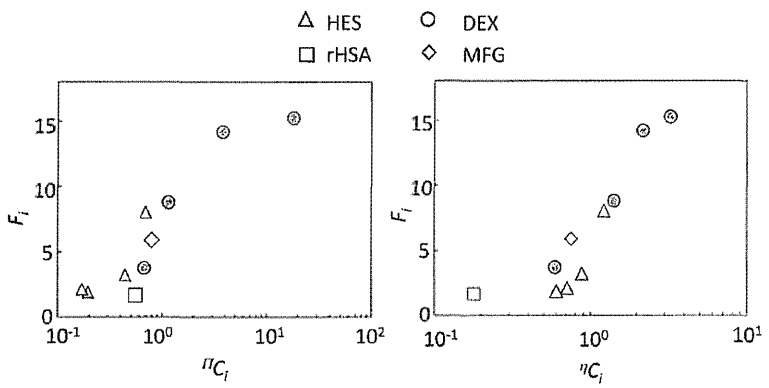

Fig. 3. Flocculation index (F_i) increases with the addition of dextran HES, MFG, or HSA, showing that the crowding index, C_i, of the polymer aqueous solution is the determining factor of flocculate formation.[18] We defined the flocculation index (F_i) as $F_i = (\eta_{10} - \eta_0)/(\eta_{1000} - \eta_0)$. In that equation, η_{10} and η_{1000} respectively represent the viscosity at the shear rates of 10 and 1000 s^{-1}. Crowding index ($^{\eta}C_i$, and $^{\eta}C_i$) representing the crowding level of a polymer solution is defined using R_h and R_g, respectively, as [(excluded volume of one polymer) × (molar concentration) × Avogadro's number]. Adapted with permission from Sakai H, Sato A, Takeoka S, Tsuchida E. (2009) Mechanism of flocculate formation of highly concentrated phospholipid vesicles suspended in a series of water-soluble biopolymers. *Biomacromolecules* **10:** 2344–2350. Copyright (2009) American Chemical Society.

or R_g. All polymers' flocculation level increases when C_i approaches 1: when the theoretical total excluded volume approaches the entire solution volume, the excluded HbV particles are forced to flocculate.

2.3. *In Vivo Study of Co-Injection of HbV and a Series of Plasma Expanders*

It remained unknown whether such flocculate formation of HbV in blood might affect an animal's hemodynamics. Using a rat model, we tested infusion of a series of plasma expanders (MFG, HES_{670}, HES_{130}, HES_{70}, rHSA) to maintain the blood volume (level of blood exchange led to 60%) at repeated hemorrhages and the subsequent infusion of HbV (20 mL/kg, 36% of blood volume).[19] (In this experiment we

did not use dextran because rats show anaphylactic reaction to dextran.[20]) All rats survived for 4 hr after the infusion of HbV; hemodynamic and respiratory functions were preserved, indicating that the flocculation does not induce capillary embolism. Blood exchange with rHSA and subsequent infusion of HbV showed more stable systemic parameters because of the longer retention of rHSA in blood than other plasma substitutes, indicating that rHSA is suitable for combination with HbV in this experimental model.

2.4. *Solution Properties Affects on Reactions of Hb and NO*

It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower "plasma viscosity" decreases shear stress on the vascular wall, causing vasoconstriction and reducing the functional capillary density.[21] Therefore, an appropriate viscosity might exist which maintains the normal tissue perfusion level. The large molecular dimension of HbV can result in a transfusion fluid with high viscosity. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors.[22–25]

Increased fluid viscosity of a solution of hemoglobin-based oxygen carriers (HBOCs) reduces vasoconstrictive effects because increased shear stress on the vascular wall enhances the production of vasorelaxation factors such as NO. Nevertheless, on a microcirculatory level, it remains unclear how viscosity affects the reaction of HBOCs and NO. To clarify the effect of viscosity on the NO-binding, different HBOCs were perfused through narrow gas-permeable tubes (25 μm inner diameter at 1 mm/s centerline velocity; hemoglobin concentration [Hb]=5 g/dL).[26] The reaction was examined microscopically based on the Hb visible-light absorption spectrum. When immersed in a NO atmosphere, the NO-binding of deoxygenated Hb solution (viscosity, 1.1 cP at 1000 s^{-1}) in the tube occurred about twice as rapidly as that of red blood cells (RBCs): 1.6 cP (Fig. 4). Binding was reduced by PEGylation (PEG-Hb, 7.7 cP), by addition of a high molecular weight hydroxyethyl starch (HES) (2.8 cP), and by encapsulation to

