enging. 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 [20]. Oversupply of O_2 induces autoregulatory vasoconstriction to regulate the O_2 supply [42]. Injection of small HBOCs induces vasoconstriction, probably because of the facilitated O_2 transport [43]. These reports imply the importance of studying the reaction profiles of HBOCs with NO, CO, and O_2 in microvessels.

Stopped flow-rapid scan spectrophotometry and flash photolysis are common methods to define the binding and dissociation rate constants of Hb [39,40,44,45]. However, the Hb concentration in a cuvette must be diluted extremely, e.g., to 2 μM heme concentration ([Hb] = 0.003 g/dL). In contrast, a gas-permeable narrow tube (a model of microvessel) enables the measurement of the O_2-releasing rates of HBOCs and RBCs during their flow through the tube at a practical Hb concentration (e.g. 6–13 g/dL) [43,46,47] (Fig. **4**). We used gas-permeable narrow tubes (25 μm inner diameter) made of perfluorinated polymer to study not only O_2-release but also NO-binding and CO-binding profiles. We examined these gas reactions when Hb-containing solutions of four kinds were perfused through the tubes at a practical Hb concentration (10 g/dL). Purified Hb solution, $Poly_BHb$, encapsulated Hb (HbV, 279±95 nm), and RBCs were perfused 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 and $Poly_BHb$ in the tube were faster than those of HbVs and RBCs, and HbVs and RBCs showed almost identical binding rates. When the tube was immersed in a N_2 atmosphere, oxygenated Hb and $Poly_BHb$ showed much faster O_2-release than did either HbVs or RBCs. $Poly_BHb$ showed a faster reaction than Hb because of the lower O_2 affinity of $Poly_BHb$ than that of Hb [48].

The diffusion process of the particles was simulated using Navier-Stokes and Maxwell-Stefan equations (Fig. 4). Results clarified that small Hb (6 nm) diffuses laterally and mixes rapidly. However, the large-dimension HbVs show no such rapid diffusion. 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, fast mixing would be effective to create more binding sites 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. Fast mixing would create a higher concentration gradient and rapid O_2 transfer.

It is also speculated that there is a threshold particle diameter to penetrate 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), where CO or NO is produced as a vasorelaxation factor [20,36]. Because the particle size of HbV is obviously much larger than the Hb tetramer both the retardation of NO reaction and the larger particle diameter are inferred to be keys to suppress vasoconstriction and hypertension induced by HBOCs.

The purely physicochemical differences in diffusivity of the particles and the resulting reactivity with gas molecules are factors supporting the biological vasoconstriction of HBOCs.

5. HBOCS AS CARRIERS OF CO AND O_2

Actually, CO, biliverdin, and bilirubin are all produced during oxidative heme degradation that is catalyzed by a single stress protein: heme oxygenase (HO). They mediate anti-oxidative, anti-proliferative, and anti-inflammatory effects [49]. Endogenous CO shows a vasorelaxation effect, as does NO [50]. Motterlini *et al.* synthesized a series of CO-releasing metal complexes and clarified some pharmacological effects [51, 52]. Despite the poisonous effect of CO gas, low-concentration CO inhalation (250 ppm) was tested in animal models of hemorrhagic shock, septic shock, and ischemia-reperfusion [53]. Some cytoprotective effects were obtained. Their mechanisms have been studied extensively. Cabrales et al. [54] reported CO-bound RBC injection to hemorrhaged hamsters and clarified its cytoprotective effect in subcutaneous microcirculation. These studies have led us to test intravenous injection of CO as a ligand of heme in HBOCs that have been studied extensively as transfusion alternatives.

A traumatic hemorrhage might cause a shock state, which subsequently causes a systemic inflammatory response, leading in some cases to multiple organ failure (MOF). Resuscitation with transfusion or HBOCs with an O_2-carrying capacity induces reperfusion injury [17]. Activated neutrophils and macrophages produce reactive oxygen species (ROS) [55], in which NADPH-oxidase is involved as a major source of ROS by reacting with O_2 at reperfusion. Actually, we observed elevation of plasma enzyme levels 6 hr after resuscitation from hemorrhagic shock by administration of O_2 bound RBCs and HbVs in a rat model [16]. It is expected that co-injection of cytoprotective CO would improve resuscitative effects. For this study, using the same experimental model, we tested injection of CO-bound HbVs for the first time as an exogenous CO supplier for fluid resuscitation (Amount of injection fluid, 28 mL/kg body weight; concentration of Hb in the fluid, 8.6 g/dL; concentration of CO in the fluid, 5.3 mM). As comparative experiments, we also tested empty vesicles (EV), which carry neither O_2 nor CO, and CO-bound RBC. All fluids showed restoration of blood pressure and blood gas parameters, and the rats survived for a 6 hr observation period. No remarkable difference was found among the groups, except that the EV group showed marked hypotension. Plasma enzyme levels (AST and ALT) were elevated, especially in the O_2-HbV, O_2-RBC, and EV groups. They were significantly lower in the CO-HbV and CO-RBC groups than in the O_2-bound fluids. Blood HbCO levels (26–39% immediately after infusion) decreased to less than 3% at 6 hr, whereas CO was exhaled through the lung, as detected by gas chromatography. Both HbVs and RBCs gradually gained the O_2 transport function. Accordingly, both CO-HbV and CO-RBC showed a resuscitative effect for hemorrhagic-shocked rats. They reduced oxidative damage to organs, as shown as reduced plasma enzyme levels, in comparison to O_2-HbV and O_2-RBC. Adverse and poisonous effects of CO gas were not evident in this experimental model [27]. CO reportedly interacts with heme proteins such as NADPH-oxidase and NO synthase that relate to the production of O_2^- and NO. The possibility exists that the injected CO reduces production of both NO and O_2^-, along with its resultant $ONOO^-$. Actually, our immunohistochemical observations of the liver and lung clarified that injection of CO-HbV and CO-RBC reduced the formation of nitrosotyrosine on the proteins [27].

