Hemoglobin encapsulation in vesicles retards NO and CO binding and $O_2$ release when perfused through narrow gas-permeable tubes

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Sakai H, Okuda N, Sato A, Yamaue T, Takeoka S, Tsuchida E. Hemoglobin encapsulation in vesicles retards NO and CO binding and $O_2$ release when perfused through narrow gas-permeable tubes. Am J Physiol Heart Circ Physiol 298: H956–H965, 2010. First published December 31, 2009; doi:10.1152/ajpheart.00741.2009.—Intravenous administration of cell-free Hb induces vasoconstriction and circulatory disorders, presumably because of the intrinsic affinities to endogenous nitric oxide (NO) and carbon monoxide (CO) as vasorelaxation factors and because of the facilitated $O_2$ release that might induce autoregulatory vasoconstriction. We examined these gas reactions when Hb-containing solutions of four kinds were perfused through artificial narrow tubes at a practical Hb concentration (10 g/dl). Purified Hb solution, polymerized bovine Hb (PolyBHb), encapsulated Hb [Hb-vesicles (HbV), 279 nm], and red blood cells (RBCs) were perfused through a gas-permeable narrow tube (25 μm inner diameter) at 1 mm/s centerline velocity. The level of reactions was determined microscopically based on the visible-light absorption spectrum of Hb. When the tube was immersed in NO and CO atmospheres, both NO binding and CO binding of deoxygenated Hb (deoxy-Hb) and PolyHb in the tube was faster than those of HbV and RBCs, and HbV and RBCs showed almost identical binding rates. When the tube was immersed in a $N_2$ atmosphere, oxygenated Hb and PolyHb showed much faster $O_2$ release than did HbV and RBCs. PolyHb showed a faster reaction than Hb because of the lower $O_2$ affinity of PolyHb than Hb. The diffusion process of the particles was simulated using Navier-Stokes and Maxwell-Stefan equations. Results clarified that small Hb (6 nm) diffuses laterally and mixes rapidly. However, the large-dimension HbV shows no such rapid diffusion. The purely physicochemical differences in diffusivity of the particles and the resulting reactivity with gas molecules are one factor inducing biological vasoconstriction of Hb-based oxygen carriers.

microcirculation; blood substitutes; gas biology; liposome; erythrocytes

CELL-FREE, HEMOGLOBIN-BASED oxygen carriers (HBOCs) have been developed for use as transfusion alternatives. Some examples are intramolecular cross-linked Hb, polymerized Hb, and polyethylene glycol conjugated Hbs (5). The realization of HBOCs has long been anticipated, because they are free of pathogens and blood-type antigens and are storable for a long time for using at emergency situations. Some are in the final stage of clinical trials (23). The major remaining hurdle before clinical approval of this earliest generation of HBOCs is vasoconstriction and resulting hypertension, which are presumably attributable to the high reactivity of Hb with endothelium-derived nitric oxide (NO) (26, 28, 55). It has been suggested that small molecular Hbs permeate across the endothelial cell layer to the space near by the smooth muscle and inactivate NO. However, cellular Hb-vesicles (HbV) that encapsulate concentrated Hb solution in phospholipid vesicles (37) induce neither vasoconstriction nor hypertension (32). A physicochemical analysis using stopped-flow rapid scan spectrophotometry clarified that Hb encapsulation in vesicles retards NO binding compared with molecular Hb, because an intracellular diffusion barrier of NO is formed. The requisites for this diffusion barrier are 1) a more concentrated intracellular Hb solution, and 2) a larger particle size (34, 36). Even though various kinds of liposome-encapsulated Hb have been studied by many groups (29, 38), our HbV encapsulates a highly concentrated Hb solution (>35 g/dl) with a regulated large-particle diameter (250–280 nm) and attains 10 g/dl Hb concentration in the suspension. The absence of vasoconstriction in the case of intravenous HbV injection might be related to the lowered NO-binding rate constant, although it is much larger than that of red blood cells (RBCs) (34), and the lowered permeability across the endothelial cell layer in the vascular wall.

The proposed mechanism of vasoconstriction induced by HBOCs in relation to gaseous molecules is not limited to NO scavenging (31, 46, 50). For example, endogenous carbon monoxide (CO) is produced by constitutive hemeoxygenase-2 in hepatocytes; it serves as a vasorelaxation factor in hepatic microcirculation. Small molecular Hb permeates across the fenestrated endothelium, scavenges CO, and induces constriction of sinusoids and augments peripheral resistance (8). Oversupply of $O_2$ induces autoregulatory vasoconstriction to regulate the $O_2$ supply (10, 14, 16, 19). Injection of small HBOCs induces vasoconstriction, probably because of the facilitated $O_2$ transport (1, 18).