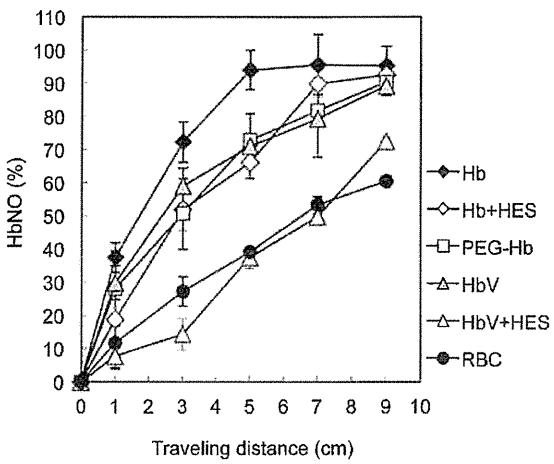

Fig. 4. Change of the level of NO-binding reactions of the Hb containing fluids, Hb solution, PEG-Hb, HbV, Hb+HES, HbV+HES, and RBC (black circles) with traveling distance.[26] (Permission obtained from Elsevier)

form Hb-vesicles (HbVs, 1.5 cP; particle size 279 nm). However, the reduction was not as great as that shown for RBCs. A mixture of HbVs and HES (6.2 cP) showed almost identical NO-binding to that of RBCs. Higher viscosity and particle size might reduce lateral diffusion when particles are flowing. The HbVs with HES showed the slowest NO-binding. Furthermore, Hb encapsulation and PEGylation, but not HES-addition, tended to retard CO-binding. Increased viscosity reportedly enhances production of endothelium NO. In addition, our results show that the increased viscosity also slows down the reaction with NO. Each effect might mitigate vasoconstriction.

3. Biocompatibility of HbV in Terms of Immunological Responses

3.1. *Complement Activation*

A so-called injection reaction, or pseudo-allergy, resulting from complement activation after injection of a small amount of liposome is well known, giving rise to anaphylatoxins, which trigger various

hypersensitivity reactions.[27–29] Transient thrombocytopenia and pulmonary hypertension in relation to complement activation is an extremely important hematologic effect observed in rodent and porcine models after infusion of LEH (containing DPPG) developed by the US Naval Research Laboratory.[30] Neo red cells (Terumo Corp.) containing stearic acid showed pulmonary hypertension in beagle and porcine models,[31] but not in monkeys. In our group, exchange transfusion of prototype HbV (containing DPPG, no PEG modification) in anesthetized rats engendered transient thrombocytopenia and slight hypertension.[32] The transient reduction in platelet counts and increase of thromboxane B_2 caused by complement-bound liposomes was also associated with sequestration of platelets in the lung and liver.[30] In the present formation of HbV, we use a negatively charged lipid (DHSG) instead of DPPG. It does not induce thrombocytopenia or complement activation in animal experiments,[33,34] probably because it contains PEGylated lipids and a different type of negatively charged lipid (DHSG), instead of DPPG or a fatty acid. The *in vitro* human blood compatibility of HbV has been extensively studied.[33,35–37] The present PEG-modified HbV containing DHSG does not affect the extrinsic or intrinsic coagulation activities of human plasma, although HbV-containing DPPG and no PEG-modification tends to shorten the intrinsic coagulation time. The kallikrein–kinin cascade of plasma was activated slightly by the prototype DPPG-HbV, but not by the present PEG–DHSG–HbV. The exposure of human platelets to high concentrations of this HbV (up to 40%) *in vitro* does not cause platelet activation and does not affect adversely the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by platelet agonists.[38] These results imply that HbV, at concentrations of up to 40%, do not have aberrant interactions with either unstimulated or agonist-induced platelets. It can be concluded that the PEG–DHSG–HbV described here have higher blood compatibility.

3.2. *RES Trap, Degradation, and Excretion*

Biodistribution of HbV was examined using ^{99m}Tc-conjugated homocysteine or glutathione containing HbV[39] and HbV containing

^{125}I-labeled Hb.[40] These experiments show that HbV are finally captured by macrophages, mainly in the spleen and liver. Electron microscopic observation can detect the presence of Hb-encapsulating particles in the phagosomes of macrophages because of the high densities of protein and electrons (derived from Fe) in the particles such as RBCs. The HbV particles disappear in one week.[41] Immunohistochemical staining with antihuman Hb antibody and antimethoxy-PEG indicates that Hb and PEG of HbV disappear in two weeks.[41–43] It was shown recently that ^{125}I-labeled Hb and ^{3}H-labeled cholesterol in HbV have identical blood clearance, indicating that HbV retains its integrity in the bloodstream, and distributes to the reticuloendothelial system together. However, ^{125}I mainly appears in urine, and ^{3}H in feces, showing different metabolic routes in the macrophages.[44]

3.3. *Transient Immunosuppressive Effect*

Accumulation of considerable amounts of liposome in a RES can affect immunologic response. Actually, phagocytic index measured by carbon particle clearance in rats showed significant reduction of phagocytic index one day after injection of HbV. While, it increased considerably three days after injection,[41] indicating the increased defense function. On the other hand, HbV showed transient suppressive effect on the proliferation of rat splenic T cells. Takahashi *et al.* of Hokkaido Red Cross Blood Center[45] elucidated the mechanism underlying that phenomenon and its effect on both local and systemic immune response. HbV was injected intravenously at a volume of 20% of whole blood into rats. Then their spleens were removed, and T cell responses to concanavalin A (Con A) or keyhole limpet hemocyanin (KLH) were evaluated by measuring the amount of [^{3}H]thymidine incorporated into DNA. Results showed that T cell proliferation in response to Con A or KLH was inhibited from 6 hr to 3 days after the liposome injection. The phagocytosis of the large load of liposomes by rat CD11b/c+, class II immature monocytes temporarily renders them highly immunosuppressive, but most importantly, the systemic immune response was unaffected.