The HbCO levels in the CO-HbV and the CO-RBC groups immediately after infusion were 26%–39%, but they decreased rapidly and became less than 3% within 6 hr (Fig. **5**). The equilibrium constant of HbCO is well known as 200 times greater than that of HbO_2. However, this is calculated under equal concentrations of O_2 and CO. A rapid ligand-exchange reaction from HbCO to HbO_2 occurs because the rats inhale atmospheric air; fundamentally, the amount of O_2 is much greater than that of CO in the rat circulating blood. The *in vitro* rapid CO exchange reaction, which is shown to occur within 1 min from HbV to RBC, also supports an *in vivo* rapid ligand exchange among Hbs in HbVs and RBCs and heme proteins in tissues.

This study is the first reported to use an HBOC to administer CO in a shock state for a pharmacological effect. Although further research is necessary to clarify the mechanism and clinical relevance of our experimental results obtained using small animals, the data suggest that both RBCs and HBOCs can be effective CO carriers. Vandegriff et al. also reported that CO-bound PEG-Hb reduces

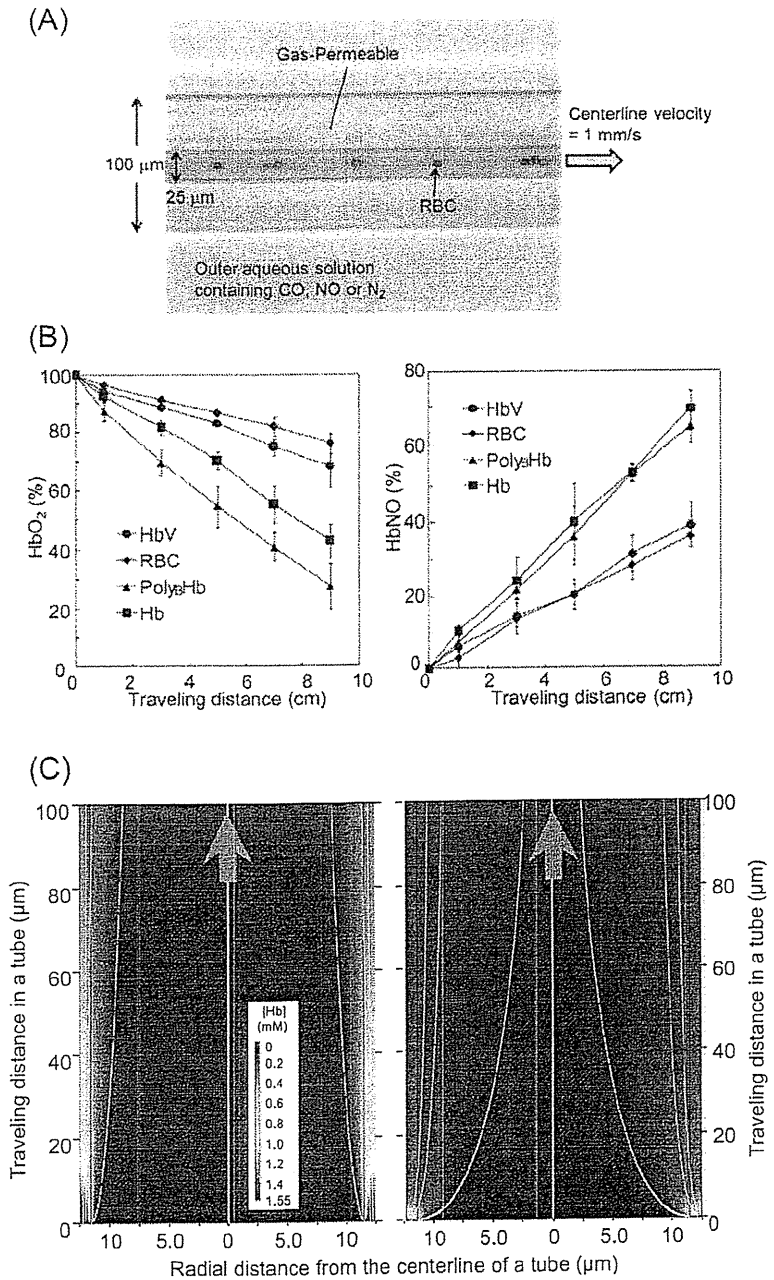

Fig. (4). (A) Left: Experimental setup of a gas-permeable artificial narrow tube immersed in a water bath made by the gap between two transparent acrylic plates with a rubber supporting plate. One end of the narrow tube was connected to a reservoir of the Hb-containing suspension. Then the suspension was perfused through the tube at 1 mm/s. **(B)** The change of the level of ligand reactions of the Hb containing fluids, HbV, $Poly_{B}Hb$ solution, Hb solution, and RBC with traveling distance; (left) Levels of O_2 release; (right) Levels of NO-binding. **(C)** Schematic representation of the simulated density distribution and track of HbV (left) and Hb (right) 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 tube radius 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, 11–12.5μm). Finally, both components are mixed completely. The diffusivity of HbVs is much slower than that of Hb, resulting in the retarded gas reactions in microvessels. The Hb concentration is expressed as the heme concentration ([Hb] = 1.55 mM at 10 g/dL) [48]. *(Reproduced with permission from Am J Physiol Heart Circ Physiol 2010; 298: H956-H965).*

