waseda University

2) Research Institute for Science and Engineering, Waseda University

Abstract

Hemoglobin-vesicles (HbV) are artificial oxygen carriers. The HbV suspension has an oxygen-carrying capacity that is comparable to that of blood. Since HbV suspension does not possess a colloid osmotic pressure, it should be suspended in or co-injected with an aqueous solution of a plasma substitute (water-soluble polymer), which might interact with HbV. This article describes the rheological properties of HbV suspended in a series of plasma substitute solutions and their mixtures with human blood. HbV suspended in a recombinant human serum albumin solution was nearly Newtonian and that mixtures with blood exhibited very low viscoelasticity. On the other hand, HbV suspended in a solution of dextran, modified fluid gelatin, or high-molecular-weight hydroxyethyl starch exhibited higher viscoelasticity. However, it decreased with increasing the volume ratio of blood. Microscopically, the flow pattern of the mixture of blood and flocculated HbV perfused through microchannels (4.5 μm wide, 20 cmH_2O applied pressure) showed few plugging. Furthermore, the time required for passing was simply proportional to the viscosity. This result indicates that flocculated HbVs would not affect the plugging factor of blood when perfusing through microchannels. The HbV suspension would be useful as various clinical applications in addition to its use as a transfusion alternative by manipulating the rheological property.

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NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers

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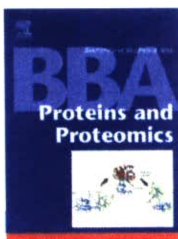

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NO and CO Binding Profiles of Hemoglobin Vesicles as Artificial Oxygen Carriers

Hiromi Sakai[a], Atsushi Sato[b], Peter Sobolewski[c,d], Shinji Takeoka[b], John A. Frangos[c], Koichi Kobayashi[e], Marcos Intaglietta[c,d], and Eishun Tsuchida[a]*

[a] *Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan;*
[b] *Graduate School of Science and Engineering, Waseda University, Tokyo 169-8555, Japan;*
[c] *La Jolla Bioengineering Institute, La Jolla, CA, 92037, USA;*
[d] *Department of Bioengineering, University of California, San Diego, La Jolla, CA, 92093-0412, USA*
[e] *Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan*

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* To whom correspondence should be addressed.
Eishun Tsuchida, Professor
Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan.
Phone +81-3-5286-3120; Fax +81-3-3205-4740; e-mail eishun@waseda.jp

Abbreviations: HbV, hemoglobin-vesicles; RBC, red blood cells; $k'_{on}{}^{(NO)}$, apparent NO binding rate constant; $k'_{on}{}^{(CO)}$, apparent CO binding rate constant; apparent CO binding rate constant; $k'_{OX}{}^{(NO)}$, apparent oxidation rate by NO; PLP, pyridoxal 5'-phosphate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine

Summary

Hemoglobin vesicles (HbVs) are artificial oxygen carriers encapsulating purified and concentrated Hb solution in phospholipid vesicles (liposomes). We examined in-vitro reaction profiles of a formulation of HbV with NO and CO in anaerobic and aerobic conditions using stopped-flow spectrophotometry and a NO electrode. Reaction rate constants of NO to deoxygenated and oxygenated HbV were considerably smaller than those of cell free-Hb because of the intracellular NO-diffusion barrier. The reaction of CO with deoxygenated HbV was slightly slower than that of cell free-Hb solely because of the co-encapsulated allosteric effector, pyridoxal 5'-phosphate. The NO depletion in an aerobic condition in the presence of empty vesicles was monitored using a NO electrode, showing that the hydrophobic bilayer membrane of HbV, which might have higher gas solubility, does not markedly facilitate the O_2 and NO reaction, and that the intracellular Hb is the major component of NO depletion. In conclusion, HbV shows retarded gas reactions, providing some useful information to explain the absence of vasoconstriction and hypertension when they are intravenously injected.

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1. Introduction

A physiologically important aspect of the structure of red blood cells (RBCs) is the management of the transport of endogenous gaseous messenger molecules (NO and CO) [1-3]. Conditions of hemolysis [4] and studies related to the development of hemoglobin-based oxygen carriers (HBOCs) [5-11] have shown that the entrapment of endothelium-derived NO induces vasoconstriction, hypertension, reduced blood flow, and vascular damage. Physiological doses of CO are a vasorelaxation factor, especially in the hepatic microcirculation [12] and its entrapment by cell-free hemoglobin (Hb) solutions induces constriction of sinusoidal capillaries [13]. These side effects due to the presence of molecular Hb in plasma suggest that the cellular structure of RBCs plays a role in insuring the bioavailability of NO and CO.

Several mechanism have been proposed to explain NO binding retardation by Hb encapsulation in RBCs [14-16], namely: (i) an unstirred layer forming an extracellular diffusion barrier surrounding the RBC [2,14]; (ii) a protein-rich RBC cytoskeletal submembrane constituting a physical barrier against NO diffusion [17,18]; and (iii) retardation of gas diffusion due to the viscosity of the Hb solution in RBCs [19].

Hemoglobin has been encapsulated using lipid bilayer membranes to form of Hb vesicles (HbV), in order to produce a blood like HBOC where the oxygen carrying Hb is not dissolved in plasma [20,21]. In this configuration Hb is not vasoactive [9,10], an effect in part due to the retardation of NO binding by Hb encapsulation in phospholipid vesicles [22]. Furthermore, using results of stopped-flow spectrophotometry and computer simulation, we recently determined that the major component of retardation is the intracellular rather than the extracellular diffusion barrier. This conclusion is also supported by considering that: (i) the

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large binding rate constant of NO to a heme in an Hb molecule, (ii) the numerous hemes as sites of gas entrapment at a high intracellular Hb concentration $[Hb]_{in}$, (iii) the slowed gas diffusion in the intracellular viscous Hb solution; and (iv) the lipid bilayer membrane of HbV would not possess any barrier function to the NO diffusion into the particles [23].

On the other hand, the hydrophobic part of a cell membrane reportedly possesses higher gas solubility than the bulk aqueous phase. Both NO and O_2 dissolve to a greater extent in the hydrophobic part, which facilitates the reaction of NO with O_2 to produce NO_2^- [24]. HbV mimics the structure of RBCs, however the particle diameter is much smaller than that of RBCs, and the surface to volume ratio is larger. Therefore for the same oxygen carrying capacity, or equivalent cell concentration by volume there is proportionally greater amount of membrane material for HbVs than for RBCs providing a greater hydrophobic domain. This structural difference is important because it is likely that there is NO depletion in the lipid bilayer membrane of HbVs.

In the present study we analyzed the NO and CO binding rates of HbV with the present formulation, along with the possibility of accelerated NO depletion in the hydrophobic domain of the HbV lipid bilayer membrane. We compared our findings to the corresponding binding rates of a purified human Hb solution suspended in phosphate buffered saline solution (PBS) mixed with pyridoxal 5'-phosphjate (PLP), a solution of polymerized bovine Hb ($poly_BHb$) used for veterinary purposes (Oxyglobin; Biopure Corp. Cambridge, MA) [25], and vesicles containing saline solution.