4. Conclusion

Liposomes are clinically utilized for cancer and antifungal therapies, and other usages as a vehicle of functional molecules are developed aggressively. HbV is one liposomal product, but the differences from such conventional liposomal products are that it is a highly concentrated fluid, and it inevitably requires a massive dose (like 20 mL/kg body weight) as it will be utilized as a substitute for a RBC concentrate. Therefore, injection of HbV would affect spontaneously on hemorheology, hemodynamics, immune system, phagocytosis, gas exchange reactions between tissue and blood, etc. It is also important to have stability as a capsule during storage and during blood circulation to shield a toxic effect of molecular Hb. It also requires instability to be decomposed by macrophages and complete excretion from a body. In this chapter we discuss such important biocompatibilities of HbV. We believe the above mentioned biocompatibility of HbV guarantees the safety of HbV and a potential for versatile clinical application.

Acknowledgments

The author greatly appreciates the late Emeritus Professor Eishun Tsuchida, Waseda University, for his support for the Project of Oxygen Infusion. Research of Hb-vesicles has been conducted by an academic consortium comprising many domestic and overseas research institutes. The author acknowledges the contribution of the collaborators. This research has been supported by Health and Sciences Grants from Ministry of Health, Labour and Welfare, Japan; and a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Sciences (JSPS).

Disclosure

Hiromi Sakai is an inventor holding some patents related to the production and utilization of Hb-vesicles.

References

1. Chang TMS. (1997) *Blood Substitutes: Principles, Methods, Products and Clinical Trials.* S. Karger AG, Basel.
2. Djordjevich L, Miller IF. (1977) Lipid encapsulated hemoglobin as a synthetic erythrocyte. *Fed Proc* **36:** 567.
3. Sakai H, Sou K, Tsuchida E. (2009) Hemoglobin-vesicles as an artificial oxygen carrier. *Methods Enzymol* **465:** 363–383.
4. Nolte D, Pickelmann S, Lang M, *et al.* (2000) Compatibility of different colloid plasma expanders with perflubron emulsion: an intravital microscopic study in the hamster. *Anesthesiology* **93:** 1261–1270.
5. Jouan-Hureaux V, Audonnet-Blaise S, Lacatusu D, *et al.* (2006) Effects of a new perfluorocarbon emulsion on human plasma and whole-blood viscosity in the presence of albumin, hydroxyethyl starch, or modified fluid gelatin: an *in vitro* rheologic approach. *Transfusion* **46:** 1892–1898.
6. Sakai H, Yuasa M, Onuma H, *et al.* (2000) Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjugate Chem* **11:** 56–64.
7. Vandegriff KD, McCarthy M, Rohlfs RJ, Winslow RM. (1997) Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated. *Biophys Chem* **69:** 23–30.
8. Cabrales P, Tsai AG, Intaglietta M. (2008) Isovolemic exchange transfusion with increasing concentrations of low oxygen affinity hemoglobin solution limits oxygen delivery due to vasoconstriction. *Am J Physiol Heart Circ Physiol* **295:** H2212–H2218.
9. Nosé Y. (1998) Oxygen-carrying macromolecules: therapeutic agents for the treatment of hypoxia. *Artif Organs* **22:** 618–622.
10. Sakai H, Masada Y, Horinouchi H, *et al.* (2004) Hemoglobin vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* **32:** 539–545.
11. Terajima K, Tsueshita T, Sakamoto A, Ogawa R. (2006) Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. *Shock* **25:** 184–189.
12. Sakai H, Takeoka S, Park SI, *et al.* (1997) Surface-modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. *Bioconjugate Chem* **8:** 23–30.
13. Sakai H, Tsai AG, Kerger H, *et al.* (1998) Subcutaneous microvascular responses to hemodilution with red cell substitutes consisting of polyethylene glycol-modified vesicles encapsulating hemoglobin. *J Biomed Mater Res* **40:** 66–78.
14. Kobayashi K. (2006) Summary of recombinant human serum albumin development. *Biologicals* **34:** 55–59.