These reports imply the importance of studying the reaction profiles of HBOCs with NO, CO, and $O_2$. Stopped flow-rapid scan spectrophotometry and flash photolysis are common methods to define the binding and dissociation rate constants of Hb (25, 26, 31, 34, 36, 42). However, the Hb concentration in a cuvette must be diluted extremely, e.g., to 2 μM heme concentration ([Hb] = 0.003 g/dl), which is much lower than the practical concentration of HBOC injections ([Hb] = 4–13 g/dl). Moreover, the results of flash photolysis depend on the quantum yield (Φ = 0.5 for CO; 0.002 for NO) (43). This is not practical for larger particles, such as HbV, because the photodissociated gas molecule would remain in the particles before the rebinding profiles are observed (11). The results do not necessarily reflect the in vivo physiological circulatory condition, especially in small arteries and arterioles, so-called resistance vessels, where blood flow is strictly regulated. We are interested in the reaction profiles of HBOC fluids of a practical concentration without considering any biological effect, such as permeation across the endothelial cell layer in the vascular wall.
Gas-permeable narrow tubes enable the measurement of the O₂-releasing rates of HBOCs and RBCs during their flow through the tubes at a practical [Hb] (6–13 g/dl) (18, 27, 40, 48). As described in this paper, we used gas-permeable narrow tubes made of perfluorinated polymer to study not only O₂ release, but also NO-binding and CO-binding profiles, all of which should relate to the mechanisms of vasooactive properties of cell-free HBOCs and the vasoinactive properties of cellular HbV proposed above.

**MATERIALS AND METHODS**

**Preparations of HbV, Stroma-free Hb, Polymerized Hb, and Human RBCs**

HbV was prepared as reported previously (33, 40, 45, 47), with slight modifications. Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). Then Hb was stabilized by carbonylation (HbCO) and concentrated by ultrafiltration to 38 g/dl. Subsequently, pyridoxal 5-phosphate (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, because 2,3-diphosphoglyceric acid is chemically unstable (53). 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, cholesterol, 1,5-O-dihexadecyl-N-succinyl-1-glutamate (Nippon Fine Chemical, Osaka, Japan), and 1,2-distearoyl-sn-glycerol-3-phosphatidylethanolamine-N-PEG5000 (NOF, Tokyo, Japan) at a molar composition of 5:5:1:0.033. The particle diameter was regulated using the extrusion method (45). The encapsulated HbCO was converted to oxyhemoglobin (HbO₂) by exposing the liquid membrane of HbV to visible light under an O₂ atmosphere. Finally, the Hb concentration of the suspension was adjusted to 10 g/dl. The particle size distribution was measured using a light-scattering method (Submicron Particle Size Analyzer, model N4 PLUS; Beckman Coulter, Fullerton, CA).

Purified human Hb solution suspended in phosphate-buffered saline (PBS) solution was prepared and mixed with PLP at molar ratios of PLP/Hb tetramer = 4 [Hb] = 10 g/dl). We also used polymerized bovine Hb (PolyHb) solution (PolyHbHb) designed for veterinary use (oxygloin; Biopure, Cambridge, MA) (2), which is a mixture of nonpolymerized tetrameric Hb (37.2%) and PolyHbHb with a broad molecular weight distribution. The PolyHbHb solution (13 g/dl) was diluted to 10 g/dl using PBS. PolyHbHb was only one chemically modified Hb that was commercially available, and its physicochemical properties and the presence of vasoconstrictive effect were documented (2, 3). We used this product as the standard product as a negative control.

For this study, we used fresh human blood specimens. The study was approved by Waseda University’s Ethics Committee on Medical Research Involving Human Subjects and performed according to the World Medical Association Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects (revised Nov. 13, 2001). A blood specimen was withdrawn after obtaining written, informed consent from donors. It was mixed immediately with an anticoagulant, and RBCs were pelleted at 800 g for 30 min. Then they were resuspended and washed twice with PBS. The suspension was then filtered through a leukocyte removal filter (Pall, East Hills, NY). The RBC suspensions were prepared at a Hb concentration of 10 g/dl.

The values of the oxygen partial pressure at which Hb is half-saturated and Hill numbers of HbV, Hb solutions, and RBCs were obtained from the oxygen equilibrium curve measured using a Hemox Analyzer (TCS Medical Products, Philadelphia, PA) at 37°C (Table 1). Steady-shear viscosity measurements were performed using a rheom (Physica MCR 301; Anton Paar, Graz, Austria) at 25°C.