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2. Materials and Methods

2.1. Hb-vesicles (HbV), Hb solutions, empty vesicles (EV), and RBCs

HbVs were prepared as reported previously [26-29], with slight modifications. Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). Hb was stabilized by carbonylation (HbCO) and concentrated by ultrafiltration to 38 g/dL. PLP (Sigma, St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 2.5. We use PLP instead of 2,3-diphosphoglyceric acid (2,3-DPG) or inositol hexahosphate (IHP) because 2,3-DPG is very unstable and expensive, and the interaction of IHP and Hb is so strong that the oxygen dissociation curve of Hb is distorted and the Hill number becomes nearly 1 [30].

The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG_{5000} (DSPE-PEG; NOF Corp., Tokyo, Japan) at a molar composition of 5/5/1/0.033. Particle diameter was regulated by extrusion. The encapsulated HbCO was converted to HbO_2 by exposing the liquid membrane of HbV to visible light under an O_2 atmosphere. The particle size distribution was measured using a light-scattering method (Submicron Particle Size Analyzer, model N4 PLUS; Beckman-Coulter, Inc., Fullerton, CA). An empty vesicle (EV) suspension was prepared using the same lipids through hydration with a saline solution. The lipid concentration (6.8 g/dL) and the particle diameter (ca. 250 nm) were almost identical to those of HbV.

A purified human Hb solution suspended in phosphate buffered saline solution (PBS)

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was prepared and mixed with PLP at molar ratios of PLP/Hb tetramer = 0 and 2.5. We also used the of polymerized bovine Hb solution (poly$_B$Hb) (Oxyglobin; Biopure Corp. Cambridge, MA) [25]. This solution is a mixture of nonpolymerized tetrameric $_B$Hb (37.2%) and polymerized $_B$Hb with a wide molecular weight distribution [25]. The P_{50} values and Hill numbers of HbV were Hb solutions were obtained from the oxygen equilibrium curve measured using a Hemox-Analyzer (TCS Medical Science, Philadelphia, PA) at 37°C [23,31] **(Figure 1)**.

Blood was harvested from female C57BL/129 mice in accordance with IACUC protocol. RBCs were pelleted at 800g for 30 min and resuspended and washed twice with a 40 mM-HEPES buffer (pH = 7.4, 5 mM glucose, Sigma, St. Louis, MO; tonicity, 285 mOms regulated with NaCl). The RBC suspensions were prepared at concentration of 2×10^6 cells/mL ([Hb] = 0.93 μM, [heme] = 3.72 μM) in the same HEPES buffer.

2.2. Stopped-flow spectrophotometry

The time course of the ligand binding was analyzed during rapid mixing of the deoxygenated HbV and Hb solutions and a NO- or CO-containing solution using a stopped-flow rapid scan spectrophotometer (RSP-1000; Unisoku Co. Ltd., Osaka, Japan) [22,23]. Solutions in the two gas-tight reservoirs (A and B) are mixed rapidly applying a pressure of 0.3 - 0.6 MPa; the dead time for mixing being < 1.5 ms. All measurements were performed at 25 °C. A PBS solution (3 ml each) was poured into both reservoirs, which were both sealed using septum rubber seals. The reservoirs were deoxygenated by N_2 bubbling for more than 30 min. A stock solution of HbV or Hb (ca. 30 μl, [heme] = 300 μM) was injected into Reservoir A to adjust [heme] to 3 μM and the N_2 bubbling was changed to a flow to avoid denaturation of the solutes. Complete deoxygenation was confirmed using preliminary stopped-flow measurements (wavelength: 385 - 593 nm), where the Soret band showed a

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maximum absorbance (λ_{max}) at 430 nm because of the presence of deoxyHb. To make the reservoirs deoxygenated, we did not use sodium dithionate because it is well known that an excess amount of this reagent sometimes possibly induces chemical modifications of heme or globin [32, 33]. In Reservoir B, NO or CO gas bubbling was started while a gentle N_2 flow was maintained in Reservoir A. The mixed gases for NO binding (NO, 0.2029%; N_2, 99.7971%) and for CO binding (CO, 14.14%; N_2, 85.86%) were purchased from Takachiho Chemical Industrial Co., Ltd. (Tokyo).

Stopped flow measurement was initiated after about 10 min bubbling. The sampling interval and the exposure time were set as 1 ms and measurement time was 210 ms. All measurements were performed 3 times and the change of absorbance at 430 nm was plotted as a function of time. The apparent binding rate constants of NO and CO ($k'_{on}{}^{(NO)}$ and $k'_{on}{}^{(CO)}$, respectively) were calculated using Eq. 1 under the assumption of homogeneous distribution of Hb, irreversible second order reaction, and a pseudo-first order reaction when gas molecules are abundant.

$$\ln\frac{\Delta A_t}{\Delta A_0} = -k'_{on} \cdot C_{Gas} \cdot t \qquad (1)$$

In this equation, ΔA_t is the change of absorbance at 430 nm at time t (= $A_t - A_{t=\infty}$) and ΔA_0 is the absorbance at the initial time (= $A_{t=0} - A_{t=\infty}$), and C_{Gas} is the initial gas concentration. NO (1.9 μM) was not much more abundant than the heme concentration (1.5 μM) in the NO-reaction experiments, therefore the apparent binding rates we calculated from the slopes of the initial phase of reactions.

The time courses of the reaction of NO and O_2-bound HbV or HbO_2 solutions were analyzed using stopped-flow spectrometry in the same manner, except that an air-equilibrated

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PBS solution was introduced into Reservoir A to ensure the O_2-binding state. The absorption changes were monitored at 408 nm.

2.3. Measurement of NO depletion using a NO electrode

The rate of NO depletion in a test solution after adding NO in an aerobic condition was monitored via an amino-700 probe and an inNO-T sensor (Innovative Instruments, Inc.) connected to a Dell PC running InNO-T software (Innovative Instruments, Inc.) [34]. In a room atmosphere, 3 mL of a PBS solution containing HbV, EV, or mice RBCs was pipetted into a cuvette and stirred using a magnetic stir bar. The NO sensor was introduced into the cuvette for continuous monitoring. After a steady condition was confirmed, a stock NO solution (100 μL) was injected, yielding the concentration of 2 μM NO. The NO level rapidly increases and then decreases with the reaction with O_2 and Hb. Concentrations of the specimens were as follows: HbV ([heme] = 4 μM, [Hb] = 65 mg/L, [Lipid] = 36 mg/L), EV ([lipid] = 36 mg/L), and mice RBC ([heme] = 3.72 μM). Data were fitted and basic statistics and graphs were obtained using Synergy KaleidaGraph 3.6. Correlation was determined using Spearman's rank correlation test, with StatView 5.