15. Meyuhas D, Nir S, Lichtenberg D. (1996) Aggregation of phospholipid vesicles by water-soluble polymers. *Biophys J* **71:** 2602–2612.
16. Neu B, Meiselman HJ. (2002) Depletion-mediated red blood cell aggregation in polymer solutions. *Biophys J* **83:** 2482–2490.
17. Sakai H, Sato A, Takeoka S, Tsuchida E. (2007) Rheological property of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma substitute aqueous solutions. *Langmuir* **23:** 8121–8128.
18. Sakai H, Sato A, Takeoka S, Tsuchida E. (2009) Mechanism of flocculate formation of highly concentrated phospholipid vesicles suspended in a series of water-soluble biopolymers. *Biomacromolecules* **10:** 2344–2350.
19. Sakai H, Miyagawa N, Horinouchi H, *et al.* (2011) Intravenous infusion of Hb-vesicles (artificial oxygen carriers) after repetitive blood exchange with a series of plasma expanders (water-soluble biopolymers) in a rat model. *Polymer Adv Technol* **22:** 1216–1222.
20. Koller ME, Reed RK. (1992) Increased negativity of interstitial fluid pressure in rat trachea in dextran anaphylaxis. *J Appl Physiol* **72:** 53–57.
21. Tsai AG, Friesenecker B, McCarthy M, *et al.* (1998) Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. *Am J Physiol Heart Circ Physiol* **275:** H2170–H2180.
22. Goda N, Suzuki K, Naito M, *et al.* (1998) Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J Clin Invest* **101:** 604–612.
23. Sakai H, Hara H, Yuasa M, *et al.* (2000) Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* **279:** H908–H915.
24. Sakai H, Sato A, Masuda K, *et al.* (2008) Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J Biol Chem* **283:** 1508–1517.
25. Nakai K, Ohta T, Sakuma I, *et al.* (1996) Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. *J Cardiovasc Pharmacol* **28:** 115–123.
26. Sakai H, Okuda N, Takeoka S, Tsuchida E. (2011) Increased viscosity of hemoglobin-based oxygen carriers retards NO-binding when perfused through narrow gas-permeable tube. *Microvasc Res* **81:** 169–176.
27. Chonn A, Cullis PR, Devine DV. (1991) The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J Immunol* **146:** 4234–4241.
28. Loughrey HC, Bally MB, Reinish LW, Cullis PR. (1990) The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. *Thromb Haemost* **64:** 172–176.
29. Szebeni J, Baranyi L, Savay S, *et al.* (2005) Complement activation-related cardiac anaphylaxis in pigs: role of C5a anaphylatoxin and adenosine in

liposome-induced abnormalities in ECG and heart function. *Am J Physiol Heart Circ Physiol* **290:** H1050–H1058.

30. Phillips WT, Klipper R, Fresne D, *et al.* (1997) Platelet reactivity with liposome-encapsulated hemoglobin in the rat. *Exp Hematol* **25:** 1347–1356.
31. Pape A, Kertscho H, Meier J, *et al.* (2008) Improved short-term survival with polyethylene glycol modified hemoglobin liposomes in critical normovolemic anemia. *Intensive Care Med* **34:** 1534–1543.
32. Izumi Y, Sakai H, Takeoka S, *et al.* (1997) Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J* **43:** 289–297.
33. Abe H, Azuma H, Yamaguchi M, *et al.* (2007). Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. *Artif Cells Blood Substit Immobil Biotechnol* **35:** 157–172.
34. Sou K, Tsuchida E. (2008). Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: effect of the structure of acidic groups. *Biochim Biophys Acta* **1778:** 1035–1041.
35. Abe H, Fujihara M, Azuma H, *et al.* (2006) Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: effects on coagulation, kallikrein-kinin, and complement systems. *Artif Cells Blood Substit Immobil Biotechnol* **34:** 1–10.
36. Wakamoto S, Fujihara M, Abe H, *et al.* (2001) Effects of poly(ethylene glycol)-modified hemoglobin vesicles on agonist-induced platelet aggregation and RANTES release *in vitro*. *Artif Cells Blood Substit Immobil Biotechnol* **29:** 191–201.
37. Wakamoto S, Fujihara M, Abe H, *et al.* (2005) Effects of hemoglobin vesicles on resting and agonist-stimulated human platelets *in vitro*. *Artif Cells Blood Substit Immobil Biotechnol* **33:** 101–111.
38. Ito T, Fujihara M, Abe H, *et al.* (2001) Effects of poly(ethylene glycol)-modified hemoglobin vesicles on N-formyl-methionyl-leucyl-phenylalanine-induced responses of polymorphonuclear neutrophils *in vitro*. *Artif Cells Blood Substit Immobil Biotechnol* **29:** 427–437.
39. Sou K, Klipper R, Goins B, *et al.* (2005). Circulation kinetics and organ distribution of Hb vesicles developed as a red blood cell substitute. *J Pharmacol Exp Ther* **312:** 702–709.
40. Taguchi K, Maruyama T, Iwao Y, *et al.* (2009). Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J Control Release* **136:** 232–239.
41. Sakai H, Horinouchi H, Tomiyama K, *et al.* (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* **159:** 1079–1088.

42. Sakai H, Masada Y, Horinouchi H, *et al.* (2004). Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* **311:** 874–884.
43. Sakai H, Seishi Y, Obata Y, *et al.* (2009). Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days. *Shock* **31:** 192–200.
44. Taguchi K, Urata Y, Anraku M, *et al.* (2009) Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin-vesicles as an artificial oxygen carrier. *Drug Dispos Metabol* **37:** 1456–1463.
45. Takahashi D, Azuma H, Sakai H, *et al.* (2011) Phagocytosis of liposome particles by rat splenic immature monocytes makes them transiently and highly immunosuppressive in *ex vivo* culture condition. *J Pharmacol Exp Ther* **337:** 42–49.