myocardial infarction [56]. Advantages of CO-bound HBOCs injection are the following: (i) the oxygen carrying function, which is unnecessary at the beginning of resuscitation, is masked by carbonylation; (ii) carbonylhemoglobin is sufficiently stable for longer term storage; (iii) special equipment to inhale CO gas is unnecessary in emergency situations; (iv) the CO dosage is strictly definable; and (v) the fluid functions initially as a CO-carrier to prevent pro-oxidative damage, after which it functions in succession as an O_2-carrier.

6. CONCLUDING STATEMENT

Historically, the starting point of the development of HBOCs was the simple aim of transporting oxygen to peripheral tissues as

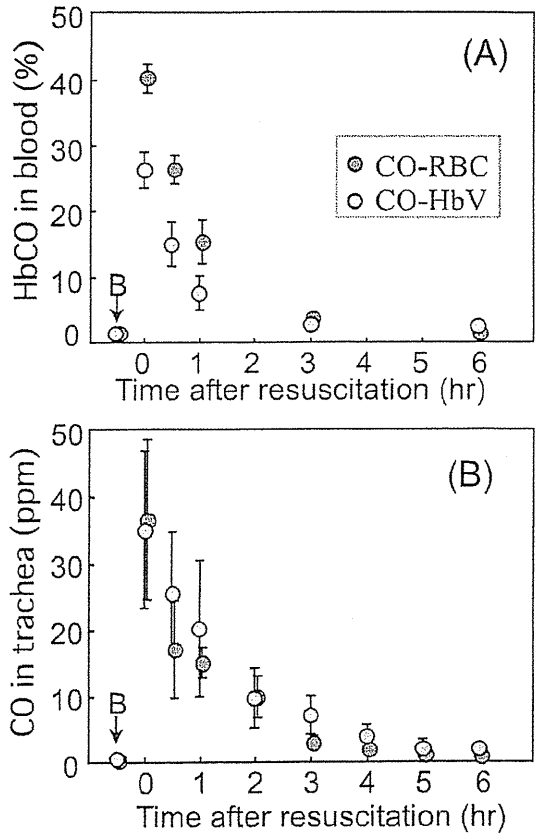

Fig. (5). Time course of HbCO levels in blood **(A)** and CO levels in the trachea **(B)** before and after injection of CO-HbV as a resuscitative fluid for hemorrhagic shock in rats. B denotes the baseline. CO-HbV released CO and gradually became O_2 carriers. In addition, some released CO was exhaled through a lung and detected in the trachea. Most of the injected CO became undetectable in the body within 6 h [27]. *(Reproduction permission obtained from Lippincott Williams & Wilkins).*

blood does. However, its development became complicated after the discovery of endogenous NO and CO, which have strong affinity to HBOCs, and which influence their safety and efficacy as oxygen carriers. Actually, RBCs are evolutionally designed to "retard" the gas reactions. Therefore, we must reconsider the physiological importance of the RBC structure when designing HBOCs. Oxygen should be transported to a site where oxygen is necessary. Oversupply engenders other problems. "Targeted oxygen delivery" using a HBOC is desirable by shifting the oxygen equilibrium curve to the left (high oxygen affinity) [57,58]. In addition to HbVs, new encapsulated Hbs without liposomes, such as polymersomes [59] and PEG-poly(ε-caprolactone) copolymer nanoparticles, have emerged recently from the use of advanced nanotechnologies [60]. Encapsulation of Hb can reduce the toxicity of cell-free Hbs by regulating the reactions with gaseous molecules. However, numerous hurdles must be surmounted to realize encapsulated Hbs because of the components of the capsules themselves and their structural complexity as a molecular assembly, especially on blood compatibility (absence of complement activation, platelet activation, aggregation, embolism, etc.). It is also important to consider the larger dosage requirement of encapsulated Hb for blood substitution than those of conventional drug delivery systems, which require no large dosage. In fact, the main concern of the side effect of HbV is splenohepatomegaly caused by the accumulation of a large amount of HbV in RES [33,61]. We confirmed it is transient and reversible without inducing systemic immunosuppression. This is a common issue of injecting a large amount of nanoparticle suspension, and we have to observe carefully the impact of this side effect in the ongoing preclinical research. Even so, we believe HbV is the most advanced cellular type HBOCs in terms of safety and stability of the capsules. We also demonstrated the possibility of HBOCs as a CO-carrier. Although CO is a toxic gaseous molecule, it shows a cytoprotective effect depending on the dose.

Gas bioengineering using HbVs can supply, adjust, or prevent gaseous molecules that are vital or toxic to the human body. Our research specifically emphasizes the discovery of new clinical applications of HbVs.