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3. Results

3.1. Stopped-flow spectrophotometry of NO and CO bindings in an anaerobic condition

Complete deoxygenation of HbV was confirmed using the characteristic wavelength of the maximum absorption (λ_{max}) at 430 nm. Because of the stronger light-scattering effect of HbV suspension than that of the Hb solution and RBCs [35], the absorption peaks in the *Q* band region were not well defined in this measurement. After rapid mixing with NO, the immediate absorption reduction at 430 nm and the increase at 418 nm that correspond to the formation of HbNO were confirmed (**Fig. 2a**). In the case of mixing with CO, the immediate absorption increase at 419 nm was confirmed (**Fig. 2b**). The change of absorption at 430 nm in both reactions was indicative of the formation of HbNO or HbCO in the vesicles.

The spectrophotometric scans presented in **Figs. 2a and 2b** were performed 3 times and the averaged level of reaction was plotted as a ratio of absorbance at 430 nm (ΔA_t) at time t, to the initial absorbance (ΔA_0) at time 0 (**Figs. 3a and 3b**). Data of Hb solutions are also plotted in those figures. The graph clearly shows that both NO binding and CO binding were slower for HbV than for the human Hb solution without PLP. However, Hb with PLP showed almost identical CO binding to HbV. The NO binding rates of Hb solution with and without PLP and poly$_B$Hb were almost identical. The apparent binding rate constants of $k'_{on}{}^{(NO)}$ for NO and $k'_{on}{}^{(CO)}$ for CO are presented in **Table 1**.

3.2. Stopped-flow spectrophotometry of the reaction of NO and O_2-bound HbV and Hb solutions in an aerobic condition

The oxygenated HbV showed the absorption maximum at 415 nm (**Fig. 2c**). Mixing with NO showed the immediate reduction of the absorption maximum, although an increase at 405 nm showed attributes of the metHb formation. The spectrophotometry scans portrayed in

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Fig. 2c were performed 3 times and the average level of reaction was plotted as a ratio of absorbance at 408 nm ($\Delta A_t = A_t - A_{t=\infty}$) at time t, to the initial absorbance ($\Delta A_0 = A_{t=0} - A_{t=\infty}$) at time 0 **(Fig. 3c)**. The graph shows that the reaction of NO with oxygenated HbV was considerably slower than that of human Hb solutions with and without PLP. The rate of poly$_B$Hb was almost identical to that of human Hb solutions. The apparent oxidation reaction rate constants $k'_{OX}{}^{(NO)}$ are summarized in **Table 1**.

3.3. NO depletion in an aerobic condition in the presence of HbV, RBC, and EV

When the stock NO solution was simply injected into a PBS solution, the NO electrode showed an immediate increase to about 2000 nM, even in the aerobic condition. Then it gradually decreased according to the reaction of NO and O_2 to produce NO_2^- **(Fig. 4)**. From the slope after 200 s, the apparent decay constant is calculated as 3.7×10^{-3} s^{-1}. In the case of the presence of EV, the increase in NO concentration immediately after injection and the decay curve were almost identical, and the apparent decay constant was 3.9×10^{-3} s^{-1}. From results of this experiment, it is not clear that the hydrophobic part of the lipid bilayer membrane facilitates NO depletion. The reactions of NO in the aqueous phase are still the prominent ones.

In the presence of HbV, the first injection of NO (2000 nM) was decayed much faster than the sensor was able to record, and only a part of the second NO injection was recorded. The decay curve was finally obtained at the third NO injection when the heme of HbV was totally inactivated with NO and converted to the ferric state. On the other hand, one injection of NO into the RBC suspension clearly showed the decay curve. However, the rate of decay is extremely fast, as indicated by the need for a different time scale of the abscissa. The NO decay constant of HbV was not obtained from this experiment, which has to be analyzed by stopped flow spectrophotometry.

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4. Discussion

Our primary finding is that NO binding is considerably retarded by encapsulation into phospholipid vesicles, and that CO binding is not influenced by encapsulation, but is slightly retarded by co-encapsulation with PLP as an allosteric effector. In the aerobic condition, the hydrophobic part of the lipid bilayer membrane does not seem to be involved in NO depletion (facilitated reaction with O_2), which is mostly attributable to the reaction with the intracellular Hb solution.

We recently showed that NO binding of Hb is retarded by encapsulation in phospholipid vesicles because of the intracellular diffusion barrier [23]. Intrinsically rapid NO binding causes Hb to become a sink of NO in the interior surface region of HbV, thereby hindering further NO diffusion into the core of HbV, in combination with the lowered diffusion constants of NO in a highly concentrated Hb solution. Using results described in a previous report, we compare two kinds of HbV with different P_{50} values (25 and 14 Torr), and showed that the $k'_{on}{}^{(NO)}$ might change slightly according to P_{50} (0.61×10^7 vs. 0.88×10^7 $M^{-1}s^{-1}$). Accordingly, the major cause of retardation is Hb encapsulation. Cell-free Hb removes NO much faster (cf. $k'_{on}{}^{(NO)}$ of cell-free Hb solution, 2.4 - 2.6×10^7 $M^{-1}s^{-1}$). Cell-free Hb solutions with and without PLP did not differ greatly. The results coincide well with those in precedent reports: that NO binding to α and β subunits and that the T-state and R-state Hbs are almost identical [36,37]. Poly$_B$Hb that contains high concentration of terameric $_B$Hb and intermolecularly crosslinked tetrameric $_B$Hb are known to induce vasoconstriction and hypertension [38]. In the present study, such Poly$_B$Hb showed identical rapid NO binding.

For CO, we previously reported that CO binding to Hb is intrinsically much slower than NO binding by two orders of magnitude, which allows more time for CO diffusion into the

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HbV core. Therefore, $k'_{on}{}^{(CO)}$ is not influenced by the intracellular Hb concentration and particle size smaller than 500 nm [23]. Results of the present study show the presence of a slight retardation of CO binding by HbV in comparison to the cell-free Hb solution, which is solely attributable to the co-encapsulation of PLP as an allosteric effector. The effector tends to stabilize the T-state and retards CO binding. In contrast to NO, it is well known that cooperative CO binding to four subunits of Hb occurs, and that the binding rate constants to α and β subunits, or T-state and R-state Hbs, are different [39,40]. According to Kwansa et al. [41], the value of $k_{on}{}^{(CO)}$ of unmodified $_B$Hb measured using stopped flow is 1.3×10^5 $M^{-1}s^{-1}$. Polymerization of $_B$Hb by glutaraldehyde apparently facilitates CO binding ($k_{on}{}^{(CO)}$ of Poly$_B$Hb = 2.7×10^5 $M^{-1}s^{-1}$).

