

Review

Comparison of the Pharmacokinetic Properties of Hemoglobin-Based Oxygen Carriers

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Academic Editor: Hiromi Sakai

Received: 11 January 2017; Accepted: 15 March 2017; Published: 18 March 2017

Abstract: Hemoglobin (Hb) is an ideal material for use in the development of an oxygen carrier in view of its innate biological properties. However, the vascular retention of free Hb is too short to permit a full therapeutic effect because Hb is rapidly cleared from the kidney via glomerular filtration or from the liver via the haptoglobin-CD 163 pathway when free Hb is administered in the blood circulation. Attempts have been made to develop alternate acellular and cellular types of Hb based oxygen carriers (HBOCs), in which Hb is processed via various routes in order to regulate its pharmacokinetic properties. These HBOCs have been demonstrated to have superior pharmacokinetic properties including a longer half-life than the Hb molecule in preclinical and clinical trials. The present review summarizes and compares the pharmacokinetic properties of acellular and cellular type HBOCs that have been developed through different approaches, such as polymerization, PEGylation, cross-linking, and encapsulation.

Keywords: hemoglobin; oxygen carrier; liposome; pharmacokinetic

1. Introduction

Oxygen (O₂) is an essential element for most living organisms to maintain their biological activities. In the body, hemoglobin (Hb) in red blood cells (RBC) plays a central role in the delivery O₂ from the lung to individual cells. As a result, the concentration of Hb in the blood circulation is maintained at 12–17 g/dL, in order to maintain a sufficient O₂ supply. However, when unexpected massive bleeding caused by severe injury, an accident, or a surgical procedure occur, the Hb concentration drops to a lower level that may perturb various biological activities. In these cases, a RBC transfusion is the gold standard and is currently in widespread use for treating patients with massive hemorrhages. Nevertheless, RBC transfusions have some undesirable side effects associated with them, such as blood-type mismatching and infections caused by the presence of unrecognized pathogens. In addition, conventional RBC has a limited short storage period (up to 21–42 days in the world [1]) under cold storage conditions, and frozen storage remains unpractical. These problems make it difficult to use RBC, particularly in pre-hospital conditions and to maintain stable supplies of RBC in remote locations or in an emergency situation such as a disaster or a pandemic.

Researchers have attempted to prepare purified Hb from human- or animal-derived RBC in order to use them as an alternative to the conventional RBC transfusion. Purified Hb has some advantages, in that infectious viruses can be removed and they can be stored for much longer periods of time than

RBC and that the removal of the cellular membrane allows for transfusions to patients without the need for any cross-match testing. However, the vascular retention of free Hb is not sufficiently long to fully achieve therapeutic effects. The reason for this is that Hb is rapidly cleared from the blood circulation via two pathways (as described in Section 2). To overcome these problems, attempts have been made to develop various types of modified Hb, which are referred to as acellular or cellular type Hb based O₂ carriers (HBOCs) [2–4]. These HBOCs have a longer blood retention than free Hb by virtue of their original pharmacokinetic properties. Here, we briefly summarize the pharmacokinetic properties of acellular or cellular type HBOCs that have been used in research dealing with preclinical and clinical studies.

2. Stroma-free Hb

The Hb molecule is composed of four hemes (Fe-protoporphyrin IX) and a pair of identical $\alpha\beta$ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) that associate to form the Hb tetramer. Because of this, it is not possible for Hb to circulate in blood vessels for a long time (~0.5–1.5 h) [5]. Most of the Hb is packed inside RBCs in vivo because Hb can have several adverse effects. For example, Hb captures nitric oxide (NO), a vasorelaxation factor, from endothelial cells in blood vessels causing the induction of systemic or pulmonary hypertension [6]. Furthermore, although RBCs are equipped with an imbedded redox system and RBC contains enzymes that inhibit the formation of metHb or toxic substances, iron in the heme moiety of free Hb reacts easily with hydrogen peroxide or NO, to ultimately produce toxic substances such as oxoferryl Hb and transient free radical intermediates [6]. Endogenous- and exogenous-derived Hb is rapidly cleared from the body via two pathways. However, this degradation mechanism is prevented when Hb is packed in RBCs which remain in the blood circulation for longer periods of time and allows for a free exchange of O₂. One of the clearing pathways is glomerular filtration in the kidneys [7]. When Hb is released into the blood circulation, the tetramer configuration of Hb spontaneously dissociates into two dimer configurations ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$), which can easily pass through the glomerule because of its low molecular weight (approximately 32 kDa). Another clearing pathway is the identification by the Hb scavenger receptor (CD163) in the liver [8]. When Hb is released into the blood circulation, it is rapidly captured by the acute phase protein haptoglobin (Hp). The resulting Hb-Hp complex is removed from the circulation by CD163 recognition in the liver.