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27

Hemoglobin Vesicles as a Cellular-type Hemoglobin-based Oxygen Carrier

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27.1 Introduction

Hemoglobin (Hb) is the most abundant protein in blood (12–15 g/dL), and should be the most essential protein. However, Hb becomes toxic once it is released from red blood cells (RBCs), which is evident in some pathological hemolytic diseases. Chemically modified cell-free Hb-based oxygen carriers (HBOCs), such as intramolecularly crosslinked, polymerized, and polymer-conjugated Hbs, have been synthesized to prevent the toxic effect of cell-free Hbs. However, no product is commercially available yet. Some safety issues arose during the final stage of clinical trials. It seems difficult to completely eliminate the side effect of cell-free Hbs by chemical modification. Now is the time to reconsider the physiological importance of the cellular structure of RBCs. Why is Hb compartmentalized in RBCs with such a complicated corpuscular structure? Hb vesicles

* Emeritus Professor Eishun Tsuchida passed away during the submission of this manuscript.

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(HbVs) are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane. HbVs are designed to mimic or overcome the function of RBCs. In this chapter, we focus on the concept of Hb encapsulation and recent topics concerning HbVs, especially reactions with gaseous molecules (O_2, NO, CO), which greatly relate to its safety and a new application.

27.2 The Concept of Hb Encapsulation in Liposomes

Hb encapsulation was first performed by Chang in the 1950s [1], using a polymer membrane. Some Japanese groups also tested Hb encapsulation with gelatin, gum Arabic, silicone, and so on. Nevertheless, it was extremely difficult to regulate the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 [2] that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevici and Miller [3] prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, and so on. Since then, many groups have tested encapsulated Hbs using liposomes [4–7]. Some failed initially, and some are progressing with the aim of clinical usage. The Naval Research Laboratory presented remarkable progress on LEH [8], but it suspended development about 10 years ago. What we call HbVs with high-efficiency production processes and improved properties have been established by our group, based on nanotechnologies of molecular assembly and pharmacological and physiological aspects [9]. In spite of such a large number of studies of HBOCs in general, no product has so far been tested clinically because of the difficulty of the production method. Chemically modified cell-free HBOCs are much easier to produce, therefore more researchers have tested the cell-free types, and they have been more advanced than the cellular type in entering clinical trials. However, during the long history of R&D, some unexpected problems arose for cell-free HBOCs, presumably due to the direct exposure of Hb to vasculature.

It has been well understood that the compartmentalization of Hb in RBCs is important for: (i) prevention of extravasation or excretion through renal glomeruli; (ii) preservation of the chemical environment in cells, such as the concentrations of electrolytes and enzymes; and (iii) rheology control of blood, an RBC dispersion, to a non-Newtonian viscous fluid. Moreover, for us it seems that RBCs are evolutionally designed for: (iv) retardation and targeting of O_2 unloading at microcirculation to avoid autoregulatory vasoconstriction; (v) reduction of a high colloidal osmotic pressure of Hb solution to zero, to increase blood Hb concentration; and (vi) modulation of reactions with NO as an endothelium-derived relaxation factor (EDRF). Now we have to consider the physiological importance of RBC structure, and mimic the structure to design the optimal HBOCs.

Our HbVs are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane [7]. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, [lipids] = 6 g/dL, volume fraction ~40%). HbV has an oxygen-carrying capacity that is comparable to that of blood. HbV is much smaller than RBCs (250 vs 8000 nm), but it recreates the functions of RBCs, as has been confirmed by many animal experiments testing its effectiveness as a resuscitative

Table 27.1 *Preclinical studies of HbV as a transfusion alternative and for other therapeutics.*

	Indication	Ref.
1.	Resuscitative fluid for hemorrhagic shock	[11–13]
2.	Hemodilution	[14]
3.	Priming fluid for extracorporeal membrane oxygenator (ECMO) for cardiopulmonary bypass	[10]
4.	Perfusate for resected organs	[15, 16]
5.	Oxygenation of ischemic brain (stroke)	[17]
6.	Oxygenation of ischemic skin flap (plastic surgery)	[18, 19]
7.	Tumor oxygenation for irradiation sensitization	[20]
8.	CO carrier for cytoprotection at reperfusion	[21]
9.	Measurement of brain oxygen consumption by positron emission tomography (PET)	[22]

fluid for hemorrhagic shock, hemodilution, and a prime for cardiopulmonary bypass [10–12] (Table 27.1). Other characteristics similar to those of RBCs include: (i) the rate of O_2 unloading is slower than Hb solution [23]; (ii) colloid osmotic pressure is zero at [Hb] = 10 g/dL, and it has to be co-injected with or suspended in a plasma substitute such as albumin or HES [24]; (iii) the resulting viscosity of an HbV suspension is adjustable to that of blood [25]; (iv) HbV is finally captured by RES and the components are degraded and excreted [13, 26, 27]; (v) the HbV particle of itself is not eliminated through glomeruli [28]; (vi) PLP is co-encapsulated as an allosteric effector, instead of 2,3-diphosphoglyceric acid, to regulate oxygen affinity [18, 29]; (vii) no hemolysis occurs during circulation and the lipid-bilayer membrane prevents direct contact of Hb and vasculature; and (viii) reaction of NO is retarded to some extent by an intracellular diffusion barrier, and HbV does not induce vasoconstriction [30–32].

In the next section we focus on the gas reactions of cell-free Hb and cellular HbV.