### Table 1. Physicochemical properties of HbV, PolyHbHb, Hb, and RBC

<table>
<thead>
<tr>
<th></th>
<th>HbV</th>
<th>PolyHbHb</th>
<th>Hb</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb], g/dl</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Hb/PLP by mol</td>
<td>1/2.5</td>
<td>1/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_{\text{CO}}) (10^{-4} M^{-1} s^{-1})</td>
<td>2.1a</td>
<td>2.7a</td>
<td>2.1a</td>
<td>0.65b</td>
</tr>
<tr>
<td>(k_{\text{NO}}) (10^{-7} M^{-1} s^{-1})</td>
<td>0.61a</td>
<td>2.4a</td>
<td>2.4a</td>
<td>0.012b</td>
</tr>
<tr>
<td>(k_{\text{O}}) (10^{-4} M^{-1} s^{-1}), in 50 mM Na₂S₂O₄</td>
<td>2.14a</td>
<td>2.14a</td>
<td>2.14a</td>
<td>0.014b</td>
</tr>
<tr>
<td>P₅₀, Torr</td>
<td>25–28</td>
<td>54</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>Size</td>
<td>279 nm</td>
<td>87–502 kDa</td>
<td>65 kDa</td>
<td>8 µm</td>
</tr>
<tr>
<td>Viscosity, mPa·s, at 10 s⁻¹</td>
<td>3.75</td>
<td>1.48</td>
<td>1.35</td>
<td>2.46</td>
</tr>
<tr>
<td>Viscosity, mPa·s, at 1 s⁻¹</td>
<td>6.88</td>
<td>1.54</td>
<td>1.35</td>
<td>3.18</td>
</tr>
<tr>
<td>Perfusion pressure, kPa</td>
<td>21.0–23.0</td>
<td>7.0–8.0</td>
<td>6.0–7.0</td>
<td>9.0–10.0</td>
</tr>
</tbody>
</table>

HbV, Hb-veicles; PolyHbHb, polymerized bovine Hb solution; RBC, red blood cells; [Hb], Hb concentration; PLP, pyridoxal 5'-phosphate; \(k_{\text{CO}}\), apparent NO-binding rate constant; \(k_{\text{NO}}\), apparent CO-binding rate constant; \(k_{\text{O}}\), apparent O₂-releasing rate constant; P₅₀, oxygen partial pressure at which Hb is half-saturated. *See Ref. 36; **see Ref. 4; ***see Ref. 2; ****see Ref. 42; measured using a stopped-flow rapid scan spectrophotometer (RSP-1000; Unisoku, Osaka, Japan) by rapidly mixing the PolyHbHb solution (10 µM in PBS) and a 50 mM Na₂S₂O₄ solution in PBS; ***see Ref. 52.

### Perfusion of Hb-containing Fluids Through Narrow Tubes

Narrow, gas-permeable tubes (25-µm inner diameter; 37.5-µm wall thickness; 150-mm length) were made of a fluorinated ethylene-propylene copolymer (Hirakawa Hewtech, Ibaraki, Japan), as described in previous reports (15, 35, 39, 48) (Fig. 1). One end of the narrow tube was connected to a reservoir of the Hb-containing suspension. The narrow tube was immersed in a water bath (12 cm long × 3 cm width, 0.3 mm depth) made by two acrylic plates with a rubber supporting plate in between, and it was placed horizontally on the stage of an inverted microscope (IX-71; Olympus, Tokyo, Japan). The suspension in the reservoir was mixed gently and continuously with a magnetic stirrer (CC 301; AS One, Tokyo, Japan) and pressurized using a syringe connected to a syringe pump (FP-100; Toyo Sangyo, Tokyo, Japan). The perfusion pressure was monitored using a digital pressure sensor (AP-C30; Keyence, Tokyo, Japan). The centerline flow velocity was analyzed using photodiodes and the cross-correlation technique (Velocity Tracker Mod-102 B; Vista Electronics, Ramona, CA) (13). This method usually requires a significant change of contrasts because of the RBCs passing. However, stroma-free Hb (SFHb), PolyHbHb, and HbV are distributed homogeneously in the tube; no change of contrast is obtainable. Therefore, we added a small amount of RBCs (5 vol%) to enable centerline velocity measurements. This level of addition would not influence the reaction rate of the whole solution, because we confirmed that perfusion of saline with 5 vol% RBCs (without Hb or HbV) provided negligibly small light absorption spectrum. The centerline velocity was adjusted to 1 mm/s by changing the pressure that was applied to the reservoir. We selected the velocity 1 mm/s in the tube and the gas concentrations (see below) to obtain the absorption changes in the tube of 12 cm length, according to our laboratory’s previous paper (39) and a reference paper of Tateishi et al. (48). The water bath was filled with saline containing 10 mM sodium hydrosulfite (Na₂S₂O₄; Wako Pure Chemical Industries, Tokyo, Japan), bubbled with pure N₂, low-concentration NO (NO, 4.7%; N₂, 95.3%), or CO (CO, 14.14%; N₂, 85.86%). Na₂S₂O₄ is effective to eliminate trace amount of remaining oxygen that might affect the reactions of Hb and CO or NO. The wall of the narrow tube is made of perfluorinated polymer and is permeable only for gas molecules, not for Na₂S₂O₄. The entire perfusion experiment was performed at 25°C. In our experiment, the inner volume of the narrow tube is 5.9 × 10⁻⁵ cm³, which is much smaller (1/180,000) than that of the exterior water bath (11 cm³). We assumed that O₂ is change of contrasts because of the RBCs passing. However, stroma-free HBOCs and the vasoinactive properties of cellular HbV proposed above.
Fig. 1. Top: experimental setup of a gas-permeable artificial narrow tube immersed in a water bath made by the gap between two transparent acrylic plates with a rubber supporting plate. One end of the narrow tube was connected to a reservoir of the Hb-containing suspension. The reservoir is pressurized by N2 gas for perfusion of a fluid through the tube. Bottom: microscopic view of a gas-permeable artificial narrow tube. The tube, made of perfluoro polymer, is gas permeable. The tube is immersed in water equilibrated with N2, low-concentration carbon monoxide (CO), or nitric oxide (NO) gases. The wall thickness, (100 – 25)/2 = 37.5 μm, is important, not only for regulation of measurable gas permeability, but also for the stiffness of the tube of greater than 12 cm traveling distance. The centerline flow velocity was adjusted to 1 mm/s. To monitor the velocity, a small amount of red blood cell (RBC) solution is mixed. Measurements of absorption spectrophotometry of the fluid in the tube were performed at several traveling distances.