3. Recombinant Hb (rHb1.1, Optro[®])

rHb1.1 is a molecular mutant human Hb produced in *Escherichia coli* in which the amino acid located at position 108 of both β chains has been changed ($\beta 108\text{Asn} \rightarrow \text{Lys}$) [9]. Furthermore, two α globin chains are genetically fused with a glycine bridge between the C-terminal and the N-terminal amino acid of each α globin to prevent the dissociation into two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$). The half-life of the rHb1.1 molecule in vivo is prolonged by 3–4 fold compared to native Hb by virtue of the two α globin units being fused [9]. In a clinical trial (Phase I), the half-life of rHb1.1 was found to increase (2.4–18.9 h) with increasing dose (0.015–0.32 g/kg) [10]. Although rHb1.1 actually advanced to Phase II, it was discontinued due to adverse effects [11]. A second generation product, rHb2.0, has been developed and its efficacy is being evaluated in preclinical trials [12,13]. Furthermore, Marquardt and colleagues prepared di-Hb in which two rHb1.1 units are covalently cross-linked by gene fusion using a peptide linker and they also prepared tetra-Hb that is chemically cross-linked with bismaleimido-hexane between a cysteine residue into di-Hb [14]. A pharmacokinetic study in rats demonstrated that the half-life of di-Hb and tetra-Hb are approximately 1.4 and 1.6 times longer than that of rHb1.1 [14,15].

4. Acellular Type HBOCs

Since the 1980s, many types of acellular HBOCs, including polymerized Hb, intramolecularly cross-linked Hb, and poly(ethylene glycol)-conjugated (PEG-conjugated) Hb, have been developed and examined in preclinical and clinical trials [3,16]. Their modification (PEGylation, cross-linking,

and polymerization) was found to prolong vascular retention compared to free Hb [17]. In this section, we introduce some of the developed acellular HBOCs and summarize their pharmacokinetic results obtained in preclinical and clinical trials.

4.1. HbNFPLP

2-nor-2-formylpyridoxal 5'-phosphate (NFPLP) can form a cross link between the two β chains of Hb, resulting in an intramolecular cross-linked form of Hb [18]. Using this characteristic, Bakker et al. prepared an Hb that was modified by cross-linking with NFPLP (HbNFPLP) in the 1980s [19,20]. They investigated the vascular retention of HbNFPLP in blood exchange model rats and rabbits using a unique method in which a 1:1 mixture of unmodified Hb and HbNFPLP were transfused [21]. This method enables the blood retention between unmodified Hb and HbNFPLP to be compared in the same animals. As a result of this study, the half-life of HbNFPLP was found to be approximately 3 and 2.7 times longer than that of unmodified Hb in rats and rabbits, respectively (1.1 h and 3.2 h in rats, 2.7 h and 7.2 h in rabbits, for unmodified Hb and HbNFPLP, respectively). Similar results were reported by another group using tritium (^3H) labeled HbNFPLP in healthy rats [22]. Furthermore, they evaluated the difference in biodistribution between unmodified Hb and HbNFPLP using a technetium 99m ($^{99\text{m}}\text{Tc}$) technique [23]. Although the $^{99\text{m}}\text{Tc}$ activity was mainly detected in the liver and kidney at 2 h after $^{99\text{m}}\text{Tc}$ -labeled HbNFPLP administration (5% and 4% of the dose in the kidney and liver, respectively), it was much less than that for the $^{99\text{m}}\text{Tc}$ -labeled unmodified Hb (15% and 6% of the dose in the kidney and liver, respectively). In addition, light microscopy observations after staining with diaminobenzidine showed that strong Hb accumulations were seen in the lumina of many proximal and distal tubules after the administration of unmodified Hb, but rarely after the administration of HbNFPLP. These results indicate that intramolecular cross-linking prevents glomerular filtration resulting from maintaining the tetramer configuration of Hb. These pharmacokinetic results are summarized in Table 1.

4.2. Diasprin Cross-Linked Hb (DCLHb, HemAssist[®])

Diasprin cross-linked Hb is an intramolecular cross-linked HBOC (DCLHb), in which human Hb is cross-linked between the α -chain with bis(3,5-dibromosalicyl)fumarate. In the early 1980s, researchers began working on the development of this product. Its pharmacokinetic properties were evaluated in preclinical and clinical trials (Table 1). Keipert et al. reported on its pharmacokinetic properties, such as plasma retention, tissue distribution, and excretion, in a rat model of exchange transfusion using carbon 14 (^{14}C)-labeled DCLHb (the total administered dose was 2400 mg/kg) [24,25]. They demonstrated that the half-life for DCLHb was 5 h and tissues containing high ^{14}C radioactivities were the kidney, spleen, bone marrow, and liver. In addition, they monitored ^{14}C radioactivities in tissues for as long as 14 days, and demonstrated that 2 weeks were required for the metabolic degradation and elimination of a large dose of DCLHb. The pharmacokinetic properties of DCLHb have also been evaluated in a rat model of sepsis [26] and hepatic cirrhosis [27]. In a human study, the half-life of DCLHb was 2.5–3.3 h in healthy volunteers [28] and 2.1–4.3 h in patients who were receiving chronic hemodialytic therapy [29] at a dose of 25, 50, and 100 mg/kg. The changes in the plasma concentration of DCLHb in large blood loss surgical patients and patients with cardiac surgery have also been investigated [30,31]. However, its development for clinical use was stopped in 1999 [3].