27.3 Hb Encapsulation Retards Gas Reactions

The major remaining hurdle before clinical approval of the earliest generation of HBOCs is vasoconstriction and resulting hypertension, which is presumably attributable to the high reactivity of Hb with NO [33]. It has been suggested that Hbs permeate across the endothelial cell layer to the space near the smooth muscle and inactivate NO. However, cellular HbVs induce neither vasoconstriction nor hypertension [30]. A physicochemical analysis using stopped-flow rapid-scan spectrophotometry clarified that Hb encapsulation in vesicles retards NO binding in comparison to Hb because an intracellular diffusion barrier of NO is formed. The requisites for this diffusion barrier are (i) a more concentrated intracellular Hb solution and (ii) a larger particle size [31, 32] (Figure 27.1a). Even though various kinds of liposome-encapsulated Hb have been studied by many groups [7], 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

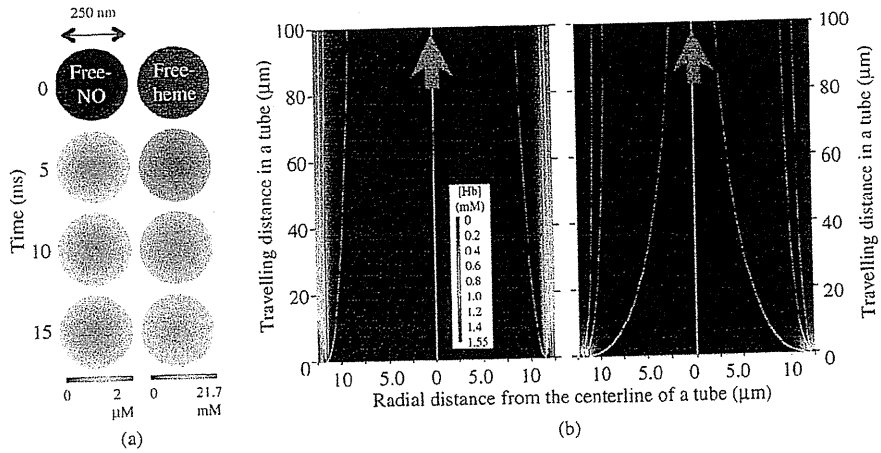

Figure 27.1 *Encapsulation of Hb in vesicles retards NO binding. (a) Schematic two-dimensional representation of the simulated time courses of distributions of unbound free NO and unbound free heme (deoxy form) in one HbV (250 nm) after immediate mixing of NO and HbV by stopped-flow method. Computer simulation shows that both free NO and unbound hemes are distributed heterogeneously. The concentration changes gradually from the surface to the core. The determinant factor of retardation of NO binding should be the intracellular diffusion barrier, which was induced by: (i) intrinsically larger binding rate constant of NO to a heme in an Hb molecule; (ii) numerous hemes as sites of gas entrapment at a higher Hb concentration; (iii) a slowed gas diffusion in the intracellular viscous Hb solution; and (iv) a longer gas diffusion distance in a larger capsule [31]. (b) Schematic representation of the simulated density distribution and track of HbV (left) and Hb (right) 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. The diffusivity of HbV is much slower than that of Hb, resulting in the retarded gas reactions in microvessels. The concentration of Hb is expressed as heme concentration ([Hb] = 1.55 mM at 10 g/dL) [39]. (Reproduced with permission from* Am J Physiol. Heart Circ. Physiol., ***298****, H956–H965 (2010)).*
For a better understanding of the figure, please refer to color plate 6.

be related to the lowered NO binding rate constant, though it is much larger than that of RBCs, 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. For example, endogenous carbon monoxide (CO) is produced by constitutive hemeoxygenase-2 in hepatocytes, serving as a vasorelaxation factor in hepatic microcirculation. Hb permeates across the fenestrated endothelium, scavenges CO, and induces constriction of sinusoids and augments peripheral resistance [15]. Oversupply of O_2 induces autoregulatory vasoconstriction to regulate the O_2 supply [34]. Injection of small HBOCs induces vasoconstriction, probably because of the facilitated O_2 transport [35].

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 of defining the binding and dissociation rate constants of Hb [31, 32, 36, 37]. However, Hb concentration in a cuvette must be diluted extremely, for example to 2 μM heme concentration ([Hb] = 0.003 g/dL). On the other hand, gas-permeable narrow tubes enable the measurement of the O_2-releasing rates of HBOCs and RBCs during their flow through the tubes at a practical Hb concentration (6–13 g/dL) [23, 35, 38]. We used gas-permeable narrow tubes made of perfluorinated polymer to study not only O_2-release but also NO-binding and CO-binding profiles. 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 ($Poly_BHb$), encapsulated Hb (HbV, 279 nm), and RBCs were perfused through a gas-permeable narrow tube (25 μm inner diameter) at 1 mm/second 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 and $Poly_BHb$ in the tube were faster than those of HbV and RBCs, and HbV and RBCs showed almost identical binding rates. When the tube was immersed in an N_2 atmosphere, oxygenated Hb and $Poly_BHb$ showed much faster O_2 release than did HbV and RBCs. $Poly_BHb$ showed a faster reaction than Hb because of the lower O_2 affinity of $Poly_BHb$ than of Hb [39].

The diffusion process of the particles was simulated using Navier–Stokes and Maxwell–Stefan equations (Figure 27.1b). Results clarified that small Hb (6 nm) diffuses laterally and mixes rapidly. However, the large-dimension HbV shows no such rapid diffusion. 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 in creating more binding sites of these gas molecules. In the case of O_2 release, 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. The purely physicochemical differences in diffusivity of the particles and the resulting reactivity with gas molecules are one factor inducing biological vasoconstriction of HBOCs.