Equipment to Monitor Gas Reactions in Narrow Tubes

The apparatus consisted of an inverted microscope with an objective lens of ×40 magnification (ULWD CDPlan 40PL; Olympus), a spectrophotometer (Photicn multichannel analyzer, model PMA-11; Hamamatsu Photonics K.K., Hamamatsu, Japan) connected through a C-mount, a thin optical guide, and a computer (FMV BIBLIO MG50R; Fujitsu, Tokyo, Japan). The microscope’s light source (a halogen lamp) intensity was controlled using a current stabilizer (TH4-100; Olympus). The scanned wavelength was 194–956 nm with a gate time of 100 ms/scan; data were obtained every 0.2 nm. One spectrum from a 25-μm-diameter spot over the centerline of the narrow tube was recorded, and 100 scans were accumulated in 10 s. A measuring spot on the narrow tube within the visual field of the microscope was fixed on a monitor (PVM-14L2; Sony, Tokyo, Japan) through a charge-coupled device camera (model CS 230B; Olympus) by sliding the microscope stage.

Measurement of O2-releasing Rates of Oxygenated Hb-containing Solutions

To measure the O2 releasing rate, the gas-permeable tube was immersed in deoxygenated saline solution containing 10 mM Na2S2O4. The spectroscopic measurements were performed at traveling distances of 10, 30, 50, 70, and 90 mm. After a steady flow was attained, three measurements were performed. Because of the light scatter by fine particles, the absorbance of the HbVs at a shorter wavelength was slightly higher than that at a longer wavelength (41). In the spectra of the 100% deoxy-Hb and 100% oxygenated Hb-containing samples, two isosbestic points (522 and 586 nm) in the Q band of Hb were connected by a straight line as the baseline (Fig. 2A). The absorbances at 555 nm [A555; maximum absorbance (λmax) of deoxy-Hb] and 576 nm (A576, λmax of oxyHb) from the baseline were obtained to produce a calibration line that shows the relation between the O2 saturation (in %) and the ratio of the two absorbances (R = A555/A576) (39). The O2 saturation values of each sample were averaged and shown vs. the traveling distance (n = 3, mean ± SD).

Measurement of CO-binding Rate of Deoxy-Hb-containing Solutions

To measure the CO-binding rate, the tube was immersed in a saline solution containing 10 mM Na2S2O4, which had been previously bubbled with a gas of 14.14% CO/N2 balance (Takachiho Chemical Industrial, Tokyo, Japan). The resultant CO concentration outside of the tube was ~135 μM. The spectroscopic measurements were performed by the same manner. In the spectra of the 100% deoxy-Hb and 100% carboxylated Hb-containing samples, two isosbestic points (454 and 578 nm) were connected by a straight line as the baseline (Fig. 2B). The A555 (λmax of deoxy-Hb) and A569 (λmax of HbCO) from the baseline were obtained to make a calibration line that shows the relation between the CO saturation (in %) and the ratio of the two absorbances (R = A555/A569). The CO saturation values of each sample were averaged and shown vs. the traveling distance (n = 3, mean ± SD).

