Chapter 37
Vasoconstriction, Hypertension and Oxidative Toxicity are Regulated by Polymerized Hemoglobin Size

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37.1 Negative Side-Effects of Early Generation HBOCs

37.1.1 Cell-Free Hb

Hb was one of the first HBOCs to be evaluated as a RBC substitute (Savitsky et al. 1978). Unfortunately, Hb is able to extravasate through endothelial cell–cell junctions and scavenge NO several orders of magnitude more than RBCs (Suaudeau et al. 1979; Nakai et al. 1998; Dull et al. 2004; Liu et al. 1998; Liao et al. 1999). This is attributed to the smaller molecular dimensions of cell-free Hb compared to the RBC, which allows it to extravasate through the endothelial cell–cell junctions in capillaries into the surrounding tissue space (Suaudeau et al. 1979; Nakai et al. 1998; Dull et al. 2004). Consequently, vasoconstriction (i.e. reduction in blood vessel diameter) ensues, due to scavenging of the gaseous signaling molecule nitric oxide (NO) by the extravasated cell-free Hb (Vogel et al. 1986; Kavdia et al. 2002). Vasoconstriction at the microcirculatory level then leads to systemic hypertension, or high blood pressure (Doherty et al. 1998). In a human clinical trial, human Hb (HbA) induced mild hypertension in patients (Savitsky et al. 1978). HbA has also been shown to elevate the mean arterial blood pressure in hemorrhaged pigs and rats (Hess et al. 1993; Thompson et al. 1994). Therefore in light of these vascular side-effects, Hb is not used as a RBC substitute. Table 37.1 lists the biochemical and biophysical properties of Hb and various HBOCs.

In addition to its' vascular side-effects, cell-free Hb can also elicit tissue toxicity due to its ability to induce oxidative stress. In the blood stream, tetrameric Hb easily dissociates into two $\alpha\beta$ dimers (Alayash 1999; Bunn et al. 1969). The ferrous heme iron of the $\alpha\beta$ dimer autoxidizes to the ferric or methemoglobin

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Table 37.1 Biochemical and biophysical properties of various HBOCs

HBOC	MW (kDa)	P_{50} (mm Hg)	n	[Hb] (g/dl)	Viscosity (cP)
Bovine RBCs	[8 μm] (Rameez and Palmer 2011)	26.97 (Palmer et al. 2009b)	2.72 (Palmer et al. 2009b)	N/A	N/A
Human RBCs	[8 μm] (Rameez and Palmer 2011)	32.6 (Gelderman et al. 2010)	2.23 (Palmer et al. 2009b)	13 (Napolitano 2009)	5–10 (Napolitano 2009)
bHb	65.3 (Zhou et al. 2011)	26.02 (Rameez and Palmer 2011), 26.03 Palmer et al. (2009b), 27.37 (Zhou et al. 2011)	2.50 Palmer et al. (2009b), 2.75 (Rameez and Palmer 2011), 2.84 (Zhou et al. 2011)	10 (Zhou et al. 2011)	1.6 (Zhou et al. 2011)
HbA	62.29 (Zhang et al. 2011)	13.00 (Rameez and Palmer 2011), 13.27 (Zhang et al. 2011), 13.57 (Palmer et al 2009b)	2.43 Palmer et al. (2009b), 2.58 (Rameez and Palmer 2011), 2.59 (Zhang et al. 2011)	10 (Zhang et al. 2011)	1.6 (Zhang et al. 2011)
rHb 1.1	64 (Doyle et al. 1999)	32 (Doherty et al. 1998; Doyle et al. 1999), 33 (Looker et al. 1992)	2.2 (Doyle et al. 1999), 2.3 (Doherty et al. 1998)	5 (Stetter et al. 1997),10 (Doyle et al. 1999)	0.8 (Stetter et al. 1997)
DCLHb	65 = 96 % (Yu et al. 1997) 130 = 2–3 % (Yu et al. 1997)	27.0 (Cashon and Alayash 1995), 32 (Nagababu et al. 2002), 33.9 (Winslow et al. 1998)	2.07 (Nagababu et al. 2002), 2.4 (Winslow et al. 1998)	7.9(Winslow et al. 1998)	1.00 (Winslow et al. 1998)
Oxyglobin®	200 (Rentko et al. 2006; Day 2003)	34.8 (Alayash et al. 2001), 38.4 (Buehler et al. 2005), 40 (Rentko et al. 2006)	1.3 (Alayash et al. 2001), 1.4 (Buehler et al. 2005)	12–14(Rentko et al. 2006)	1.3 (Rentko et al. 2006)
Hemopure®	250 (Rentko et al. 2006; Day 2003)	38 (LaMuraglia et al. 2000; Napolitano 2009), 40 (Rentko et al. 2006)	1.4(Napolitano 2009)	12–14(Rentko et al. 2006), 13(LaMuraglia et al. 2000; Napolitano 2009)	1.3(Rentko et al. 2006; LaMuraglia et al. 2000; Napolitano 2009)
PolyHeme®	150 (Day 2003)	26–32 (Gould et al. 2002; Napolitano 2009), 28–30 (Gould et al. 1998)	1.7 (Napolitano 2009)	10(Gould et al. 2002; Napolitano 2009)	2.1 (Napolitano 2009)
Hemolink™	32 ≤ 5 % (Adamson and Moore 1998) 64 = 33 % (Adamson and Moore 1998) 128-600 = 63 % (Adamson and Moore 1998) >600 ≤ 3 % (Adamson and Moore 1998)	34 (Adamson and Moore 1998), 50.9 (Jia et al. 2004), 52(Nagababu et al. 2002), 52.6(Rohlfs et al. 1998)	0.97 (Nagababu et al. 2002) 1 (Adamson and Moore 1998), 1.0 (Jia et al. 2004), 1.01 (Rohlfs et al. 1998)	10 (Adamson and Moore 1998)	[1 cs] (Adamson and Moore 1998)