4.3. Polymerized Bovine Hb (HBOC-201, Hemoglobin Glutamer-250 (Bovine), Hemopure[®])

HBOC-201 (hemoglobin glutamer-250 (bovine), Hemopure[®]) is bovine Hb that has been polymerized with glutaraldehyde. Although a number of preclinical studies of HBOC-201 have been performed and its efficacy has been demonstrated [32], to best of our knowledge, pharmacokinetic studies of HBOC-201 in preclinical trials are limited, due to the fact that the half-life of HBOC-201 is around 22 h in a 40% hemorrhagic shock model of a Yucatan mini-pig [33] (or have not been published). On the other hand, the safety and efficacy of HBOC-201 has been examined in more than 20 clinical

trials [34], some in which the half-life of HBOC-201 in humans were reported (Table 2). Hughes et al. studied the pharmacokinetics of HBOC-201 in normal healthy men who underwent a 15% blood volume phlebotomy, followed by volume replacement with lactated Ringer’s solution, and then an infusion of HBOC-201 with dose-escalation (16.5–45 g Hb/person) [35,36]. In these studies, a dose-dependent increase in the plasma Hb peak was observed and the half-life was found to be 16–20 h. Furthermore, the half-life of HBOC-201 was similar in men and women (men; 20.0 ± 2.4 h, women; 20.3 ± 1.2 h) [37]. In addition, another prospective, randomized study in which HBOC-201 was administered to patients undergoing a liver section at a dose of 400 mg/kg showed that the half-life of HBOC-201 in these patients was about 8.5 h [38]. These long half-lives of HBOC-201 can be attributed to preventing filtration from glomerul due to its increase in molecular weight. In fact, no detectable hemoglobinuria were observed in clinical trials [37]. HBOC-201 is currently approved for the treatment of anemia and for use during surgery in South Africa since 2001 [39,40].

Table 1. Pharmacokinetic summary of intramolecular cross-linking HBOCs.

Characteristic	DCLHb	NbNFPLP
Modification		
cross-linker	bis(3,5-dibromosalicyl)fumarate	2-nor-2-formylpyridoxal 5'-phosphate
cross-linked amino acid	Lys99 residue in the two α-chain	Val1-Lys82 in the two β-chain
Pharmacokinetic studies		
Animal	Blood exchange model–rat [24,25] Half-life: 5 h (2400 mg/kg) Distribution: kidney, spleen, bone marrow, liver	Healthy rat [22,23] Half-life: 2.7 h (145 mg/kg) Distribution: kidney, spleen, liver
	Sepsis model–rat [26] Half-life: 4.2 h (300 mg/kg)	Blood exchange model–rat [21] Half-life: 3.2 h (~2000 mg/kg)
	Hepatic cirrhosis model–rat [27] Half-life: 4.7 h (400 mg/kg)	Less excretion into urine than Hb Blood exchange model–rabbit [21] Half-life: 7.2 h (1400 mg/kg)
Human	Healthy volunteer [28] Half-life: 2.5–3.3 h (25, 50, 100 mg/kg)	No clinical study
	Hemodialysis patient [29] Half-life: 2.1–4.3 h (25, 50, 100 mg/kg)	
	Large blood loss surgical patient [31] Half-life: 10 h (658–1500 mg/kg)	
	Cardiac surgery patient [30] Half-life: ~24 h (~7500 mg/person)	

HBOCs: Hb based O₂ carriers, DCLHb: Diasprin cross-linked Hb, NFPLP: 2-nor-2-formylpyridoxal 5'-phosphate

4.4. Hb-raffimer (Hemolink®)

Hb-raffimer (Hemolink®) is an o-raffinose cross-linked and oligomerised form of human Hb. The molecular weight of Hb-raffimer ranges from 32 kDa to 500 kDa, indicating that a Hb-raffimer solution is a mixture that contains heterogeneous Hb up to nanomer-sized molecules [41]. Hsia et al. investigated the pharmacokinetic difference of Hb-raffimer among different molecular weights using an ³H labeled Hb-raffimer in a rat model of isovolemic 50% exchange transfusion [42]. Although the half-life of the total Hb-raffimer was around 10 h, the half-life values for the individual molecular weight of Hb-raffimer were prolonged, ranging from 0.6 h to 13 h with increasing molecular weight. Since the pharmacokinetic evaluation in healthy rats indicated that the Hb-raffimer was mainly distributed in the kidney and liver [43], they determined the hepatic and renal distribution of the Hb-raffimer with different molecular weights. The distribution in the kidney was decreased with increasing molecular weight of Hb-raffimer, while the distribution in liver was increased with increasing molecular weight of Hb-raffimer. Another study reported the half-life of Hb-raffimer preparations with different molecular weights in a healthy dog that was administered a 25% top load infusion of Hb-raffimer [44]. Along with results obtained in an isovolemic 50% exchange transfusion model rat, the half-life values of individual molecular weights of Hb-raffimer were prolonged with increasing molecular weight (total Hb-raffimer; 25.4 h, Hb-raffimer (64 kDa); 11 h, Hb-raffimer (128 kDa); 25 h, Hb-raffimer (>128 kDa; 42 h). In addition to animal studies, Carmichael et al. reported that the half-life of total Hb-raffimer was approximately 14 h, and that for the oligomer (>64 kDa) it was

18–20 h in Phase I study [45]. Although the Hb-raffimer has been evaluated in Phase III trials [46–48], its development for clinical use has been suspended.