In the circulation where O_2 is abundant NO is inactivated mainly by NO dioxygenation by O_2-bound HBOCs and RBC. Results of the present study confirmed that the cell-free human Hb solutions with our without PLP showed $k'_{ox}{}^{(NO)}$ of 7.4 - 8.7 × 10^7 $M^{-1}s^{-1}$, which was faster than that of the reaction of deoxyHb and NO ($k'_{on}{}^{(NO)}$ = 2.4 - 2.6 × 10^7 $M^{-1}s^{-1}$), a tendency that coincided with the results of Herold et al. [42]. Encapsulation of HbO_2 in vesicles significantly retarded the reaction with NO, and $k'_{ox}{}^{(NO)}$ was 0.88 × 10^7 $M^{-1}s^{-1}$. However, we observed the change of heme ligation state (HbO_2 → metHb) and we were unable to observe the NO concentration using stopped-flow spectrophotometry. In an aerobic condition, NO reacts slowly with O_2 to produce NO_2^- in water.

The weight ratio of lipids to Hb of HbVs is about 0.5 - 0.6, this ratio being as low as 0.15 for RBCs. We anticipated that the hydrophobic part of the lipid bilayer membrane of HbV would be involved considerably in NO depletion, as reported by Liu et al. [24], which might affect the results of stopped flow spectrophotometry. We monitored the NO decay in the presence of empty vesicles (without Hb) in an aerobic condition using a NO electrode to

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clarify the facilitation of NO depletion. Results show that NO decay in the presence of empty vesicles was almost identical to that in the PBS solution, indicating that the facilitated reaction of NO and O_2 in a hydrophobic part of the lipid bilayer membrane is negligibly small compared to the reaction with the intracellular Hb.

This finding may be related to the lipid composition of the membrane. The saturated phospholipid (DPPC, phase transition temperature = 41 °C) with cholesterol in HbV might be more resistant to oxidative damage than is soy phosphatidylcholine reported by Liu et al. [24], which probably contains many unsaturated fatty acids. Moreover, nitration of unsaturated bonds might occur, which is another pathway for NO depletion [43]. Conversely, our lipid mixture of DPPC comprises saturated fatty acids and cholesterol providing a hydrophobic rigid domain that would differ from the liposomes made of soy phospholipids. If the aqueous reaction of NO and dioxygen occurs with overall stoichiometry of $4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-$ [24,44], then H_2O is required even in the hydrophobic part. The unstable soy phospholipid vesicles in the literatures would provide more water molecules than does the rigid and stable bilayer membrane of our HbV. Accordingly, we conclude that NO depletion in aerobic conditions in the presence of HbV is caused primarily by the encapsulated Hbs, and the contribution of the bilayer membrane of HbV is negligibly small.

The NO decay in HbV was much faster than that for RBCs. In addition, measurements using the NO electrode were unable to detect the reaction rate of O_2-HbV and NO. These results demonstrate that NO mainly reacts with Hb in HbV. Given the rate of NO decay shown in Figure 4, we can assume that less than 0.1% of total NO (1.9 μM) reacts with the physically-dissolved O_2 in PBS in 200 ms after the rapid mixing by stopped flow spectrophotometry.

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In a previous report on the relative magnitude of the binding rate constant of NO (fast) and CO (slow) to deoxygenated HbV, we predicted that the strength of the diffusion barrier induced in HbV becomes more significant with the faster ligand binding reaction with Hb [23]. According to our results and Herold et al. [42], the elementary reaction of NO and HbO_2 is much faster than that of NO with deoxyHb. As shown in Figure 2, Hb encapsulation in vesicles retarded NO binding to deoxyHb by 1/4, and NO reaction with HbO_2 by 1/8. The impact of Hb encapsulation on the reaction with NO is strengthened in the aerobic condition.

We previously reported that HbV does not induce hypertension. However, present results, which show retardations of NO binding by encapsulation and CO binding by co-encapsulation of PLP in comparison to cell-free Hb solutions and poly$_B$Hb, cannot fully explain the absence of vasoconstriction or hypertension after intravenous injection because these reactions of HbV are much greater than those of RBCs (10^4 - 10^5 $M^{-1}s^{-1}$) [1,2,37,45]. Any Hb-based oxygen carrier (HBOC) is much smaller than an RBC and is distributed homogeneously in the plasma layer [46]. Consequently, an RBC-free zone at the blood/endothelium interface becomes an HBOC-containing zone, and might be a sink of NO [47,48]. Rohlfs et al. [49] reported that NO binding rate constants of a series of chemically modified HBOCs (diameter, 6 - 28 nm), measured using the laser flash photolysis, were identical to that of an unmodified Hb solution: 3.0×10^7 $M^{-1}s^{-1}$. They concluded straightforwardly that NO uptake was unrelated to vasoconstriction because PEG-modified Hb did not induce vasoconstriction, other mechanisms being suggested as determinants of vasoconstriction such as molecular recognition, oxygen affinity [7,50] and facilitated diffusion. Poly$_B$Hb is larger than monomeric $_B$Hb, but may be vasoactive because of the presence of monomeric $_B$Hb and its high P_{50} [25,31].

It is also speculated that there is a threshold particle diameter to penetrate across the

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perforated endothelial cell layer to approach a space (such as the space of Disse near the sinusoidal endothelial layer in a hepatic microcirculation, or the space between the endothelium and the smooth muscle), where CO or NO is produced as a vasorelaxation factor. Because the particle size of HbV (250 nm) is obviously much larger than Hb tetramer and its polymerized form as shown in **Figure 5**. Both the retardation of NO reaction and the larger particle diameter are inferred to be keys to suppress vasoconstriction and hypertension induced by HBOCs.

In conclusion, the source of vasoactivity of HBOCs has been extensively reviewed [7,9,13,38,51-54] and it is generally agreed that NO scavenging is a factor in this process, although it does not provide an all encompassing explanation. In terms of HbV, the intrinsically fast reactions of NO with the cell-free Hb solutions in both aerobic and anaerobic conditions are considerably retarded by the encapsulation process. Therefore, since NO bioavailability results from the balance of the rate of NO production and NO entrapment or scavenging, the finding that the latter processes are significantly retarded in HbVs provides some meaningful information to explain their vasoinactivity.

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Figure Legends

Figure 1. Oxygen dissociation curves of Hb solutions and HbV with or without PLP measured with Hemox-Analyzer at 37°C. The P_{50} values and Hill numbers are listed in Table 1. The data of HbV (w/o PLP) is cited from our previous study [23].