Chapter 12
Cellular-Type Hemoglobin-Based Oxygen Carriers to Mimic the Red Blood Cell Structure

Hiromi Sakai

Abbreviations

Hb	Hemoglobin
HBOCs	Hb-based oxygen carriers
RBC	Red blood cell
HbV	Hb-vesicles
LEH	Liposome-encapsulated Hb
HbCO	Carbonylhemoglobin

12.1 Chemically Modified Cell-Free Hb and Encapsulated Hb

The concentration of hemoglobin (Hb) in healthy human blood is around 12–15 g/dL, making Hb the most abundant protein in blood. Hb is an oxygen binding protein that is compartmentalized in red blood cells (RBCs) with an intracellular Hb concentration of about 35 g/dL. Packed RBCs derived from blood donation can be stored only for 6 weeks in the US and for 3 weeks in Japan. Historically, a crude Hb solution was tested as a substitute for RBCs in (Von Stark 1898), but it was not successful because of various side effects. Since the late 1960s, chemically modified Hb solutions have been developed (Vandegriff and Winslow 1991). Many materials have progressed to use in clinical studies, but many such studies have been suspended because of side effects (Natanson et al. 2008).

H. Sakai (✉)
Department of Chemistry, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8521, Japan
e-mail: hirosakai@naramed-u.ac.jp

H. Sakai
Organization for University Research Initiatives, Waseda University, Tokyo 162-0041, Japan

H. W. Kim and A. G. Greenburg (eds.), *Hemoglobin-Based Oxygen Carriers as Red Cell Substitutes and Oxygen Therapeutics*, DOI: 10.1007/978-3-642-40717-8_12,

Recombinant human Hb was also tested, but it failed in clinical trials (Murray et al. 1995). Actually, an earthworm, as a lower organism, has no RBCs, but it does have gigantic Hb molecules. Mammalians, as higher animals, have RBCs for several physiological reasons. It seems difficult to create an RBC substitute with cell-free Hb solutions. Even though Hb is the most abundant protein in blood, it becomes toxic once released from RBCs.

We believe in the physiological importance of the cellular structure of RBCs, and continue to develop Hb-vesicles (HbV) as a cellular-type HBOC (Sakai et al. 2008a; Tsuchida et al. 2009). By considering the physiological importance of RBCs, it is easy to understand the side effects of cell-free HBOCs. An RBC has a biconcave disk structure with 8 μm long-axis diameter, encapsulating about two million Hb molecules (Mw. 64500) at a concentration of about 35 g/dL. The physiological reasons for Hb compartmentalization in RBCs are the following: (i) shielding direct contact of toxic Hb and vasculature (Burhop et al. 2004); (ii) prevention of extravasation of dissociated Hb dimers through renal glomeruli, and prolonged circulation time; (iii) circumvention of high colloid osmotic pressure and viscosity of concentrated Hb solution (Sakai et al. 2000); (iv) co-encapsulation of electrolytes, ATP, glycolytic, and metHb reducing enzymatic systems, etc.; (v) retarded reaction of Hb with NO and CO as vasorelaxation factors and retarded O_2-release in the vasculature (Sakai et al. 2008, 2010); (vi) RBCs tend to flow near the centerline in vasculature (centralization), avoiding contact with vascular walls where shear stress is the greatest. This flow style is appropriate for preventing hemolysis (Sakai et al. 2009); (vii) Moreover, the high viscosity of blood is mainly attributable to the presence of RBCs, producing a non-newtonian fluid, which is important for blood circulation, especially in microcirculation, from a physiological perspective.

12.2 Attempts to Produce Cellular Type HBOCs Using Polymeric Materials

Chang (McGill University) was the first to test encapsulation of Hb solution with a polymer membrane in 1957 (Chang 2007) as one example of "artificial cells". In Japan, Kimoto and his colleagues tested Hb encapsulation from around 1961 using polystyrene, gelatin, and rubber membranes (Toyoda 1966; Kimoto et al. 1968; Kitajima et al. 1970). Although their attempts were original, they were unsuccessful: the particle size could not be reduced to less than capillary diameter (<4 μm). Later, polymeric materials of various kinds with biodegradable properties became available through the use of polypeptides (Arakawa et al. 1975; Palath et al. 2007), polycaprolactone, and polylactide (Zhao et al. 2007; Zhang et al. 2008) with much smaller diameters. These capsules have permeability of small ionic molecules, which would be advantageous for the reduction of intracellular methemoglobin by reducing agent dissolved in plasma. However, it is speculated that hydrolysis of the polymeric materials during preservation (before

injection) and during blood circulation might induce hemolysis: leakage of the encapsulated Hb. Polymersomes are new materials for encapsulation of Hb solution (Rameez et al. 2008). Kishimura et al. (2007) reported encapsulated myoglobin using PEGylated polyion complex vesicles (Table 12.1). These new materials have been mostly described in reports published in chemistry journals. They await detailed in vivo and in vitro examination to assess their safety and efficacy.