4.5. Pyridoxal PolyHb (PolyHeme®)

Pyridoxal PolyHb is a chemically modified human Hb solution. The developers began developing this preparation in 1969 in conjunction with the U.S. Army, and Northfield Laboratories then began work on its development for clinical use. This product is pyridoxylated to increase O₂ affinity (P₅₀) and polymerized with glutaraldehyde. Although pyridoxal PolyHb has already completed Phase III trials [49,50], as far as we know, reports of its pharmacokinetic properties are limited. Sehgal et al. compared the vascular retention between stroma free Hb and pyridoxal PolyHb in adult baboons (exchange transfusion model) [51]. After a 900 mL partial exchange, the half-life of the stroma free Hb and pyridoxal PolyHb were determined to be around 6 h and 46 h, respectively. In addition, some articles reported that the half-life of pyridoxal PolyHb in humans is around 24 h based on data obtained from clinical trials [49,52]. Unfortunately, the development of pyridoxal PolyHb for clinical use was stopped in 2009 [3].

Table 2. Summary of the pharmacokinetics of polymerized HBOCs.

Characteristic	HBOC-201	Hb-Raffimer	Pyridoxal PolyHb
Modification			
Species of Hb	bovine	human	human
Modification	Glutaraldehyde polymerization	Cross-linking with o-raffimer	Pyridoxylation and glutaraldehyde polymerization
Molecular weight	Average; 250 kDa	32–500 kDa	No reported
Pharmacokinetic studies			
Animal	Hemorrhagic shock model–pig [33] Half-life: 22 h (1300–3200 mg/kg)	Healthy rat [43] Half-life: 5 h (65 mg/kg) Healthy dog [44] Half-life: 25.4 h (1.8 g/kg) Blood exchange model–rat [42] Half-life: 10 h (dose: unknown)	Blood exchange model–baboon [51] Half-life: 46 h (90 g/ baboon?)
Human	Healthy volunteer [35,36] Half-life: 16–20 h (16.5–45 g/person) Patient undergoing a liver section [38] Half-life: 8.5 h (400 mg/kg)	Healthy volunteer [45] Half-life: 18–20 h (25–600 mg/kg)	Healthy volunteer (?) [49,52] Half-life: ~24 h (dose: unknown)

4.6. MP4 (Hemospan®)

MalPEG-hemoglobin (MP4) is maleimide-PEG modified human Hb developed by Sangart Inc. (San Diego, CA, USA). The molecular weight of MP4 is about 100 kDa, because a mean of 7–8 PEG₅₀₀₀ is conjugated to one human Hb [53]. Animal studies with MP4 indicate that the half-life of MP4 is about 24 h in rats at a dose of 2600 mg/kg and about 18 h in monkeys at a dose of 840 mg/kg [54,55] (Table 3). MP4 has been undergoing clinical safety evaluations since 2002. A phase I study indicated that the half-life of MP4 in healthy volunteers was 66.2 and 42.8 h at a dose of 50 mg/kg and 100 mg/kg, respectively [56]. Interestingly, serum Hp completely disappeared immediately after MP4 administration, suggesting that MP4 was captured by Hp and eliminated via CD163, similar to endogenous Hb [8], albeit PEG would be expected to prevent the interactions between Hp and Hb. Olofsson et al. reported two randomized Phase II studies that were carried out to evaluate the safety and efficacy of MP4 at a dose of 100–500 mg/kg during spinal anesthesia in patients who were undergoing orthopedic surgery [57,58]. The half-life of MP4 in these patients ranged from 14 to 23 h, which was prolonged with increasing dose. Urinary Hb measured by the dipstick method showed an equivalent value to that for control patients [57,58]. In addition, Hp levels fell to 0 in these patients as well as healthy volunteers [57]. Taking these findings into consideration, MP4 appears to be mainly eliminated via CD163 after binding with Hp, even in orthopaedic surgery patients with spinal anesthesia.

Table 3. Summary of the pharmacokinetics of PEGylated HBOCs.

Characteristic		MP4	PEG-Hb	SB-1
Modification				
Species of Hb	human		bovine	bovine
Number of PEG	7–8		12	8
Size of PEG (Da)	5000		5000	5000
Pharmacokinetic studies				
Animal	Healthy rat [54] Half-life: 24 h (2600 mg/kg)		Healthy rat [61] Half-life: 17 h (25 mL/kg)	Healthy rat [67] Half-life: 9.6–10.6 h (5, 12.5 mL/kg)
	Healthy monkey [55] Half-life: 18 h (840 mg/kg)		Healthy rabbit [63] Half-life: 43 h (25 mL/kg)	Healthy dog [68] <i>Single injection (Male)</i> Half-life: 7.7–17 h (2.5–10 mL/kg)
Human	Healthy volunteer [56] Half-life: 42.8–66.2 h (50–100 mg/kg)		Blood exchange model-rat [62] Half-life: 15 h	<i>Multiple injection (Male)</i> Half-life: 16.7–38 h (2.5–10 mL/kg)
	Patient with orthopedic surgery [57,58] Half-life: 14–23 h (100–500 mg/kg) No hemoglobinuria	No clinical study	No hemoglobinuria Blood exchange model-dog [64] Half-life: 58 h Hemorrhagic shock model-dog [65] Half-life: 18 h	<i>Single injection (Female)</i> Half-life: 6.2–14.4 h (2.5–10 mL/kg) <i>Multiple injection (Female)</i> Half-life: 17.7–28.5 h (2.5–10 mL/kg)