Figure 2. Representative profiles of the reactions of NO or CO HbV using stopped-flow spectrophotometry. (A) A NO-bubbled PBS ([NO] = 3.8 μM) and deoxygenated HbV in PBS ([heme] = 3.0 μM) were mixed rapidly; the absorption spectra were collected every millisecond for 0.2 s after mixing. In this figure, the spectroscopic curves of every 10 ms are selected. The figure shows clearly that the spectrum of deoxyHbV is mostly converted to NO-HbV during 0.2 s. (inset) The time course of the measured absorbance at 430 nm. **(B)** A CO-bubbled PBS ([CO] = 135 μM) and deoxygenated HbV in PBS ([heme] = 3.0 μM) were mixed rapidly using a stopped-flow spectrophotometer. This figure clearly shows that the spectrum of deoxyHbV is mostly converted to CO-HbV in 0.2 s. (inset) The time course of the measured absorbance at 430 nm. **(C)** A NO-bubbled PBS ([NO] = 3.8 μM) and oxygenated HbV in PBS ([heme] = 3.0 μM) were mixed. The spectrum of oxygenated HbV is mostly converted to metHbV during 0.2 s. (inset) The time course of the measured absorbance at 408 nm. The optical path length was 1 cm. In these experimental conditions, the CO-binding in (C) seems faster than the NO reactions (A & B), due to the higher concentration of CO (135 μM) than that of NO (3.8 μM). However, the calculated reaction rate constant of the CO binding is much smaller than those of NO reactions as shown in Table 1.

Figure 3. Time courses of NO and CO reactions by HbV, human Hb solutions, and Poly$_B$Hb. The level of reaction was plotted on a semi-logarithmic graph as a ratio of

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absorption at 419 or 430 nm (ΔA_t) at time t, to the initial absorption (ΔA_0) at time 0. **(A)** A NO-bubbled PBS ([NO] = 3.8 μM) and deoxygenated-Hb-containing solutions in PBS ([heme] = 3.0 μM) were mixed. **(B)** A CO-bubbled PBS ([CO] = 135 μM) and deoxygenated-Hb-containing solutions in PBS ([heme] = 3.0 μM) were mixed. **(C)** A NO-bubbled PBS ([NO] = 3.8 μM) and an oxygenated-HbO_2-containing solution in PBS ([heme] = 3.0 μM) were mixed.

Figure 4. NO decay in an aerobic condition in PBS solution after injection of NO yielding a concentration of 2000 nM NO. (A) PBS solution, as a standard curve of NO decay by the dissolved O_2. The decay rate is calculated as 3.7×10^{-3} s^{-1}. **(B)** PBS solution containing EV ([lipids] = 36 mg/mL). The decay rate is calculated as 3.9×10^{-3} s^{-1}. **(C)** PBS solution containing HbV ([heme] = 4 μM, [Hb] = 65 mg/L, [Lipid] = 36 mg/L). The arrows indicate the points of three injections. At the first and the second injections, HbV consumed NO so rapidly that the NO electrode was undetectable. At the third injection, Hb of HbV would be totally inactivated, and the NO level increased, and subsequently decreased gradually through reaction with the physically dissolved O_2. **(D)** A PBS solution containing mice RBCs ([heme] = 3.72 μM). In contrast to HbV, the NO level increased at the first NO injection, although it did not reach to 2000 nM, and the NO decay was much faster than that in (A).

Figure 5. Comparison of particle size between HbV (250 nm), Hb (5 nm) and polymerized Hb. According to the molecular weight distribution of Poly$_B$Hb [31], the molecular weight (Mw) of the largest polymerized Hb is 502 kDa, which corresponds to 8 Hbs. One HbV contains about 30,000 Hbs. It is obvious that HbV is much larger than the largest fraction of Poly$_B$Hb. For the retardation of NO binding, both Hb concentration in the particle and the particle size are important [23].

Table 1. Apparent reaction rate constants of Hb, HbV, and polyHb with different P_{50}. The data of HbV without PLP were referred from a previous paper [23].

	HbV (with PLP)	HbV [a] (w/o PLP)	Hb (with PLP)	Hb (w/o PLP)	$poly_B Hb$
$k'^{(CO)}_{on}$ (10^5 $M^{-1}s^{-1}$)	2.1	3.4 [a]	2.1	3.4	2.7
$k'^{(NO)}_{on}$ (10^7 $M^{-1}s^{-1}$)	0.61	0.88 [a]	2.4	2.6	2.4
$k'^{(NO)}_{OX}$ (10^7 $M^{-1}s^{-1}$)	0.88	-	8.7	7.4	6.6
P_{50} (Torr)	25 – 31	14 [a]	23	14	54 [b]
Hill number	2.1	2.1	1.9	2.2	1.2 [b]
Size	279 nm	265 nm [a]	65 kDa	65 kDa	87–502 kDa [c]

[a] Ref. [23]; [b] Ref. [31]; [c] Ref. [25]

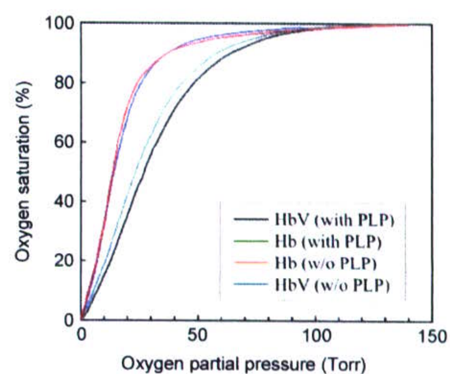
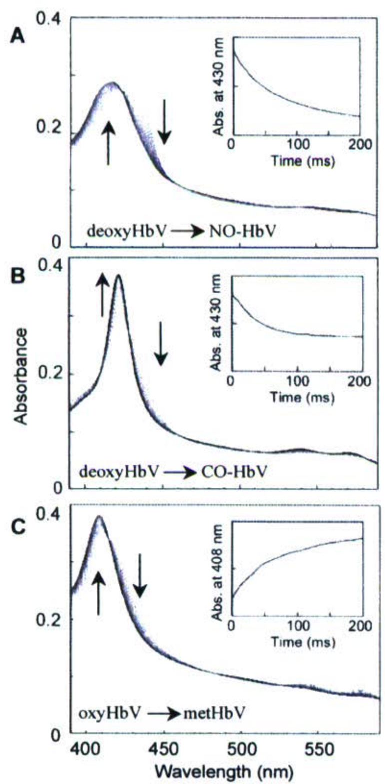


Figure 1
Sakai et al.



