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

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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).

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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	HSPC/cholesterol/myristic acid/α-tocopherol/DPPE-PEG	Microfluidizer
Takahashi 1995		
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	Gamma-ray polymerization	
Akama et al. 2000		
Liu and Yonetani 1994	EYL/cholesterol/dicetylphosphate/α-tocopherol	Freeze–thaw method
Sakai et al. 1996	DPPC/cholesterol/DPPG or palmitic acid	HbCO, extrusion
Takeoka et al. 1996		
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.

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