MP4: MalPEG-hemoglobin, PEG-Hb: PEG conjugated bovine Hb, SB-1: SunBio1

4.7. PEG-HbTM

Enzon Inc. (Piscataway, NJ, USA) developed a PEG conjugated bovine Hb (PEG-HbTM) in which each bovine Hb is modified with approximately 12 succinimidyl carbonate PEG₅₀₀₀. PEG-HbTM has been previously reported to be safe and effective in a number of animal species models [59–62]. Changes in the plasma concentration of PEG-HbTM after a top-load infusion (25 mL/kg) was examined in rats and rabbits, and the findings showed that the mean half-life was approximately 17 h and 43 h in rats and rabbits, respectively [61,63]. In addition, PEG-HbTM was shown to have a half-life of 15 h and 58 h in rat and dog models of 30% blood volume exchange transfusion [62,64]. Furthermore, PEG-HbTM showed a longer half-life than native Hb in a canine hemorrhagic shock model [65]. These long half-lives, as summarized in Table 3, indicate that PEG modification can prevent excretion via glomerular filtration due to the increase in overall molecular weight. In fact, less hemoglobinuria were observed in animals that had been injected with PEG-HbTM than those injected with native Hb [61,63]. Although PEG-HbTM had earlier proceeded to Phase Ib, its development for clinical use was stopped more than 20 years ago [3].

4.8. SB-1

SunBio1 (SB-1) is a PEG-conjugated bovine Hb [66]. The purified Hb derived from bovine Hb was reacted with activated methoxy PEG₅₀₀₀ to prepare SB-1 in which each bovine Hb is PEGylated with an average of 8 PEG. The resulting SB-1 has a molecular weight of 104.5 kD with a size of 30–50 nm. Pharmacokinetic studies of SB-1 have only been evaluated in animals (Table 3). A pharmacokinetic study of SB-1 using iodine-125 (¹²⁵I)-labeled SB-1 showed that the half-life of SB-1 is 9.6 h and 10.6 h in male rats at a dose of 5 mL/kg and 12.5 mL/kg, respectively [67]. Furthermore, pharmacokinetic analyses after single and multiple injections in male beagle dogs showed that the half-life for a single injection increased with the dose (7.7 h, 9.7 h, and 17 h for 2.5 mL/kg, 5 mL/kg, and 10 mL/kg, respectively). On the other hand, the half-life values for multiple injections were about two times higher than that of the single dose in male beagle dogs. However, no significant difference in pharmacokinetic properties was found between male and female beagle dogs [68].

5. Newer Generated Acellular Type HBOCs

In 2008, Natanson et al. reported the results of a meta-analysis that used 16 randomized controlled trials of five types of acellular HBOCs (DCLHb, HBOC-201, Hb-raffimer, pyridoxal PolyHb, and MP4) [69]. This report concluded that using acellular HBOCs is associated with a significantly increased risk of death and myocardial infarct compared to a control solution. After releasing this

meta-analysis, the development of new acellular type HBOCs was accelerated [70]. In this section, we discuss the development of new types of acellular HBOCs and summarize their pharmacokinetic results obtained in preclinical and clinical trials.

5.1. Oxy Vita-Zero-Link Polymerized Hb (Oxy Vita[®]Hb)

Oxy Vita-Zero-Link polymerized Hb is a novel type of acellular HBOC, the design and approach of which was initially carried out by Dr. Bucci using zero-linked polymerized technology [71]. Each intramolecular cross-linked bovine Hb (cross-linker: bis (3,5 dibromosalicyl-adipate)) is polymerized via a stable amide bond between the carboxylate groups of C-terminal or side chains of glutamic acid (Glu) and aspartic acid (Asp) and N-terminal amino groups or amino side chains of the lysyl residue [72]. The mean molecular weight of Oxy Vita[®]Hb is about 17 MDa, substantially larger than that for other acellular HBOCs, and its physicochemical properties are also well defined [73–76]. Matheson et al. reported on the intravascular retention of Oxy Vita[®]Hb in rats and cats [77]. The half-life of Oxy Vita[®]Hb was approximately 7 h and 10 h in rats and cats, respectively. Furthermore, Oxy Vita[®]Hb with a large radius showed an 8–12 times longer half-life than that with a small radius [72]. This product is still in the preclinical trial stage of development. The pharmacokinetic data in clinical trials are being awaited.