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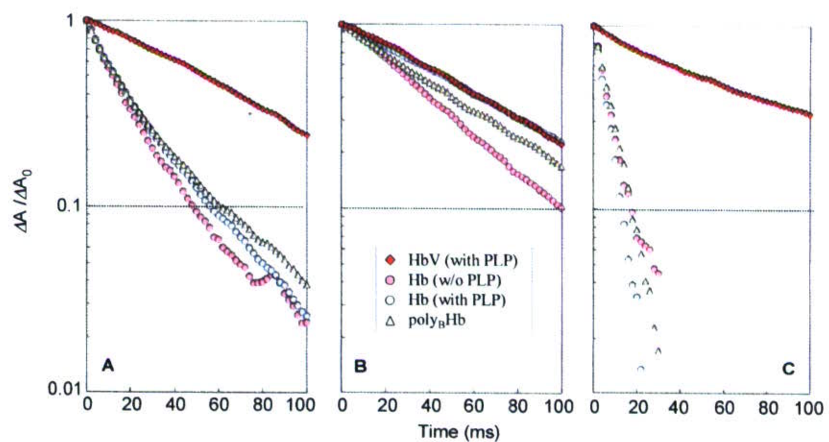

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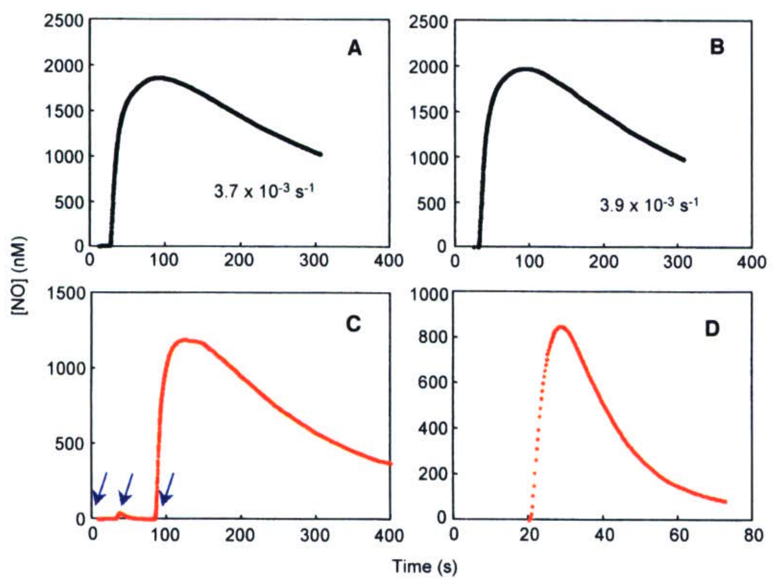

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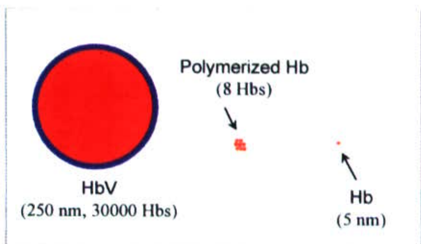

Figure 5
Sakai et al.

Review

Hemoglobin Vesicles to Treat Critical Ischemia

Dominique Erni, Reto Wettstein, Claudio Contaldo, Jan A. Plock, Nassim Rafatmehr, Hiromi Sakai* and Eishun Tsuchida*

Abstract

The initital purpose for developing artificial oxygen carriers was to replace blood transfusions in order to avoid their adverse effects such as immunologic reactions, transmission of infectious diseases, limited availability and restricted storage conditions. With the advent of new generations of artifical oxygen carriers, a shift of paradigm evolved that considers the artificial oxygen carriers as oxygen therapeutics re-distributing oxygen delivery in the favor of tissues in need. This function may find a particular application in tissues rendered hypoxic due to arterial occlusive diseases. This review, based on a large series of intravital microscopy studies in a hamster skin flap model, outlines the optimal design of hemoglobin vesicles (HbVs) given for the above intention. In summary, the HbV should be of a large diameter, and oxygen affinity, colloid osmotic pressure and viscosity of the HbV solution should be high.

Keywords artificial oxygen carrier, intravital microscopy, skin flap, hypoxia, hemodilution

Introduction

The initital drive to develop artificial oxygen carriers was to reduce the need of blood transfusions in order to circumvent their drawbacks such as immunologic reactions, blood-borne transmitted diseases, limited availability and restricted storage time[1-3]. The first generation artificial blood substitutes included modified hemoglobin (Hb) molecules and perfluorocarbons. The Hb-based oxygen carriers (HBOCs) were designed to mimick the physiological function of red blood cells in terms of oxygen uptake, transport and release. Accordingly, their physicochemical properties were targeted to that of human blood.

However, on a clinical level, the artificial blood substitutes have failed to meet the expectations so far. Clinical trials yielded serious adverse effects, which were mainly related to an unwanted vasoconstrictor response to the Hb molecules[1-4]. There was an urge to invent new HBOCs, which was achieved by introducing a variety of structural modifications of the hemoglobins and the rheological properties of the solvent[1,2,5]. This evolution was paralleled by a shift of paradigm regarding the function of artificial oxygen carriers: they were no longer seen as mere oxygen transporters and suppliers but rather as oxygen therapeutics in a sense that they would influence the distribution of RBC-bound oxygen in favor of the tissues in need[2,5].

This new paradigm went along with the idea of using artificial oxygen-carrying solutions as drugs instead of blood substitutes, thus revealing new therapeutic strategies. One of them consists in the treatment of local tissue hypoxia caused by arterial occlusion. Although occlusive vascular diseases account for the highest causes of death and some of the highest rates of morbidity and health care costs in the industrialized countries, little emphasis has been put on translational research focused on this indication for artificial oxygen carriers. Symptomatically, this is not either the issue in any of the ongoing clinical trials[3]. Some experimental experience with this therapeutic strategy exists mostly for first generation HBOCs and perfluorocarbons, both of which yielded beneficial effects in acute ischemic hypoxia in cerebral[6-11], myocardial[12-15] and peripheral[16] tissues. On the other hand, a clinical study testing the effect of diaspirin cross-linked Hb on acute ischemic stroke revealed that treatment with this first generation HBOC was an independent predictor of a worse outcome[4].

In collaboration with Waseda University, we have investigated the efficacy of hemoglobin vesicles (HbVs), a second generation HBOC, in critically ischemic skin flaps. Our goal was to ameliorate hypoxia by improving oxygen delivery with the infusion of HbV solutions. This treatment is supposed to serve as an ancillary measure until adequate

Department of Plastic Surgery, Inselspital, University of Berne, CH-3010 Berne, Switzerland
*Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan
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perfusion is re-established surgically or spontaneously by neo-vascularization. We favored the use of an HBOC because unlike perfluorocarbons, HBOCs do not require high pressure oxygen ventilation, which may be toxic if applied over the prolonged period of time that may be necessary to bridge the ischemic condition[17]. This article reviews our experimental experience with HbVs used for this purpose. Our studies were focused on investigating the effects of the various physicochemical properties of the HbV solutions in order to optimize their design.