27.4 HBOCs as a Carrier of not only O_2 but also CO

CO, biliverdin, and bililubin are produced during oxidative heme degradation that is catalyzed by a stress protein: heme oxygenase (HO). They mediate antioxidative, antiproliferative, and anti-inflammatory effects [40]. Endogenous CO shows a vasorelaxation effect, as does NO [41]. Motterlini *et al.* [42] synthesized a series of CO-releasing metal complexes; subsequent *in vivo* studies clarified some pharmacological effects. Despite the poisonous effect of CO gas, low-concentration CO inhalation (250 ppm) was tested in animal models of hemorrhagic shock, septic shock, and ischemia-reperfusion [43]. Some cytoprotective effects were obtained and the mechanism has been studied extensively. Cabrales *et al.* [44] recently reported CO-bound RBC injection to hemorrhaged hamsters and clarified its cytoprotective effect in subcutaneous microcirculation. These studies have led us to test intravenous injection of CO as a ligand of heme in HBOCs that have been extensively studied as transfusion alternatives.

A traumatic hemorrhage might cause a shock state, which subsequently causes a systemic inflammatory response, in some cases leading to multiple organ failure (MOF). Resuscitation with transfusion or HBOCs with an O_2-carrying capacity induces reperfusion injury, as evidenced by elevations of plasma enzyme levels and tissue cytokine levels [12, 45, 46]. Actually, we observed elevation of plasma enzyme levels 6 hours after resuscitation from hemorrhagic shock by administration of O_2-bound RBCs and HbVs in a rat model [11]. It is expected that co-injection of cytoprotective CO would improve resuscitative effects. For this study, using the same experimental model, we tested injection of CO-bound HbVs for the first time as an exogenous CO supplier for fluid resuscitation. In comparative experiments, we also tested empty vesicles (EVs) which carry neither O_2 nor CO, and CO-bound RBCs. All fluids showed restoration of blood-pressure and blood-gas parameters, and the rats survived for 6 hours of observation period. No remarkable difference was found among the groups, except that the EV group showed significant hypotension. Plasma enzyme levels (AST and ALT) were elevated, especially in the O_2-HbV, O_2-RBC, and EV groups. They were significantly lower in the CO-HbV and CO-RBC groups than in the O_2-bound fluids. Blood HbCO levels (26–39% immediately after infusion) decreased to less than 3% at 6 hours, while CO was exhaled through the lung, as detected by gas chromatography. Both HbV and RBC gradually gained the O_2 transport function. Accordingly, both CO-HbV and CO-RBC showed a resuscitative effect for hemorrhagic-shocked rats. They reduced oxidative damage to organs in comparison to O_2-HbV and O_2-RBC. Adverse and poisonous effects of CO gas were not evident in this experimental model [21].

Hemorrhagic shock and resuscitation typically entail systemic ischemia-reperfusion injury. Activated neutrophils and macrophages produce reactive oxygen species (ROS) [47], with NADPH-oxidase involved as a major source. This enzyme contains two hemes that catalyze the NADPH-dependent reduction of oxygen to form O_2^- [48]. However, CO can bind to the hemes and modulate the enzymatic activity [49]. During hemorrhagic shock, there should be an initiation of inflammatory cytokine production and NO release from the inducible form of NO synthase (NOS) in organs such as the liver and lung. In fact, CO gas potently inhibits the conversion of L-arginine to NO and citrulline by neuronal and macrophage NOS because two heme moieties are contained in the active enzymes. CO would modulate overproduction of NOS-derived NO [50]. Together, O_2^- and NO react to form peroxynitrate, $ONOO^-$, a potent cytotoxic molecule that promotes nitration of tyrosyl residues in proteins [51]. The possibility exists that the injected CO reduces production of both NO and O_2^-, and the resultant $ONOO^-$. Actually, our immunohistochemical observations of the liver and lung clarified that injection of CO-HbV and CO-RBC reduced the formation of nitrosotyrosine on the proteins.

To our knowledge, the present study is the first to use an HBOC to administer CO in a shock state for a pharmacological effect. Although further research is definitely necessary to clarify the mechanism and clinical relevance of our experimental results using small animals, the data would suggest that both RBCs and HBOCs can be effective CO-carriers. Vandegriff *et al.* also reported that CO-bound PEG-Hb reduces myocardial infarction [52]. The advantages of CO-bound HBOC injection are: (i) carbonylhemoglobin is stable for a longer-term storage; (ii) special equipment to inhale CO gas is not necessary in an emergency situation; (iii) the CO dosage is strictly definable; and (iv) the fluid functions initially as a CO carrier to prevent pro-oxidative damage and then as an O_2 carrier.

27.5 Conclusion

Historically, the starting point of the development of HBOCs simply aimed at transporting oxygen to peripheral tissues as blood does. However, the development became complicated after the discovery of endogenous NO and CO, which have strong affinity to HBOCs and influence on their safety. Actually, RBCs are designed to retard the gas reactions, and we have to reconsider the physiological significance of the RBC structure when designing HBOCs. In this chapter, we also demonstrated the potential of HBOCs as CO carriers. Of course, CO is a toxic gaseous molecule, but it shows cytoprotective effect depending on the dose.