5.2. PEGylated carboxyHb (SANGUINATE[™], MP4CO)

SANGUINATE[™] is a PEGylated bovine carboxyHb in which each bovine Hb molecule is modified with PG-succinimidyl carbonate PEG₅₀₀₀. This product is expected to avoid the problems associated with NO scavenging and auto-oxidation, and functions as both a carbon monoxide (CO) releasing molecule and an O₂ transfer agent [78]. No adverse effects were observed at a dosage level of 1200 mg Hb/kg in monkeys, 1600 mg Hb/kg in pigs, and 2400 mg Hb/kg in rats [78–80]. From the pharmacokinetics viewpoint, changes in plasma carboxyHb in the rat were evaluated [81]. Plasma carboxyHb increased to around 6% at 1 min after the administration and then gradually decreased to 1% at 2 h after administration. In a human study (Phase I), SANGUINATE[™] was found to be safe and well tolerated in healthy volunteers, and its half-life was dose dependent and ranged from 7.9 h to 13.8 h [82]. Interestingly, the half-life of SANGUINATE[™] at a dose of 160 mg Hb/kg was prolonged in stable sickle cell anemia patients (about 20 h) compared to that in healthy volunteers (13.8 h) [83]. Although two cases where patients received SANGUINATE[™] under emergency conditions were reported [78], large scale clinical trials in patients will be needed prior to clinical applications.

MP4 saturated with CO (MP4CO) is a PEGylated human Hb saturated with CO and an O₂ therapeutic that has shown potential in preventing and reversing RBC sickling. The efficacy of MP4CO, including anti-adhesive, anti-inflammatory, anti-oxidant, and anti-apoptotic properties was demonstrated in a murine model of sickle cell disease, subarachnoid hemorrhage, and myocardial infarct [84–86]. MP4CO has already completed safety and tolerability studies in patients with sickle cell disease (Phase Ib) [87]. The carboxyHb level was observed to be dose-dependent, and then became normalized to pre-dosing levels by 8 h. Unfortunately, to the best of our knowledge, this is only report related to the pharmacokinetics of MP4CO. However, since the basic structural and physicochemical properties of MP4CO are very similar to those for MP4, its pharmacokinetic properties would also be similar to MP4 as shown in Section 4.6 in this article.

5.3. Hb–Albumin Clusters (HemoAct[™])

Komatsu et al. recently developed a novel type of acellular HBOCs, referred to as Hb–albumin clusters [88]. The Hb–albumin cluster is composed of one Hb in the center and three or four human serum albumins (HSA) in the periphery, and was prepared by a covalent linkage between the Cys34 residue of HSA and the surface Lys amino groups of Hb via a heterobifunctional cross-linker. HemoAct showed good blood compatibility in vitro, and it induced neither hypertension nor toxicity in vivo [89]. Komatsu and colleagues prepared three types of Hb-HSA clusters, Hb-HSA₃ (Hb

was enclosed by three HSA molecules), Hb-HSA₄ (Hb was wrapped by four HSA molecules), and XLHb-HSA₃ (intramolecularly crosslinked Hb was enclosed by three HSA molecules), and compared their pharmacokinetic properties [89,90]. The half-lives of all types of Hb-HSA clusters were more than 20-times and 1.5 times longer than that of Hb and HSA, respectively. This is why Hb-HSA clusters prevent not only extravasation through the vascular endothelium, but also filtration via the renal glomerulus. When the half-life among Hb-albumin clusters was compared, the half-life of Hb-HSA₄ was comparable to that for Hb-HSA₃ in spite of the large molecular weight. This suggests that an increase in the molecular weight of the cluster (number of albumin) has no influence on extending the circulation time for the preparation. In fact, a similar phenomenon was observed in a study using albumin clusters [91]. On the other hand, the half-life of XLHb-HSA₃ was decreased compared to Hb-HSA₃. The reason for this phenomenon is unclear but it appears to be attributable to differences in the morphologies of the clusters.

In addition to the Hb-Albumin clusters mentioned above, Komatsu's group also prepared a Hb-HSA₃ cluster in which a Pt nanoparticle was incorporated into the exterior HSA unit of the cluster, to add antioxidant activity, which would be expected in many clinical situations involving ischemia-reperfusion injuries [92]. Furthermore, Oxyglobin[®] (polymerized bovine Hb) is available for the treatment of anemia in dogs [93]. Yamada et al. are attempting to prepare new Hb-Albumin clusters using Hb and albumin derived from different mammalian species for use in veterinary medicine [94]. Their unique ideas may be able to realize HBOCs for not only human but also for animal pets.

6. Cellular Type HBOCs

Historically, many types of cellular type HBOCs have been developed since 1957 (see the review by Sakai et al. [95,96]). The concept of cellular types of HBOCs is to mimic the cellular structure of the RBC, which can retard binding between Hb and gaseous messenger molecules (NO and CO) in blood vessels. Additionally, the cellular type of HBOCs can also prevent the dissociation of tetrameric Hb subunits into two dimers. These effects would be expected to shield against some adverse effects derived from Hb such as hypertension and renal failure. From the pharmacokinetic viewpoint, cellular type HBOCs showed different properties than acellular type HBOCs because their pharmacokinetic properties would be more strongly influenced by the characteristics of the nanoparticles than that of Hb. The current tendency for cellular type HBOCs is liposome-encapsulated Hb composed of phospholipids, cholesterol, fatty acids, etc. In the following section, we summarize the pharmacokinetic properties of two different kinds of liposome-encapsulated Hb, which have the potential to proceed to the clinical trial stage.