Standardized model and HBOC preparation allow for comparable and reproducible findings

In our experiments we used a hamster skin flap model that was derived from the well-established dorsal skinfold chamber, a model that allows for monitoring the hemodynamics and tissue oxygenation on a microscopic level (Fig. 1.)[18,19]. The model simulates critical ischemia in peripheral tissue after acute vascular obstruction of the anatomical, axial blood supply, which renders this tissue dependent on a collateral vascularization. In our model, the

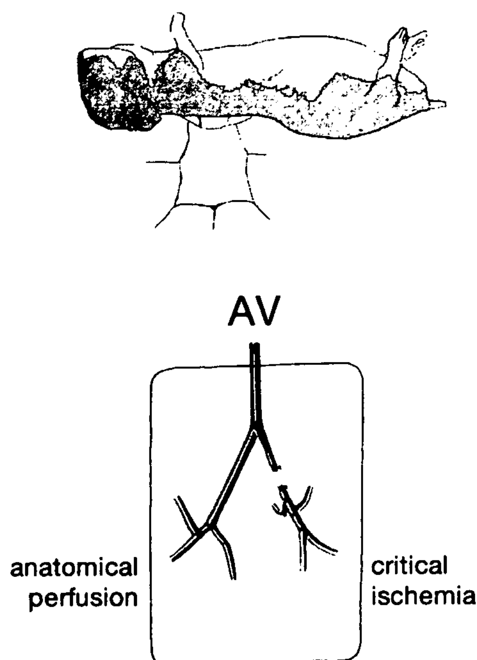

Fig. 1. Schematic view of animal model (*above*). A flap is dissected from the epilated back skin of anesthetized hamsters and mounted on a platform for intravital microscopy. Schematic view of preparation (*below*). The flap is nourished by one artery and vein, which bifurcate into two branches. After transecting the right branch, the corresponding tissue is perfused via collateral vessels connecting the two vascular beds and becomes critically ischemic. Redrawn from Erni et al[18]

critically ischemic tissue showed hypoxia[20], hypoxia-related inflammation[21] and incresaed rates of cell death[22].

The HbVs were produced and provided by Waseda University[23]. They consisted of isolated, purified human Hb originating from outdated RBC concentrates. The Hbs were encapsulated with a double-layer phospholipid membrane that was coated with polethylene-glycol in order to avoid agglutination. The diameter of the HbVs was approximately 250 nm. The oxygen affinity (P50) was regulated by adding the co-encapsulated allosteric effector pyridoxal 5'-phosphate.

To improve microcirculation: a prerequisite

Because the reason for hypoxia in critical ischemia related to arterial occlusive diseases is hypoperfusion, the effect of the oxygen-carrying solution on the microcirculation is of utmost importance. Any further reduction in microcirculatory blood flow may be deleterious. Partly severe vasoconstriction was observed after application of first generation HBOCs[1,2,24,25], which was mainly due to the NO-scavenging effect of plasma-bound Hb[15] but also due to NO-independent mechanisms[26]. The vasoconstrictor effect of the Hb compounds were largely dependent on their size[24]; its increase was therefore one of the first goals to be accomplished with the second generation HBOCs[2]. No vasoconstriction, neither in normally perfused nor in ischemic tissues, and no arterial hypertension were observed in any of the animals receiving HbVs[20,24,25,27-30] which are considered the largest HBOCs.

More than trying to avoid vasoconstriction, the primary goal should be to increase microcirculatory blood flow in the ischemic tissue, since blood flow determines oxygen delivery. In this context, homogenous distribution of blood flow, best expressed by functional capillary density (FCD), is crucial to avoid hypoxic tissue areas in spite of adequate total volumetric flow[31,32]. It has been postulated that the ischemic tissue may benefit from the small size of the artificial oxygen carrier that is still able to penetrate the vasculature through stenoses that are no longer accessible by red blood cells. Although this phenomenon is intellectually appealing and has been described *in vivo*[33], the scientific proof of its biological efficacy is lacking. On the contrary, HbVs were completely inefficient if they were given in a solution that failed to improve microcirculatory blood flow in the ischemic tissue (Fig. 2.)[20], thus emphasizing the importance of the rheological formula of the oxygen-carrying solution. Similar observations have been made in other conditions of compromized microcirculation, where re-establishing microcirculatory blood flow and FCD rather than increasing oxygen-carrying capacity were important in preventing hypoxia[32,34].

In this context, high colloidal osmotic pressure (COP) and

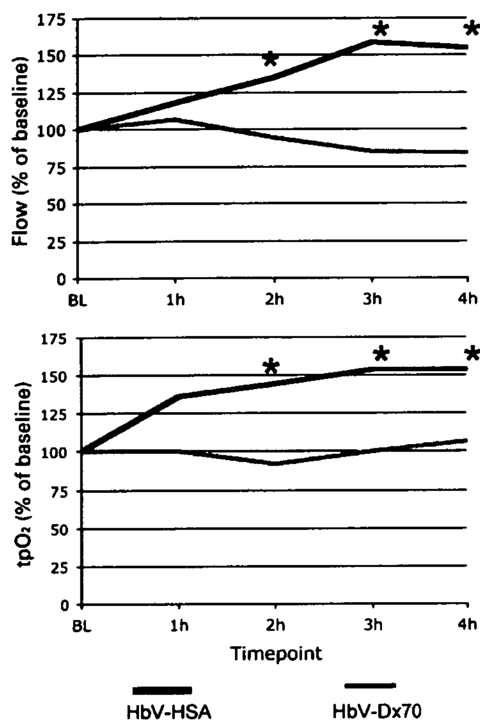

Fig. 2 Mean arteriolar blood flow and partial oxygen tension in the critically ischemic tissue before and after 50% isovolemic blood exchange with HbV dissolved in 8% human serum albumin (HbV-HSA) or 6% dextran 70 (HbV-Dx70). *p < 0.05 vs. Baseline and HbV-HSA. HbVs are not able to improve tissue oxygenation if blood flow is not increased. Redrawnfrom Erni et al[20].

high viscosity proved to be beneficial to both volumetric blood flow and FCD[2,5,20-22,25,32,34,35]. High COP leads to blood volume expansion and subsequent increase in preload, cardiac output and mean arterial blood pressure. Furthermore, hyperoncotic solutions may attenuate fluid extravasation and edema formation in the ischemic tissue, thus relieving edema-related impairment of oxygen diffusion[36-38].

The effects of the viscosity of the HBOC solution appear to be manifold. Whereas we and others have repeatedly demonstrated that the reduction in total blood viscosity during hemodilution is beneficial particularly in conditions of compromized microcirculation[19,20,22,25,30,39,40], it seems to be advantageous to enhance viscosity in the plasma phase[5,21,22,25,41,42]. To prevent from decreasing plasma viscosity below a certain level is crucial in maintaining capillary perfusion pressure, which is explained by the influence of plasma viscosity on NO-mediated, shear stress-induced arteriolar vasodilation[41,43-45]. However, raising plasma viscosity to supraphysiological levels did not reveal any arteriolar vasodilation in our model, whereas microcirculatory blood flow and capillary perfusion were improved[20-22,25,30]. Since this was accompanied by a decrease in capillary diameter, the viscosity-related microcirculatory improvement may have been achieved by a decrease in capillary intraluminal pressure due to a reduction in post-capillary resistance, which in turn is predominantly influenced by the leukocyte-endothelium interaction[46]. It has been reported that leukocyte adhesion, which is the first step in this cascade of events, may be attenuated by increasing shear stress on the endothelium[47], a scenario that is most conceivable considering the increased plasma viscosity after HbV infusion[21,22]. The assumption that HbV solutions improve capillary perfusion by reducing post-capillary leukocyte adhesion and activation was further evidenced by a decrease in inflammatory markers such as endothelial leakage, tumor necrosis factor (TNF)-alpha, interleukin (IL)-6 and tissue leukocyte counts[21,22].