(continued)

Table 37.1 (continued)

HBOC	MW (kDa)	P_{50} (mm Hg)	n	[Hb] (g/dl)	Viscosity (cP)
OxyVita™	2.5×10^4 (Bucci et al. 2007), 3.3×10^4 (Matheson et al. 2002), 4.2×10^4 (Jia and Alayash 2009)	3 (Matheson et al. 2002), 6.4(Jia and Alayash 2009; Jahr et al. 2008)	1.2 (Jia and Alayash 2009; Jahr et al. 2008)	6 (Jahr et al. 2008)	2.8 (Jahr et al. 2008)
50:1 T-PolybHb	1.659×10^4 (Cabrales et al. 2010; Buehler et al. 2010)	41 (Buehler et al. 2010)	0.9 (Cabrales et al. 2010)	10 (Cabrales et al. 2010; Buehler et al. 2010)	11.4 (Cabrales et al. 2010; Buehler et al. 2010)
40:1 T-PolybHb	5.4946×10^4 (Baek et al. 2012)	38.1 (Cabrales et al. 2009)	0.9 (Cabrales et al. 2009)	10 (Cabrales et al. 2009)	7.2 (Cabrales et al. 2009)
40:1 R-PolybHb	2.633×10^4 (Cabrales et al. 2010; Buehler et al. 2010)	0.66 (Buehler et al. 2010)	0.7 (Cabrales et al. 2010)	10 (Cabrales et al. 2010; Buehler et al. 2010)	7.8 (Cabrales et al. 2010; Buehler et al. 2010)
30:1 T-PolybHb	1.3303×10^3 (Baek et al. 2012; Zhou et al. 2011)	41.16 (Zhou et al. 2011)	1.01 (Zhou et al. 2011)	10 (Zhou et al. 2011)	14.8 (Zhou et al. 2011)
30:1 R-PolybHb	6.256×10^3 (Zhou et al. 2011)	1.84 (Zhou et al. 2011)	0.69 (Zhou et al. 2011)	10 (Zhou et al. 2011)	9.8 (Zhou et al. 2011)
20:1 T-PolybHb	749 (Baek et al. 2012; Zhou et al. 2011)	37.10 (Zhou et al. 2011)	1.05 (Zhou et al. 2011)	10 (Zhou et al. 2011)	4.8 (Zhou et al. 2011)
20:1 R-PolybHb	1.0259×10^3 (Zhou et al. 2011)	2.18 (Zhou et al. 2011)	0.56 (Zhou et al. 2011)	10 (Zhou et al. 2011)	3.6 (Zhou et al. 2011)
10:1 T-PolybHb	91 (Baek et al. 2012; Zhou et al. 2011)	29.66 (Zhou et al. 2011)	1.36 (Zhou et al. 2011)	10 (Zhou et al. 2011)	3.2 (Zhou et al. 2011)
10:1 R-PolybHb	137.950 (Zhou et al. 2011)	4.54 (Zhou et al. 2011)	0.61 (Zhou et al. 2011)	10 (Zhou et al. 2011)	2.7 (Zhou et al. 2011)
50:1 T-PolyhHb	1.844×10^4 (Zhang et al. 2011)	48.84 (Zhang et al. 2011)	0.87 (Zhang et al. 2011)	10 (Zhang et al. 2011)	9.6 (Zhang et al. 2011)
40:1 T-PolyhHb	3.68×10^3 (Zhang et al. 2011)	37.19 (Zhang et al. 2011)	0.81 (Zhang et al. 2011)	9.9 (Zhang et al. 2011)	8.7 (Zhang et al. 2011)
30:1 R-PolyhHb	5.54×10^3 (Zhang et al. 2011)	0.76 (Zhang et al. 2011)	0.91 (Zhang et al. 2011)	9.6 (Zhang et al. 2011)	6.6 (Zhang et al. 2011)
20:1 R-PolyhHb	1.10×10^3 (Zhang et al. 2011)	1.45 (Zhang et al. 2011)	0.70 (Zhang et al. 2011)	9.1 (Zhang et al. 2011)	3.2 (Zhang et al. 2011)