6.1. TRM-645 (Neo Red Cells)

Liposome encapsulated Hb (code name: TRM-645) was developed by the Terumo Corp. (Tokyo, Japan) in 1995 and its physicochemical properties are well defined [97]. The mean diameter of TRM-645 particles ranges from 200 to 250 nm and encapsulates purified and highly concentrated human Hb and inositol hexaphosphate as an allosteric effector in a multi-lamellar lipid membrane. The liposomal surface is further modified by PEG5000-DSPE, which is intended to enhance the stability of TRM-645 during both storage and in the blood circulation by preventing aggregation [98,99]. Furthermore, preclinical studies have been performed and the findings indicate that the product is efficacious and safe as a RBC substitute in some animal species including the rat, dog, and monkey [97,100].

The pharmacokinetic properties of TRM-645 were evaluated using rats and cynomolgus monkeys. Kaneda and co-workers administered TRM-645 to healthy rats and monkeys at a dose of 20 mL/kg and evaluated the changes in its concentration in the blood circulation using Enzyme-Linked Immuno Sorbent Assay (ELISA) that specifically reacts with human Hb [97]. As a result, the half-life in rats was found to be approximately 10 h and human Hb reached a non-detectable level at 72 h after administration. On the other hand, the half-life in monkeys was much longer than that in rats (half-life: 70 h), and human Hb was still observed in the blood circulation at 72 h after

administration. These results for different animals suggest a longer half-life in humans. In addition, lipid particles were observed in reticuloendothelial organs such as the spleen, liver, and bone marrow in repeated-dose toxicological studies in rats and monkeys (20 mL/kg), which indicate that TRM-645 is likely captured and degraded by the mononuclear phagocyte system (MPS) as well as other liposome preparations [101]. However, information regarding the pharmacokinetic properties of TRM-645 are limited to its retention in the blood circulation and its distribution in reticuloendothelial organs in a few animal species (Table 4). Further study will clearly be needed to determine the detailed pharmacokinetic properties such as the metabolic and accumulative properties of TRM-645 and its components in both healthy and critical conditions in which TRM-645 would be expected to be used.

Table 4. Preclinical pharmacokinetic studies of cellular type HBOCs in healthy animals.

Characteristic	TRM-645	HbV
Property		
Mean diameter (nm)	200–250	250–280
Hb concentration (g/dL)	6	10
Lipid composition	Soybean hydrogenated phosphatidylcholine, cholesterol, stearic acid, PEG ₅₀₀₀ -DSPE	DPPC, cholesterol, DHSG, PEG ₅₀₀₀ -DSPE
Pharmacokinetic studies		
	Rat [97] Half-life: 10 h (20 mL/kg) No detectable Hb in circulation at 72 h after administration	Mouse [102] Half-life: 20 h (1400 mg/kg) Distribution: spleen, liver Excretion: degraded Hb into urine lipid composition into feces
	Monkey [97] Half-life: 70 h (20 mL/kg) Detectable Hb in circulation at 72 h after administration	Rat [102] Half-life: 30 h (1400 mg/kg) Distribution: spleen, liver Excretion: degraded Hb into urine lipid composition into feces Rabbit [103] Half-life: 63 h (1400 mg/kg) Monkey [104] Half-life: 47–72 h (1400 mg/kg)

DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; DHSG, 1,5-O-dihexadecyl-N-succinyl-L-glutamate; PEG5000-DSPE, (N-[monomethoxypolyethyleneglycol-carbamyl] distearoylphosphatidyl-ethanolamine).

6.2. Hemoglobin-Vesicles (HbV)

Since around 1985, Hemoglobin-vesicles (HbV) have been developed by Tsuchida’s group at Waseda University. HbV are promising cellular types of HBOCs, in which concentrated human Hb is encapsulated and the particle surface is covered with PEG. HbV has been demonstrated to have pharmacological effects that are comparable to RBCs in hemorrhagic shock model animals [105–108]. We have accumulated data regarding the pharmacokinetic profiles of HbV in detail using healthy animals, as shown in Table 4. Based on these data, the half-life of HbV in humans was estimated to be approximately 3 to 4 days, based on the use of allometric equations [109], which is, unfortunately, much shorter than RBC (around 120 days). Detailed data on the pharmacokinetics of HbV in healthy animals are not reviewed here, since a review of this subject was previously published by our group [110].

We also investigated the pharmacokinetic profile of HbV in various animal models with some disorders that mimic a clinical setting (Table 5). In a previous study, the pharmacokinetic profiles of HbV under hemorrhagic shock conditions, in which HbV could be used, were investigated using a rat model and the results were compared to healthy conditions [109]. The half-life of HbV in hemorrhagic conditions was about 18 h, which was 0.6 times shorter than healthy conditions (about 30 h). Furthermore, our group investigated the pharmacokinetic properties of HbV using chronic liver cirrhosis model rats with fibrosis [111]. Interestingly, the pharmacokinetic parameters (the total clearance, hepatic distribution of HbV, and the amount of cholesterol excreted in feces) were negatively correlated with plasma aspartate aminotransferase levels, which reflected hepatic injury. The detailed pharmacokinetic properties of HbV in hemorrhagic shock and chronic liver failure conditions were reviewed elsewhere (see [110]).

Table 5. Pharmacokinetic studies of HbV in pathological model animals.