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29

Academia–Industry Collaboration in Blood Substitute Development: Issues, Case Histories and a Proposal

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29.1 Introduction

Over the last 30 years, researchers in academia and industry have tirelessly worked together to achieve the goal of developing successful hemoglobin-based oxygen carriers (HBOCs) as safe and clinically effective therapeutics for the treatment of hemorrhagic shock, acute anemia, ischemia, and other conditions. Some leading products have reached the final stages of the development process and are closer than ever before to regulatory approval. However, higher incidences of adverse events (AEs) in the HBOC-treated group than in controls in recent clinical trials have hampered further progress of these candidate HBOCs toward regulatory approval [1–6]. In an effort to help identify the causes of HBOC-mediated AEs and to find ways to alleviate them, a FDA–NIH-sponsored workshop was recently held [7]. Publicly available information on

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition. Edited by Andrea Mozzarelli and Stefano Bettati.

the characteristics and clinical profiles of leading HBOC products were reviewed and potential mechanisms of toxicity discussed. The workshop concluded with a recommendation that further basic research is needed to identify the cause(s) of the AEs observed with current candidate HBOC products and to develop a new generation of safer and more effective products [7].

To elucidate the pathophysiologic mechanisms of AEs observed with HBOCs, it is essential to understand how HBOCs affect key organ systems and their physiologic function, not simply in normal subjects but in patients; studies conducted in models of healthy animals have failed to predict the pathophysiologic responses observed in actual patients, who often present with multiple comorbidities. It is essential that preclinical safety studies are conducted in animal models that closely simulate target patient conditions. Further investigations are required to establish the causality of AEs to an individual HBOC tested in clinical trials and to determine the mechanism involved. Only when armed with accurate knowledge of pathophysiologic mechanisms can we make appropriate modifications to the current HBOC products or develop new, safer products. However, there are barriers to investigations. The goal of this chapter is to initiate a constructive discourse on new ideas in academia–industry collaboration, in order to break down the barriers that impede development of viable HBOC products. Toward this goal, this chapter will: (i) briefly review and discuss generic issues in current academia–industry collaboration practices in a broader field; (ii) discuss issues relevant to academia–industry collaboration in HBOC development; and (iii) propose a new conceptual model for academia–industry collaboration.

29.2 Generic Issues in Academia–Industry Collaboration

To facilitate technology transfer, in 1980 the US government enacted an amendment to the Patent and Trademark Law (commonly known as the Bayh–Dole Act) which permits universities, small businesses, and nonprofit institutions to patent inventions arising from government-funded research [8]. The Bayh–Dole Act has made a significant impact on the academia–industry relationship as industry can no longer monopolize the benefits from research inventions. The benefits of academia–industry collaboration include pooling of expertise and resources (allowing increased chance of new and improved product development) and enhanced productivity and problem-solving capability. For an academic collaborator, industrial funding also allows support for additional students and staff to advance the research and increase productivity. On the other hand, such an industrial sponsorship may have a potentially detrimental effect on free scientific communication and the objectivity of research results. Of particular concern is that industrial priority on the protection of proprietary rights ('proprietary science') may impose constraints on free communication of new knowledge to peers, the scientific community, and the general public ('public science'). Such constraints make it more difficult, if not impossible, for independent investigators to validate research results and expand and develop new applications [9]. Dissemination of study results (presentations and/or publications), even if allowed, is often delayed until well after project completion. If study results are negative or perceived as counterproductive, the sponsor may be less inclined to publish them, unless a priori agreement was explicitly made in the research contract to

allow publication regardless of study outcome. In general, industry-sponsored research contracts are short-term, often narrowly defined, and allow only limited flexibility in the conduct of research.

The primary rationale for the Bayh–Dole Act is to facilitate technology transfer and the commercialization of research results for the public good. Technology transfer is typically achieved through creation of start-up/spin-off companies or by partnering with an established industry that has the expertise and resources to quickly bring the research results to marketable products. Indeed, the enactment of the Bayh–Dole Act considerably enhanced academia–industry collaborations, resulting in a remarkable increase in the patent and licensing activities of universities and nonprofit research institutions. Recently, however, the heightened interest of academic institutions in intellectual property (IP) rights has caused an increasing number of disputes. Emphasis on IP has raised concerns regarding the core values of academic research, as securing IP rights often restricts free and objective communication of research results [10].

A variety of collaboration models (sponsored research, research partnerships/consortia, consulting, etc.) with various IP arrangements (joint ownership, licensing, first-refusal rights, etc.) are being evaluated to mitigate such conflicts and facilitate research collaboration and technology transfer. A key factor in a successful collaboration is building mutual trust based on understanding of the needs and expectations of the other party/ies) involved.

Another disadvantage of academia–industry collaboration is potential conflict of interest (COI). In a typical academia–industry research collaboration, academic investigators receive research funds from their industrial sponsor; they may thus be biased to produce and interpret research results that are more agreeable to that sponsor. In addition, academic collaborators often serve as a consultant or member of the board at a sponsoring company. In return, they may receive direct and indirect benefits (monetary and nonmonetary), raising further COI concerns. Growing emphasis on academia–industry collaboration, IP rights, and the commercialization of research findings have increased public awareness and concern about COI issues. In medical research, any serious compromise in research integrity could have grave consequences for patient treatment and public health in general. Recently, serious concerns about COI have been raised regarding pharmaceutical company-sponsored medical research/education and faculty/officers of medical institutions serving as paid consultants or as members of companies' boards [11, 12]. To protect study subjects/patients and preserve the integrity and objectivity of scientific research, the Association of American Medical Colleges (AAMC) and the Federation of American Societies for Experimental Biology (FASEB) recently issued a strong call for implementation of rigorous COI policies and guidelines in academic institutions [13, 14].