12.3 Cellular Type HBOCs Using Liposome

Bangham and Horne (1964) discovered the formation of vesicles (liposomes) when phospholipid was dispersed in aqueous phase. After this discovery, many researchers tested encapsulation of functional molecules in liposomes, especially for anticancer therapy. Djorjevici and Miller (1977) (University of Illinois, Chicago) reported encapsulation of Hb in liposomes, called "synthetic erythrocytes" (Table 12.2). Subsequently, many groups throughout the world attempted so-called liposome encapsulated Hb (LEH). However, most of those efforts were not successful because of their low encapsulation efficiency, polydispersibility of particle size, and instability. The US Naval Research Laboratory aggressively

Table 12.1 Encapsulated Hb using polymeric membrane, and polymer-embedded Hbs

Authors	Characteristics
Chang 2007	First attempt of encapsulated Hb using polymer membrane
Toyoda 1966 Kimoto et al. 1968	Encapsulated Hb using polystyrene, gelatin, rubber membranes
Arakawa et al. 1975	Encapsulation with poly(lysine membrane)
Cedrati et al. 1994	W/O emulsion using polylactide
Meng et al. 2003	Methoxypolyoxyethylene-polylactide microcapsules
Baumler et al. 2005	Polyelectrolyte microcapsules made with RBC template
Patton and Palmer 2006	Hb-poly(acrylamide) hydrogel
Zhao et al. 2007	Encapsulated with biodegradable polymers of PCL-PEG.
Palath et al. 2007	Encapsulated with polypeptide multilayer nanofilms (PLGA and PLL) using $CaCO_3$ particle template
Kishimura et al. 2007	PEGylated polyion complex vesicle encapsulating Mb
Rameez et al. 2008	Biocompatible and biodegradable polymersome encapsulated Hb
Zhang et al. 2008	Hb-loaded nanoparticles with PEG-PLP-PEG block copolymer
Shi et al. 2009	Hb-conjugated micelles based on triblock biodegradable polymers
Chauvierre et al. 2010	Hb is embedded on heparin coated poly(alkylcyanoacrylate) nanoparticles
Gao et al. 2011	Cationic amylose-encapsulated bovine Hb
Duan et al. 2012	Enclosing Hbs in $CaCO_3$ microparticles and modification with PEG.

Table 12.2 Trials of liposome encapsulated Hb

Authors	Lipid composition	Characteristic preparation methods
Djordjevich and Ivankovich 1988 (first reported in 1977)	L-α-phosphatidylcholine/cholesterol/palmitic acid	Sonication
Gaber et al. 1983	EYPC/cholesterol/bovine brain phosphatidylserine	Extrusion
Farmer and Gaber 1987	DMPC/cholesterol/dicetylphosphate	
Kato et al. 1984	EYL/carboxymethyl chitin.	Reverse phase evaporation
Hunt et al. 1985	EYPC/cholesterol/DPPA/α-tocopherol	Reverse phase evaporation and Extrusion
Hayward et al. 1985	Diacetylene phospholipid/cholesterol UV-irradiation for polymerization	HbCO, sonication
Beissinger et al. 1986	HSPC/cholesterol/dicetylphosphate or DMPG	Microfluidizer
Rudolph et al. 1988	HSPC/cholesterol/DMPG/α-tocopherol. Trehalose is added	Bovine Hb
Rabinovici et al. 1993	to store LEH as a lyophilized powder	Thin film hydration and emulsification
Jopski et al. 1989	EYL/PS (EYPA)	Detergent dialysis
Yoshioka 1991 Takahashi 1995	HSPC/cholesterol/myristic acid/α-tocopherol/DPPE-PEG	Microfluidizer
Mobed and Chang 1991	HSPC/DMPG/α-tocopherol/carboxymethyl chitin	Reverse phase evaporation
Sato et al. 1992	DODPC/cholesterol/octadecadienoic acid	HbCO, Extrusion method
Sakai et al., 1992 Akama et al. 2000	Gamma-ray polymerization	
Liu and Yonetani 1994	EYL/cholesterol/dicetylphosphate/α-tocopherol	Freeze–thaw method
Sakai et al. 1996 Takeoka et al. 1996	DPPC/cholesterol/DPPG or palmitic acid	HbCO, extrusion
Sakai et al. 1997	DPPC/cholesterol/DPPG/DSPE-PEG_{5000}	HbCO, extrusion
Phillips et al., 1999	DSPC/cholesterl/PEG_{5000}-DSPE/a-tocopherol	α-crosslinked human Hb microfluidizer

(continued)

Table 12.2 (continued)

Authors	Lipid composition	Characteristic preparation methods
Sou et al., 2003 Sakai et al. 2002	DPPC/cholesterol/DHSG/DSPE-PEG_{5000}	HbCO, extrusion
Li et al. 2005	DMPC/cholesterol/DMPG/DSPE-PEG_{2000}/actin	Extrusion
Pape et al. 2008	HSPC/cholesterol/stearic acid/DSPE-PEG_{5000}	Lipid paste rapid dispersion
Centis and Vermette 2008	DSPC/cholesterol/palmitic acid/DSPE-PEG_{2000}	HbCO, thin film hydration and extrusion
Agashe et al. 2010	DSPC/cholesterol/CHHDA/DSPE-PEG_{5000}/α-tocopherol	HbCO emulsification
Rameez et al. 2012	DSPC/cholesterol/DSPE-PEG_{5000}	Bovine HbCO Thin film hydration and emulsification