Model	Species	Dose	Pharmacokinetic Characteristic
Hemorrhagic shock [109]	Rat	1400 mg/kg	(i) half-life of HbV in hemorrhagic shock rats was reduced compared with healthy rats (ii) HbV was mainly distributed in liver and spleen as well as healthy rats
Hepatic cirrhosis [111,120]	Rat	1400 mg/kg	(i) the retention of HbV in circulation were prolonged in the chronic cirrhosis rat model compared to healthy rats (ii) the hepatic distribution of HbV was decreased in the chronic cirrhosis rat model compared to healthy rats (iii) the amount of lipid component (cholesterol) in feces was less in the chronic cirrhosis rat model than in healthy rats
Hyperlipidemia [113]	Mouse	2000 mg/kg	(i) HbV was cleared from blood circulation within 3 days after injection (ii) HbV was mainly distributed were the liver and spleen (iii) Lipid component had cleared from each organ by 14 days after injection
Pregnancy [112]	Rat	1400 mg/kg	(i) HbV was distributed mainly in spleen and liver in rat mothers (ii) no maternal/fetal transfer was occurred

The disposition of HbV for use in pregnant mothers and fetuses has also been investigated [112]. Pregnant rats were given daily repeated injections of HbV (2000 mg/kg) from days 16 to 22 of pregnancy. In a non-labeled study, the concentrations of lipids, bilirubin, and ferric iron (Fe^{3+}), which should be released as the result of the decomposition of Hb, were not significantly changed after the 7 day daily repeated injections of HbV. In addition, histological evaluations showed a slight detection of human Hb and hemosiderin in the maternal liver, spleen, kidney, and in the junctional zone or labyrinth of the placenta, but was not detected in any organs of the fetus. Furthermore, a pharmacokinetic study using ^{125}I -HbV showed that HbV was distributed mainly in the spleen and liver in rat mothers, but was not distributed in the fetal organs.

We previously investigated the pharmacokinetic properties of HbV in Apo E deficient mice (B6.KOR/StmSlc-Apoe^{shl} mice) because of the concern that the disposition of HbV, especially lipid components, may be altered due to their poor metabolic and excretion profiles [113]. Biological parameters which are related to lipid metabolites of HbV such as cholesterol were temporarily increased after an HbV injection in B6.KOR/StmSlc-Apoe^{shl} mice, but completely recovered to basal levels at 7 days after the injection. In addition, pharmacokinetic analyses using ^3H -HbV in B6.KOR/StmSlc-Apoe^{shl} mice clearly showed that the HbV had disappeared from all organs within 14 days after the HbV injection.

Taking the above findings into consideration, HbV is promptly metabolized and excreted under not only healthy conditions but also under any pathological conditions, suggesting that HbV has favorable pharmacokinetic properties as a RBC substitute. Our group is currently planning to draft a protocol for a clinical study (Phase I) according to the guidance for O_2 carrier products by The Society of Blood Substitutes Japan [114]. In addition to functioning as a RBC substitute, we and other colleagues recently showed another potential use for HbV based on its O_2 and carbon monoxide transport characteristics, which lead us to expect that it has a variety of other applications [115–119]. Thus, HbV has enormous potential for clinical applications, and the first clinical trials of HbV toward realization in clinics are being awaited.

7. Conclusions

A number of HBOCs, as described above, have been developed and their efficacy, safety, and pharmacokinetics have been evaluated in preclinical and clinical trials. Recently, Estep TN pointed out a few pitfalls with respect to the circulatory retention of HBOCs from the viewpoint of (i) methods of analysis; (ii) incorrect pharmacokinetic results due to changes in blood volume; (iii) experimental animal models and (iv) functional half-life [121]. Unlike other drugs, since the dosage volume of HBOCs is more than a hundred times larger than that of other drugs, the pharmacokinetic parameters cannot be calculated by the usual pharmacokinetic analysis methods. It may be necessary to establish a standardized pharmacokinetic analysis method for HBOCs in the future.

Since pharmacokinetic properties including blood retention depend on the dose, animal species, molecular weight, and pathological conditions, it is difficult to compare the pharmacokinetic properties and to determine the merits and drawbacks among HBOCs with different modifications from pharmacokinetic results in different experimental conditions. In any event, most acellular and cellular HBOCs showed superior therapeutic effects compared to Hb because of their longer half-life. However, nobody has yet produced HBOCs that possess a comparable half-life to RBC. In addition, although the inhibition of methemoglobin formation (functional half-life) is also important for HBOCs to function as a RBC substitute, it was quite difficult to completely reproduce the redox system of RBCs in HBOCs due to the complex biological systems of RBC. Therefore, from the viewpoint of the pharmacokinetic properties of HBOCs, HBOCs are expected to function as O₂-bridge agents before RBC transfusions, rather than as an entire RBC alternative. Needless to say, the realization of RBC substitutes is urgent, considering the medical and social problems that now face society. In the near future, it is expected that acellular or cellular HBOCs will be clinically developed for use in patients who need oxygen carrier support until appropriate units are found.

Acknowledgments: This work was supported in part by a Grant-in-Aid for Young Scientist (B) from the Japan Society for the Promotion of Science (JSPS) (KAKENHI 26860121).

Author Contributions: All of the authors generated the ideas, performed the literature search, and contributed to the preparation and editing of this paper.

Conflicts of Interest: The authors declare no conflict of interest.

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