Measurement of NO-binding Rate of Deoxy-Hb-containing Solutions

The method to measure the NO-binding rate is essentially identical with that of CO-binding rate. A gas of 4.7% NO/N2 balance (Takachiho Chemical Industrial) was used to attain the NO concentration, ~88 μM. Two isosbestic points (449 and 592 nm) of HbNO and deoxy-Hb were obtained (Fig. 2C). The A555 (λmax of deoxy-Hb) and A575 (λmax of HbNO) from the baseline were used to obtain the calibration curve of NO saturation (in %) vs. R = A555/A575.
Simulation of Diffusion Profiles of Hb and HbV in Narrow Tubes

The diffusion and distribution of Hb (6 nm) and HbV (250 nm) in a fluid flowing through a tube (25-μm diameter) were simulated to clarify the stirring effects of the fluids. The profiles of the diffusion of Hb molecules or HbV particles that were originally present just near the wall of the narrow tube (12.5-μm radius, 10-cm length, 1-mm/s centerline velocity) at the entrance are analyzed. We assumed that the two different solutions had identical physicochemical properties. Component 1 enters the core, with 0- to 11.5-μm tube radius. Then component 2 enters the peripheral, with 11.5- to 12.5-μm radius. The simulated diffusion of component 2 to the center and to the flow direction in two-dimensional imaging of the tube cross section was calculated.

Hb solution. The Hb is much smaller than the tube diameter. The Navier-Stokes equation is solved with the incompressible condition to obtain flow velocity v and pressure p.

\[
\rho(\partial v/\partial t) + (pv \cdot \nabla)v = -\nabla p + \nabla \cdot \left\{ \eta \left[ \nabla v + (\nabla v)^T \right] \right\}
\]  

where \( \rho \) denotes the density, \( \eta \) is the viscosity, and \( t \) is time. An advection-diffusion equation is used for solute transport in a flow.

\[
\partial C_i/\partial t + \nabla \cdot (-D_i \nabla C_i) = - (v \cdot \nabla) C_i
\]  

where \( C_i \) represents a solute concentration, and \( D_i \) is the diffusion constant. The flux density \( N_i \) of a solute is expressed as

\[
N_i = C_i v - D_i \nabla C_i
\]

The tube wall does not allow penetration of the solute; the Neumann condition is adopted as

\[
N_i \cdot \mathbf{n} = - D_i \nabla C_i \cdot \mathbf{n} = 0
\]

At the entrance tube, \( C_i \) is fixed. At the tube exit, the Neumann condition without the concentration gradient is adopted as \( N_i \cdot \mathbf{n} = C_i \); \( v \cdot \mathbf{n} \). In fact, \( D_i \) is expressed as

\[
D_i = kT/(6\pi\eta d)
\]

where \( d \) is particle diameter.

HbV suspension. For HbV, the particle size is 1/100 of the tube diameter and is no longer negligible. Actually, HbV is regarded as a solid particle and the fluid as a solid-liquid two-phase flow. A two-phase flow equation is solved for the velocities of the particles and the liquid phase separately, with the assumptions that 1) the difference in density between HbV and the suspending medium is not large; 2) the two phases deform and flow together; and 3) the relative velocity (slip velocity) is determined by the mechanical balance of pressure and slip force.

A mean velocity \( v \) and a pressure \( p \) is solved by a two-phase flow equation with a slide friction term expressed as Eq. 6.

\[
\rho(\partial v/\partial t) + (pv \cdot \nabla)v = -\nabla p - \nabla \cdot \left\{ \frac{1}{2} (\rho_s + \rho_d) \mathbf{v_{slip}} \mathbf{v_{slip}} \right\} + \nabla \cdot \left\{ \eta \left[ \nabla v + (\nabla v)^T \right] \right\}
\]  

Therein, \( \rho \) signifies the averaged density, \( \rho_s \) is the mass fraction of solid particles, and \( \mathbf{v_{slip}} \) is the relative velocity between the two phases. For a solid particle dispersion, \( \mathbf{v_{slip}} \) is expressed as Eq. 7.

\[
\mathbf{v_{slip}} = -\left[ (\rho - \rho_d)d^2/(18\pi\eta) \right] \nabla p
\]

Wherein, \( \rho_d \) is the density of solid particles.
The viscosity \( \eta \) of a multiphase flow depends on the volume fraction of the particles (\( \phi \)) and shear rate (\( \gamma \)) and was obtained by the experimental measurement between the volume fraction and the shear viscosity of the HbV suspension, as shown in Eq. 8.

\[
\eta(\phi, \gamma) = 0.9 \times 10^{-3}(0.2542\phi + 0.2542) \gamma^1.2784 + 1.2207\phi^2
\] (8)

The Maxwell-Stefan advection-diffusion equation was used for a highly concentrated particle dispersion (HbV) to calculate the counter diffusion.

\[
\frac{\partial \phi_i}{\partial t} + \nabla \cdot (\phi_i \rho v) = 0
\] (9)

Therein, \( j \) is the relative diffusion flux, and \( \phi_i \) is the volume fraction of the component \( i \). The diffusion flux can be expressed by external forces, such as the concentration gradient and pressure, applied to the component \( i \). Therefore, Eq. 9 is converted as follows with a self-diffusion constant of a solute \( D \).

\[
\frac{\partial \phi_i}{\partial t} + \nabla \cdot \left[ \phi_i \rho D_i (\nabla \rho_i + \frac{\rho_i - \rho_j}{\rho} \nabla \rho_j) \right] = 0
\] (10)

For calculations, we used the Comsol Multiphysics and Chemical Engineering Module (Comsol, Burlington, MA). Table 2 presents the required physicochemical parameters for simulation.