Table 37.1 (continued)

HBOC	COP (mm Hg)	k_{off}, O_2 (s^{-1})	k_{on}, CO ($\mu M^{-1} s^{-1}$)	k_{ox}, NO ($\mu M^{-1} s^{-1}$)	k_{autox}(min^{-1})
Bovine RBCs	N/A	2.01 (Rameez and Palmer 2011)	0.087 (Rameez and Palmer 2011)	0.31 (Rameez and Palmer 2011)	N/A
Human RBCs	25 (Napolitano 2009)	16.67 (Rameez and Palmer 2011)	0.130 (Rameez and Palmer 2011)	0.03 (Rameez and Palmer 2011)	N/A
bHb	38 (Zhou et al. 2011)	36.1 (Buehler et al. 2010; Zhou et al. 2011), 38.97(Rameez and Palmer 2011)	0.198 (Rameez and Palmer 2011), 0.22 (Buehler et al. 2010; Zhou et al. 2011)	18.3 (Buehler et al. 2010; Zhou et al. 2011), 28.2 (Rameez and Palmer 2011)	1.5×10^{-4} (Baek et al. 2012)
HbA	38 (Zhang et al. 2011)	38.71 (Rameez and Palmer 2011), 40.4(Zhang et al. 2011)	0.200 (Rameez and Palmer 2011), 0.214 (Zhang et al. 2011)	18.5 (Zhang et al. 2011), 27.5 (Rameez and Palmer 2011)	5.54×10^{-4} (Zhang et al. 2011)
rHb 1.1	42 (Doyle et al. 1999)	N/A	N/A	58 (Doherty et al. 1998)	8.5×10^{-4} (Doherty et al. 1998)
DCLHb	23.0 (Winslow et al. 1998)	56.0 (Cashon and Alayash 1995)	0.17 (Cashon and Alayash 1995)	31 (Rohlfs et al. 1998)	3.7×10^{-3} (Winslow et al. 1998), 5.5×10^{-3} (Cashon and Alayash 1995), 4.00×10^{-4} (Nagababu et al. 2002)
Oxyglobin®	42 (Rentko et al. 2006)	50.9 (Alayash et al. 2001), 60.0(Buehler et al. 2005)	0.15 (Alayash et al. 2001; Buehler et al. 2005), 0.27 (Sakai et al. 2008)	66 (Sakai et al. 2008)	7.8×10^{-3} (Buehler et al. 2005), 5.98×10^{-4} (Nagababu et al. 2002)
Hemopure®	17 (LaMuraglia et al. 2000), 25(Rentko et al. 2006; Napolitano 2009)	N/A	N/A	N/A	N/A
PolyHeme®	23 (Napolitano 2009)	N/A	N/A	N/A	N/A
Hemolink™	24 (Adamson and Moore 1998)	130 (Jia et al. 2004)	0.12 (Jia et al. 2004)	31 (Rohlfs et al. 1998)	6.53×10^{-4} (Nagababu et al. 2002)
OxyVita™	2.2 (Jahr et al. 2008)	27.4 (Jia and Alayash 2009)	0.28 (Jia and Alayash 2009)	30 (Jia and Alayash 2009)	3.0×10^{-4} (Jia and Alayash 2009)
50:1 T-PolybHb	1 (Cabrales et al. 2010; Buehler et al. 2010)	53.0 (Buehler et al. 2010)	0.18 (Buehler et al. 2010)	18.9 (Buehler et al. 2010)	1.23×10^{-3} (Buehler et al. 2010)
40:1 T-PolybHb	5 (Cabrales et al. 2009)	N/A	N/A	NA	N/A

(continued)

Table 37.1 (continued)