29.3 Academia–Industry Collaboration in HBOC Development

As in other fields, there are limitations and barriers to optimal academia–industry collaboration in HBOC development. First, thorough investigation of HBOC-mediated pathophysiologic responses requires full and detailed information concerning the observed AEs, including patient's clinical history, HBOC dose and rate, circumstances

around the incident, time course of events, concomitant medications, and other relevant information. Unfortunately, much of this information is often not made fully public. Of note, there is a strong ongoing ethical argument to disclose all adverse effects in clinical trials [15]. In addition, because test HBOCs are not yet commercially available, they are not generally available to independent investigators who might help raise and resolve the issues. Understandably, to protect proprietary information from competitors, HBOC producers rarely share their products with 'outsiders' beyond the circle of a small number of close collaborators who are bound by confidentiality agreements. A working group workshop organized by NIH–NHLBI in 2006 [16] explicitly discussed these issues and recommended, among other things, (i) funding of the production and distribution of highly purified 'generic' HBOC solutions in sufficient quantities to support research by independent investigators and (ii) that the FDA investigate ways of making information publicly available regarding the nature and incidence of AEs observed in clinical trials. Advancing these objectives would require breaking down the barriers between and improving the cooperation of both the HBOC producers and the academic investigators. However, because of the unique challenges involved in developing HBOCs as therapeutics, the current industry-driven collaboration model has so far not been able to produce a viable HBOC product even after decades of efforts and substantial private-sector investments.

To date, there have not been many notable IP right disputes in the HBOC field, perhaps in part due to a lack of successfully marketed products. However, COI is of concern since many academic HBOC experts often receive research funds and/or provide compensated services on behalf of one or more companies. Further, lack of transparency and clear open communications regarding some recent negative clinical results has fostered skepticism that HBOC products may have serious safety issues. Indeed, a recent study based on a meta-analysis of pooled AE data from 16 different clinical trials of five past and current HBOC products reported that use of HBOCs was associated with increased risk of serious cardiovascular events and death [17]. Pooling of data conducted with different HBOC formulations of distinct chemical compositions and physico-chemical characteristics is highly controversial. In addition, negative publicity in the media led to diminishing public trust and confidence in the whole class of HBOCs as potential oxygen therapeutics. This negative climate, coupled with recent economic downturn, seriously hampers further development of a safer new generation of HBOC products.

Notable academia–industry relationships began to develop in the early 1980s, when the first HBOC companies emerged (Baxter, Hemosol, Northfield, Biopure). Typically, researchers at academic institutions developed a noble idea of a new HBOC, did initial proof-of-concept work, and obtained IP rights. Later, the inventor(s) either started a new company or worked with an established company to develop further the work into a candidate therapeutic product, which had to meet the rigorous regulatory standards for biologic products. HBOC development efforts followed a typical academia to industry technology transfer pattern and stages. The leading role shifted from academia to industry as product development progressed from basic proof-of-concept work to developmental work for a marketable product that met GMP and other regulatory criteria (new idea/invention → proof-of-concept → further development → scale-up → product safety and effectiveness testing → regulatory approval). Both academia and industry play integral components in the development process. There can be no separate 'academic approach' and 'industrial approach' in HBOC development.

Despite the limitations, academia–industry collaborations have contributed greatly to HBOC development. Examples include: University of Chicago/Michael Reese Hospital–Northfield Inc., Letterman Army Institute of Research–Baxter International, Inc., University of California, San Diego–Sangart, Inc., University of Maryland–OxyVita, Inc., Texas Tech University–HemoBiotech, Inc., Naval Medical Research Center–Biopure Corp., Waseda and Keio Universities–industries. The achievements of the Waseda University–Keio University-initiated research collaborations have been remarkable and particularly productive. With continued government support and strong complementary expertise in sophisticated biosynthetic organic chemistry and animal experimentation, they have produced a host of promising candidate blood substitutes, including Hb-vesicles (HbVs), albumin-heme, and platelet substitutes, over the last 25 years (see Appendix). The EuroBloodSubstitutes consortium is a more recent effort, begun in 2004, consisting of 13 European academic and industrial teams aiming to develop improved blood substitutes. In just a few years, this effort produced new recombinant Hbs and Euro-PEG-Hb (see Appendix). Although these two efforts have not yet conducted any clinical trials, the entire HBOC community may benefit from their successful research collaboration experiences. Therefore, these two historic cases of HBOC research collaboration are described in more detail in the Appendix.

29.4 Proposal for a New Academia–Industry Collaboration Model in HBOC Development: an HBOC Research Consortium (a Conceptual Model)

29.4.1 Mission

- To facilitate development of candidate HBOCs to approved and clinically accepted therapeutic products.
- To resolve issues with current HBOC products collectively, and to identify clinical indications for which these products may be of therapeutic value.
- To develop a new generation of products with acceptable safety and efficacy profiles for one or more clinical indications.

29.4.2 Guiding Principles

- Sound scientific and ethical principles.
- Integrity and objectivity (avoid COI).
- Free and timely communication of research results.

29.4.3 Key Objectives

- Secure research funding (e.g. government and nonprofit foundation grants) for HBOC research.
- Develop standardized research designs/study protocols for key studies common to all HBOC products.
- Build a universally accessible repository for all consortium-generated research data and results for members of the consortium and for timely dissemination to the public.