### RESULTS

#### \( O_2 \)-releasing Profile

All Hb-containing solutions showed the change of absorption spectroscopy in the \( Q \) bands (Fig. 3A) as they traveled a longer distance and released oxygen (lines 2–6). The two characteristic peaks derived from HbO2 (top lines, 100% HbO2; \( \lambda_{max} \), 541 and 576 nm) tended to decrease and a new peak derived from deoxy-Hb (bottom lines, 100% deoxy-Hb; \( \lambda_{max} \), 555 nm) became evident, especially in the case of PolyBHb and SFHb solutions. Both HbV and RBCs showed two peaks, even at 9-cm traveling distance. Figure 4A presents the level of oxygenation vs. the traveling distance of four Hb-containing solutions. At 9-cm traveling distance, both PolyBHb and Hb solutions released \( O_2 \) considerably, and the averaged levels of HbO2, respectively, became 27 ± 8 and 43 ± 6%. On the other hand, both HbV and RBCs, respectively, remained high levels of HbO2, 68 ± 7 and 79 ± 4%. The profile of HbV resembled that of RBCs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hb</th>
<th>HbV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion coefficient*</td>
<td>9 \times 10^{-11} m²/s</td>
<td>9 \times 10^{-11} m²/s</td>
</tr>
<tr>
<td>Viscosity</td>
<td>1.0 mPa·s</td>
<td>0.9 mPa·s</td>
</tr>
<tr>
<td>Density of the solution†</td>
<td>1025 kg/m³</td>
<td>1003.3 kg/m³</td>
</tr>
<tr>
<td>[Hb]</td>
<td>10 g/dl, 1.55 mM</td>
<td>1003.3 g/dl, 1.55 mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HbV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume fraction</td>
<td>0.4</td>
</tr>
<tr>
<td>Particle diameter</td>
<td>250 nm</td>
</tr>
<tr>
<td>Viscosity of the suspending medium</td>
<td>0.9 mPa·s</td>
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<tr>
<td>Viscosity of the HbV suspension</td>
<td>Data in Table 1</td>
</tr>
<tr>
<td>Density of the suspending medium²</td>
<td>1003.3 kg/m³</td>
</tr>
<tr>
<td>Density of HbV particles‡</td>
<td>1067.05 kg/m³</td>
</tr>
</tbody>
</table>

*See Ref. 24. †Measured by a dynamic capillary rheometer (Anton Paar, Graz, Austria). ‡Calculated from the density of the HbV suspension (1028.8 kg/m³), volume fraction (0.4), and the density of the suspending medium (1003.3 kg/m³) as [1028.8 − (1.0 − 0.4) × 1003.3] / 0.4 = 1067.05 kg/m³.

#### CO-binding Profile

Figure 3B shows that all of the Hb-containing solutions showed a single peak at 555 nm attributable to deoxy-Hb (top lines). The absorption spectrum changes gradually, and two new peaks tended to appear at 540 and 569 nm (lines 2–6), indicating the conversion of deoxy-Hb to HbCO (bottom lines, 100% HbCO). The HbCO conversion rates of PolyBHb and Hb solutions were almost identical; the HbCO levels reached 94 ± 3 and 90 ± 3% at 9-cm traveling distance (Fig. 4B). On the other hand, HbV showed the lower rate of HbCO conversion, and it reached 71 ± 3% at 9-cm traveling distance, which was almost identical to the profile of RBCs (66 ± 4% at 9-cm traveling distance).

#### NO-binding Profile

Actually, HbNO has two characteristic peaks at 545 and 575 nm (bottom lines in Fig. 3C). Both PolyBHb and Hb solutions tended to show similar spectra of HbNO at 9-cm traveling distance. However, both HbV and RBC showed a slight shoulder at around 575 nm, but the new peaks were not apparent, and the change of the spectrum was not remarkable. Figure 4C portrays the NO-binding profiles of Hb and PolyBHb solutions were almost identical; they, respectively, reached HbNO levels of 70 ± 5 and 65 ± 5%. They are much faster than the profiles of RBCs (36 ± 3%) and HbV (39 ± 6%).