Abbreviations in this table
DMPC 1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine
EYPC Egg yolk phosphatidylcholine
DPPA 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid
HSPC Hydrogenated soy phosphatidylcholine
DMPG 1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol
EYL Egg yolk lecithin
PS Phosphatidylserine
DODPC 1, 2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine
DPPE 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine
DSPE 1, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine
DPPC 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
DHSG 1, 5-*O*-dihexadecyl-*N*-succinyl-L-glutamate
HbCO Carbonylhemoglobin
CHHDA 2-Carboxyheptadecanoyl heptadecylamide

developed freeze-dried powder LEH from the 1980s (Gaber et al. 1983), but the laboratory terminated its development in the late 1990s (Flower and Rudolph 1999), presumably because of low Hb encapsulation efficiency and induction of anaphylactoid reactions (Szebeni et al. 1999), despite the important LEH advantage of long-term storage as a freeze-dried powder using cryoprotectant saccharides. Terumo Corp. (Japan) started development of Neo Red Cells from around 1985 (Suzuki et al. 1988; Takahashi 1995; Pape et al. 2008) using particles that had been surface-modified with PEG chains. However, it suspended its preclinical studies in 2012. As Table 12.2 shows, most research groups use lipid composition of phosphatidylcholine, cholesterol, negatively charged lipid, and PEG-lipid. Cholesterol not only improves membrane stability; it also reduces the curvature for large unilamellar vesicles. Addition of a small amount of negatively charged lipid increases the repulsive force between the lipid membranes and reduces the lamellarity in addition to controlling the zeta potential for blood compatibility. Saturated phospholipids, such as HSPC, DSPC, and DPPC in Table 12.2, are preferred to unsaturated lipids such as EYL and soy phosphatidylcholines because of the synergistic, facilitated oxidation of both unsaturated lipids and Hb and physical instability (Szebeni et al. 1985), but cholesterol lowers such Hb denaturation to some degree. Utilization of carbonylhemoglobin (HbCO) is effective to prevent denaturation of Hb during preparation procedures.

Our academic consortium has worked to improve the encapsulation efficiency and particle size distribution from the viewpoint of molecular assembly by regulating the electrostatic and hydrophobic interactions between the components (Hb and lipids) (Sakai et al. 2009a). The resulting Hb-vesicles (HbV) encapsulate nearly 30,000 Hb molecules (35 g/dL Hb solution) within a 5 nm thin lipid membrane. The selection of lipids was also important for stability and biocompatibility. The starting material, Hb solution, is purified from outdated NAT-inspected red blood cells provided by the Japanese Red Cross. Bovine Hb and swine Hb are also available for the preparation of HbV (Sakai et al. 2002). Carbonylation of Hb (HbCO) prevents metHb formation and denaturation of Hb, and enables pasteurization at 60 °C for 10 h, thereby ensuring the utmost safety from infection. HbCO encapsulated in HbV can be converted easily to HbO_2 by photodissociation using illumination of visible light under O_2 atmosphere. We formerly used polymerizable phospholipids (containing dienoyl group in acyl chain) to stabilize the resulting encapsulated Hb because it was believed that liposome had a fragile structure. However, the problem was that the polymerized liposome was so stable that it was not degraded and it remained in the liver and spleen after intravenous administration into rats. Now we use other combination of conventional phospholipid (DPPC), cholesterol, negatively charged synthetic lipid (Sou and Tsuchida 2008), and PEG-conjugated phospholipid. The resulting liposome sufficiently prevents aggregation. Complete deoxygenation of the HbV suspension enables long-term storage for years at room temperature (Sakai et al. 2000). Without decarbonylation, HbCO is stable. It can be stored for a long time. Moreover, injection of a cellular HBOC as an HbCO form is beneficial for some pathological conditions (Sakai et al. 2009) and should be studied intensively.

Details of in vivo results of safety and efficacy of HbV are summarized in some review papers (Sakai et al. 2008; Tsuchida et al. 2009; Sakai et al. 2011). The in vivo oxygen transport capacity of HbV as a resuscitative fluid is described by Dr. Horinouchi in this book.

12.4 Advantages of Gas Reactions of Encapsulated Hbs

One important physiological aspect of cellular type HBOCs is that their particles are much larger than those of cell-free HBOCs. They do not seem to induce vasoconstriction or hypertension (Nakai et al. 1998; Sakai et al. 2000). Physiochemical analysis of NO reactions of a series of cell-free HBOCs solutions showed that NO binding rate constants are fast and mostly identical to that of stroma-free Hb (Rohlfs et al. 1988). However, one cellular type of HBOCs, Hb-vesicles (HbV), showed retarded NO binding because of the formation of intracellular diffusion barrier of NO simply by encapsulation of a concentrated Hb solution (Sakai et al. 2008b, 2009b). In fact, HbV encapsulating a diluted Hb solution provides a larger NO binding rate constant: a value similar to that of stroma-free Hb solution.

Moreover, a larger particle shows a slower lateral diffusion in an arteriole that retards the gas reaction at a vascular wall (Sakai et al. 2010). HbV showed a lower rate of NO binding, CO binding, and O_2 release in the model vessels, each of which relates to the vascular tone. In addition, the larger particles prevent penetration across the perforated endothelium to approach to a space between the endothelium and the smooth muscle where NO is produced to bind to soluble guanylate cyclase. In fact, RBCs showed the slowest rate of NO binding, CO binding, and O_2 release. These data imply that RBCs are evolutionally designed to retard gas reactions in blood circulation.