The influence of the oxygen-carrying solution on such inflammatory pathways may not be under-estimated, in particular in view of the ischemia-reperfusion effect that takes place after improving the oxygenation of this tissue. Due to the reduced arterial perfusion pressure, the influence of post-capillary resistance on capillary flow is more relevant if the tissues are indirectly perfused via the collateralized vasculature. Various authors have reported that artificial oxygen carriers may have the potential to attenuate ischemia-reperfusion injury[48,49], in particular if they are supplemented with antioxidants[50].

The principle of improving oxygen delivery by augmenting microcirculatory blood flow is based on the maintenance of adequate tissue perfusion either by the remaining blood flow an incompletely occluded arterial axis or by functional collateral vessels. Collateral vascularization varies between species, individuals, organs and even within organs[51-53]. Most of the existing experimental studies that report a beneficial effect of artificial oxygen carriers on critical ischemia due to arterial occlusion, including those cited in this review, relied on the presence of a functional collateral vasculature. Its importance was highlighted by Rebel et al who found that hemodilution with an oxygen-carrying solution after cerebral artery occlusion was able to increase oxygen delivery in the collateralized cortex but not in the caudate nucleus, which is an end artery territory[9]. The growth of the collateral vasculature, a process termed arteriogenesis, is triggered by a chronic impairment of axial, anatomical blood supply[52]. The collateral vascularization acts as a lifebelt when it comes to a complete shut down of the anatomical perfusion, and the development of therapies that aim at enhancing collateral vascularization has gained great scientific interest in the recent years[54]. It is not unlikely that artficial oxygen carriers

will be playing a pivotal role in the therapeutic concept of oxygenating critically ischemic tissue via a collateralized vasculature in the near future.

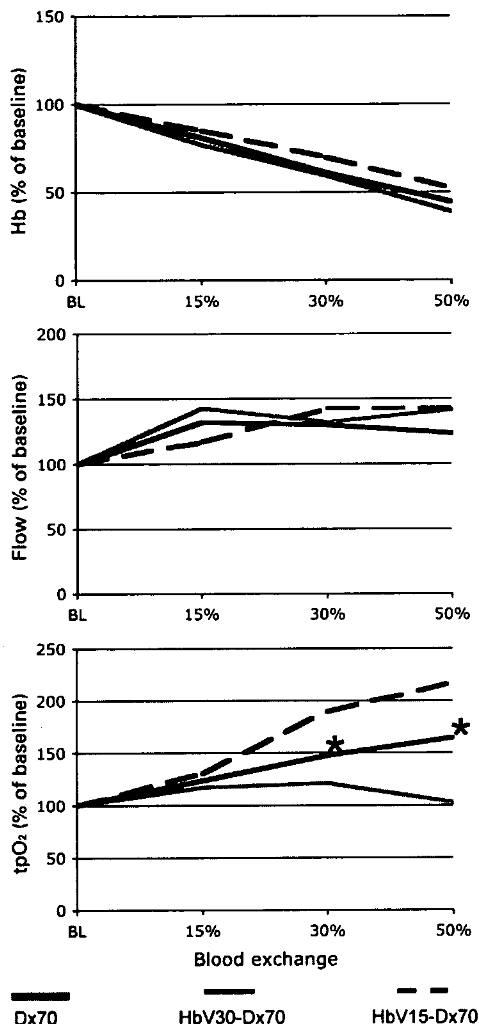

Fig. 3. Mean arterial Hb concentration, and mean arteriolar blood flow and partial oxygen tension in the critically ischemic tissue before and after stepwise isovolemic hemodilution with 6% dextran 70 (Dx70) and HbV dissolved in Dx70. The P50 of HbVs was 30 mmHg (HbV30-Dx70) or 15 mmHg (HbV15-HbV). *$p < 0.05$ vs. other groups. In spite of decreasing Hb concentrations, tissue oxygen tension increases with every step of hemodilution with HbV solutions but not with Dx70. Tissue oxygenation is better for HbV with higher oxygen affinity. Redrawn from Contaldo et al[30].

To make the HBOC increase oxygen delivery to the tissue in need

If the vesicles did not contain Hb, oxygenation and tissue survival in the critically ischemic tissue could not be improved although rheological properties and microcirculatory improvements were similar to the oxygen-carrying vesicle (HbV) solutions[22]. The oxygenation in the critically ischemic tissue was correlated with the HbV concentration in the circulating blood but not with the total Hb concentration (RBC-bound Hb plus HbV-bound Hb)[30]. Furthermore, the contribution of HbV-bound oxygen to the total oxygen extracted by the ischemic tissue was estimated to be less than 10% even at high HbV concentrations[22]. These data suggest that the HbV solution acts rather by promoting the release of RBC-bound oxygen to the ischemic tissue in terms of an oxygen therapeutic than by enhancing the oxygen-carrying capacity in terms of a RBC substitute. The HbVs circulate in the plasma phase, thus interfering with the oxygen delivery from the RBCs to the tissue[55,56]. According to the Stokes-Einstein equation, the diffusivity of oxygen is inversely proportional to the size of the plasma-bound oxygen-carrying compound and the viscosity of the plasma suspension. Furthermore, oxygen diffusivity is negatively affected by the oxygen affinity of the oxygen carrier, and high oxygen affinity shifts oxygen release towards the downstream direction[28,31,57]. In our studies, we have repeatedly demonstrated that increasing the size and oxygen affinity of the oxygen carrier as well as the plasma viscosity exerts a positive effect on the oxygenation in the critically ischemic tissue[21,22,25,30]. This strongly suggests that the benefit was obtained by the maintaining oxygen content high in the blood entering the critically ischemic tissue, which is achieved by impeding unnecessary oxygen loss to the normoxic tissue in the upstream vasculature, whereas the increased oxygen retention is over-ruled by the high oxygen tension gradient between blood and tissue being present in a hypoxic environment. The result is a net re-distribution of oxygen in favor of the critically ischemic tissue, which is the more efficient the more oxygen is kept intraluminaly upstream. In our model, the upstream oxygen loss was estimated at 40-50%[19,28]. It may be even higher in species with higher arterial oxygen saturation (SaO_2 is approximately 80% in our model), or in tissues with a higher rate of oxygen extraction.

Equipped with the above-mentioned physicochemical requirements, the HbVs were able to improve microcirculation, oxygenation and tissue survival, and to attenuate hypoxia-related inflammation in the critically ischemic tissue without necessitating the microcirculatory benefit obtained by hemodilution and volume expansion (Fig. 4.)[21]. This was accomplished in terms of a topload infusion, which is a more appropriate mode of application in the clinical setting because blood exchange and adverse effects related to hypervolemia can be avoided.