### Simulation of the Diffusion of Hb and HbV in the Narrow Tube

We assumed diffusion of two different fluids with the identical physical property in a tube, namely, HbV-1 as component 1 and HbV-2 as component 2, and simulated how the two fluids are mixed during their flow. Figure 5A shows the diffusion of HbV in the narrow tube. The red color signifies that the HbV-2 enters in the peripheral side of the tube (near the wall); the red color gradually turns to yellow, green, and light blue, progressively, with the traveling distance, indicating that the HbV-2 diffuses and is gradually mixed with the HbV-1, and that the HbV-2 concentration decreases. However, the distribution is not homogeneous, even at 100-\( \mu \)m traveling distance. The white lines show trajectory patterns of component 2. A particle of 11.5-\( \mu \)m radius located at the entrance diffuses to 9-\( \mu \)m radius from the centerline at 100-\( \mu \)m traveling distance.

The diffusion of Hb is so rapid that the color change is mostly observed only at the entrance in a short traveling distance (<10 \( \mu \)m) (Fig. 5B). It becomes homogeneous quite rapidly. The Hb-2 of 11.5-\( \mu \)m radius located at the entrance diffuses to 3-\( \mu \)m radius at 100-\( \mu \)m traveling distance. In fact, Hb reaches the centerline at 1-mm traveling distance. On the other hand, HbV reaches the centerline at 25-mm traveling distance.

### DISCUSSION

Our primary finding is that the reactions of NO binding and CO binding and the \( O_2 \) release of Hb solutions are markedly retarded for encapsulated Hb (HbV) and RBCs when perfused through artificial narrow plastic tube. These results support the physiological observation that cell-free Hb induces vasoconstriction, but not HbV and RBCs (32).

Conditions of hemolysis (20) and studies related to the development of HBOCs (9, 12, 21, 26, 30, 32, 44, 51) 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 (46). Its entrapment by cell-free Hb solutions induces constriction of sinusoidal capillaries (8). These side effects caused by the presence of molecular Hb in plasma suggest that the cellular structure of RBCs plays a role in ensuring the bioavailability of NO and CO. It has been suggested that faster O2 unloading from the HBOCs is advantageous for tissue oxygenation (27). However, this concept is controversial in light of recent findings, because an excess O2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders (31).

The presence of a plasma layer (RBC-free layer) can constitute a diffusion barrier of gas molecules between the vascular wall and RBCs. This explanation is plausible because a
cell-free Hb solution distributes homogeneously in the plasma phase and facilitates gas reactions (18, 27). The HbV is larger than these cell-free Hbs, but it is much smaller than RBCs and distributes homogeneously in the tube. However, the HbV suspension showed no such facilitated reactions. In our laboratory’s previous paper, we included speculation that the discrepancies were attributable to marked differences in the Hb and HbV sizes, which might influence the diffusion of particles and the stirring effect when the suspension flows in the tubes (39). In the present study, we clarified from the computer simulation a marked difference between Hb and HbV in the diffusion profile. The simulation showed that small Hb diffuse laterally and mix efficiently during the perfusion that facilitated the gas reactions.

Even though the apparent NO-binding rate constant $k_{on}^{(NO)}$, the apparent CO-binding rate constant $k_{on}^{(CO)}$, and the apparent $O_2$-releasing rate constant $k_{off}$ differ significantly between HbV and RBC, as shown in Table 1, the HbV fluid and RBC fluid perfused through the narrow tube showed almost identical reaction profiles. The reaction rate constants were obtained by stopped flow-rapid scan spectrophotometry, where the test solution was extremely diluted, to observe the change of absorption spectroscopy ([Hb] = 0.0024–0.032 g/dl) (34, 42). In the present experiment, the [Hb] values of the test fluids were 10 g/dl, a more practical concentration. The results imply that the size and the diffusion of particles at a high concentration would be more important than the reaction rates of the individual particles. For particles of HbV size and/or larger, including RBCs, the lateral diffusion is considerably small. For flowing RBCs, the peripheral cell-free layer can be a diffusion barrier. The slightly higher viscosity of HbV than RBC would lower the lateral diffusion of HbV. Consequently, no remarkable difference was observed between HbV and RBC in this study. It is particularly interesting that the $k_{on}^{(CO)}$ values of Hb and HbV measured using a stopped-flow spectrophotometer are almost identical. However, in this study, the CO-binding profile differs among the concentrated solutions. This also implies that the lateral diffusiveness of particles is more important in the tube than the individual binding rate constants of the particles in a cuvette. The NO and CO molecules, which diffuse through the tube wall and enter the lumen, would immediately react with Hb-containing solutions at the interface. Therefore, the fast mixing would be effective to create more binding site of these gas molecules. In the case of $O_2$ release, the $O_2$ can be removed more easily at the tube wall, where the $O_2$ concentration gradient is the greatest. The fast mixing would create a higher concentration gradient and fast $O_2$ transfer.