12.5 Intrinsic Difficulties to be Considered for Realization of Encapsulated Hb

Even though Hb encapsulation might shield all the toxic effects of cell-free Hb, cellular HBOCs have their own hurdles that impede their realization. Several are explained here.

12.5.1 Particle Size and Encapsulation Efficiency

The RBC structure is deformable, facilitating its flow through a capillary with a narrower diameter. However, that attribute of deformability is difficult to mimic artificially. Accordingly, the particle should be smaller than the capillary diameter.

It is important to encapsulate a concentrated Hb solution in the particle. To improve the particle function, the weight ratio of the encapsulated Hb to the capsular material is one parameter that must be considered. The Hb concentration in blood is around 12–15 g/dL. A fluid of a cellular HBOC dispersion should have a comparable Hb concentration if it is intended for use as a blood substitute. For this purpose, the intracellular Hb concentration must be as high as intracellular Hb concentration of RBCs, which is around 35 g/dL.

12.5.2 Stability of the Capsule

The capsule should be stable to retain Hb inside the capsules during storage for a long time, and after injection in the blood circulation until it disappears, because elimination of cell-free Hb is the purpose of Hb encapsulation. The encapsulated Hbs are usually captured by the reticuloendothelial system (RES). The capsule material should be degradable in the macrophage. Their components and their degraded or metabolic materials should never be deposited for a long time in the organs. Accordingly, the capsule material should have both stable and unstable characteristics. The pharmacokinetics of both Hb and capsule should be examined (Taguchi et al. 2009).

Trace amounts of ascorbic acid and thiol compounds are present in plasma, and oxidized cell-free HBOCs can be reduced by these compounds. Because of the stability of a capsule, ionic transport through the capsular membrane is shielded to some degree in the absence of a substitute for ion channels. Encapsulated Hb autoxidizes to form metHb and loses its oxygen binding ability. A remedy for such metHb formation must be considered, such as establishing a reduction system in the capsules (Chang T et al. 2000; Tsuchida et al. 2009).

12.5.3 Blood Compatibility of the Capsule

Some of the liposomal products for anticancer therapy induce complement activation. The so-called injection reaction is being clarified continually as clinical experience accumulates, such as dyspensa, tachypenia, tachycardia, hypotension and hypertension, chest pain, and back pain (Szebeni 2005). We confirmed that our prototype HbV, containing phosphatidyl glycerol, induced marked anaphylactoid reactions and cardiopulmonary disorders, manifested as systemic and pulmonary hypertension, increased vascular resistance, decreased cardiac output, thrombocytopenia, tachycardia, etc. (Sakai et al. 2012). Therefore, it is extremely important to confirm the absence of complement activation of the capsule material (Chang and Lister 1994).

Because the cellular type HBOCs are not dissolved but dispersed in the fluid, the particles sometimes aggregate in the presence of plasma protein by ionic

interaction, or depletion interaction. Accordingly, the particle surface would need some surface modification to prevent aggregation.

12.5.4 Influence on Clinical Instruments

Light scattering of the particle dispersion, and a stable capsule that cannot be easily destroyed by a detergent, are the chief causes of interference in clinical laboratory tests based on colorimetric and turbidimetric analysis (including quantitative measurement of Hb in blood) and in clinical diagnostic tools such as laser pulsed oxymetry. The level of interference effect should be examined carefully, and a remedy should be considered in advance (Sakai et al. 2003; Suzaki et al. 2008).

Another important point to be considered includes impacts of the RES trap after a massive dose of cellular HBOCs, which might include transient and local immunosuppression (Takahashi et al. 2011). This point was discussed at length by our collaborators in other chapter (Azuma et al.) in this book. Even though cellular HBOCs are more complicated than cell-free HBOCs, resolving the issues presented above can realize the successful development of cellular HBOC.

Acknowledgments Research of Hb-vesicles has been conducted by an academic consortium comprising many domestic and overseas research institutes. The author acknowledges the contribution of those collaborators. This research has been supported by Health and Labour Sciences Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Sciences (JSPS).

Disclosure Hiromi Sakai is an inventor holding some patents related to the production and utilization of Hb-vesicles.

References

Agashe H, Lagisetty P, Awasthi S, Awasthi V (2010) Improved formulation of liposome-encapsulated hemoglobin with an anionic non-phospholipid. Coll Surf B Biointerfaces 75:573–583

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

Arakawa M, Kondo T, Tamamushi B (1975) Flow properties of microcapsule suspensions as a model of blood. Biorheology 12:57–66

Bangham AD, Horne RW (1964) Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J Mol Biol 8:660–668

Baumler H, Kelemen C, Mitlohner R, Georgieva R, Krabi A, Schaling S, Artmann G, Kiesewetter H (2005) Micromechanical properties of newly developed polyelectrolyte microcapsules (PEMC). In: Kobayashi K, Tsuchida E, Horinouchi H (eds) Artificial oxygen carrier its front