PolyHB is larger than Hb and is expected to show a lower rate of $O_2$ release. However, the result was that PolyHB showed a faster $O_2$ release rate than Hb. PolyHB shows similar viscosity with Hb (1.48 cP for PolyHB; 1.35 cP for Hb) and contains many nonpolymerized tetrameric HB (37.2%). The possible reason is that oxygen affinity of PolyHB (54 Torr at 37°C) is much lower than that of human Hb with PLP (26 Torr), and the larger $k_{off}$ value of PolyHB than that of human Hb. It is expected that the gas reaction rates of cell-free Hb solutions, including PolyHB, can be reduced by increasing the solution viscosity or particle size and reducing $D_s$, according to the Stokes-Einstein equation (Eq. 5). In addition, an increased fluid viscosity would create a higher shear stress on the vascular wall and increase the production of vasorelaxation factor, such as NO. A combination with a high viscosity hydroxyethyl starch, or a conjugation with polyethylene glycol should be examples to retard the gas reactions and improve microcirculation (7, 54).
The limitations of our study are that 1) the gas inlet and outlet of the tube are dependent on the gas permeability of the plastic tube wall and the applied gas concentration in the outer medium; 2) the inner tube diameter was limited to 25 μm, and the tube length was as long as 12 cm; and 3) the concentrations of NO and CO are markedly higher than the physiological condition. However, these conditions are necessary to determine the difference using absorption spectrophotometry under a steady flow. A considerable amount of oxygen is known to be released from RBCs at a microvessel level. In this sense, the tube diameter, 25 μm, is appropriate because it is close to the diameter of arterioles that regulate peripheral blood flow in response to the oxygen transfer and other stimuli, such as shear stress and NO concentration. Page et al. (27) measured the O2-releasing rate using gas-permeable silicone tube (25 μm) in only 4-mm traveling distance and in <1 s. Even though they observed Soret band in the absorption spectroscopy of HBOCs, no spectra was reported in the paper. McCarthy et al. (18) measured the O2-releasing rate indirectly by observing the reduction of Po2 from inlet to outlet, without observing the flow patterns in the microscope. Our group is the first to measure the NO- and CO-binding rates of HBOCs in the artificial narrow tube by directly observing the change of absorption spectra.

One question is whether the different reaction profiles between cellular HbV and cell-free Hbs in this study are sufficient to explain the absence or presence of vasoconstriction. We infer the presence of a threshold particle diameter, not only in terms of diffusiveness in the plasma phase, as discussed herein, but also in terms of penetration across the 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). At that space, CO or NO is produced as a vasorelaxation factor to bind to soluble guanylate cyclase, which catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (8, 17, 22, 32, 49). As summarized by Olson et al. (26), both the retardation of the NO reaction (reduced NO affinity) (6) and the larger particle diameter are inferred to be keys to suppression of vasoconstriction and hypertension induced by HBOCs.

Collectively, we clarified that one physicochemical property of the HBOCs, lateral diffusivity in the tube, can explain the differences in the rates of gas binding and releasing reactions that might directly be related to the mechanism of vasoconstriction. The difference would be magnified if we additionally consider the morphological structure of in vivo vascular walls and the effect of extravasation. Several biological mechanisms of vasoconstriction other than gas reactions are reported, such as induction of hypersensitivity of adrenergic receptors and plasma endothelin-1 increase (9). Our present results cannot explain the physiological responses entirely. Nevertheless, they can at least support previous reports describing the presence and absence of HBOC-induced vasoconstriction related to Hb ENCAPSULATION RETARDS GAS REACTIONS

Fig. 5. Schematic representation of the simulated density distribution and track of Hb molecule in a narrow tube (<100 μm traveling distance). We assumed that two different solutions with the same physicochemical properties enter and flow through the same tube. The radius of the tube was 12.5 μm: component 1 (blue color) enters the core of the tube (radial distance from the centerline, 0–11 μm), and component 2 (red color) enters near the wall (radial distance from the centerline, 11–12.5 μm). Finally, both components are mixed completely, but the rate is dependent on the physicochemical properties. A: diffusivity of HbV particles as component 2. The concentration of the particle (HbV) is expressed as volume fraction (volume fraction is 0.4 at Hb concentration, [Hb], = 10 g/dl). B: diffusivity of Hb molecules as component 2. The color gradation reflects the change of the component 2 concentration; the white lines represent tracking data of representative particles. The [Hb] is expressed as heme concentration = 1.55 mM at [Hb] = 10 g/dl.
gaseous molecules after intravenous administration. In our study, the gas reactions of RBCs are always slowest. RBCs are evolutionally designed to maintain toxic Hb molecules in corpses and to retard all of the gas reactions. It is an important physicochemical requirement to design an HBOC.

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DISCLOSURES

H. Sakai, S. Takeoka, and E. Tsushida are holders of patents on the liposomes (Hb-vesicles).

REFERENCES