HBOC	COP (mm Hg)	k_{off}, O_2 (s^{-1})	k_{on}, CO ($\mu M^{-1} s^{-1}$)	k_{ox}, NO ($\mu M^{-1} s^{-1}$)	k_{autox}(min^{-1})
40:1 R-PolybHb	7 (Cabrales et al. 2010; Buehler et al. 2010)	22.0 (Buehler et al. 2010)	4.84 (Buehler et al. 2010)	17.5 (Buehler et al. 2010)	9.0×10^{-4} (Buehler et al. 2010)
30:1 T-PolybHb	2 (Zhou et al. 2011)	57.1 (Zhou et al. 2011)	0.157 (Zhou et al. 2011)	18.7 (Zhou et al. 2011)	1.5×10^{-4} (Baek et al. 2012)
30:1 R-PolybHb	14 (Zhou et al. 2011)	24.7 (Zhou et al. 2011)	5.95 (Zhou et al. 2011)	17.5 (Zhou et al. 2011)	N/A
20:1 T-PolybHb	24 (Zhou et al. 2011)	58.6 (Zhou et al. 2011)	0.183 (Zhou et al. 2011)	17.1 (Zhou et al. 2011)	1.3×10^{-4} (Baek et al. 2012)
20:1 R-PolybHb	39 (Zhou et al. 2011)	29.7 (Zhou et al. 2011)	3.92 (Zhou et al. 2011)	16.4 (Zhou et al. 2011)	N/A
10:1 T-PolybHb	42 (Zhou et al. 2011)	47.2 (Zhou et al. 2011)	0.193 (Zhou et al. 2011)	18.9 (Zhou et al. 2011)	1.2×10^{-4} (Baek et al. 2012)
10:1 R-PolybHb	48 (Zhou et al. 2011)	29.1 (Zhou et al. 2011)	6.138 (Zhou et al. 2011)	17.4 (Zhou et al. 2011)	N/A
50:1 T-PolyhHb	4 (Zhang et al. 2011)	51.7 (Zhang et al. 2011)	0.184 (Zhang et al. 2011)	20.1 (Zhang et al. 2011)	1.34×10^{-3} (Zhang et al. 2011)
40:1 T-PolyhHb	4 (Zhang et al. 2011)	49.7 (Zhang et al. 2011)	0.181 (Zhang et al. 2011)	19 (Zhang et al. 2011)	1.45×10^{-3} (Zhang et al. 2011)
30:1 R-PolyhHb	24 (Zhang et al. 2011)	25.5 (Zhang et al. 2011)	4.88 (Zhang et al. 2011)	20.2 (Zhang et al. 2011)	6.86×10^{-4} (Zhang et al. 2011)
20:1 R-PolyhHb	37 (Zhang et al. 2011)	31.4 (Zhang et al. 2011)	4.76 (Zhang et al. 2011)	21.8 (Zhang et al. 2011)	4.98×10^{-4} (Zhang et al. 2011)

(metHb) form at a higher rate than the ferrous heme of tetrameric Hb (Zhang et al. 1991; D'Agnillo 2006). MetHb formation via autoxidation produces the reactive oxygen species (ROS) superoxide anion and indirectly hydrogen peroxide (H_2O_2) (D'Agnillo 2006; Misra and Fridovich 1972). Hence, the ROS hydroxyl radical can be generated by the reaction of superoxide with H_2O_2, and this is catalyzed by iron released from degraded heme (D'Agnillo 2006; Graf et al. 1984). Hence, Hb oxidation drives the production of ROS (D'Agnillo 2006; Misra and Fridovich 1972; Graf et al. 1984). Unfortunately, in vivo exposure to hydroxyl radicals has been linked to renal failure and apoptosis of vascular cells (Walker and Shah 1988; Shah and Walker 1988; Li et al. 1997). To compound matters further, the small size of $\alpha\beta$ dimers also facilitates their filtration through the kidneys, and thus increases the opportunity for tissue exposure to ROS (Bunn et al. 1969). In clinical human trials, cell-free HbA infusion caused noticeable hemoglobinuria, indicative of HbA clearance through the kidneys (Savitsky et al. 1978). The highly reactive species ferryl Hb can also be formed from further reactions of Hb with H_2O_2 (D'Agnillo 2006; Giulivi and Davies 1990; Kanner et al. 1988). Ferrous Hb can be oxidized to ferric Hb via H_2O_2 exposure, and ferryl Hb is formed as an intermediate during this reaction (D'Agnillo 2006; Giulivi and Davies 1990). MetHb itself can also be converted to ferryl Hb in the presence of H_2O_2 (D'Agnillo 2006; Kanner et al. 1988). Ferryl Hb can elicit inflammation of vascular endothelial cells (Silva et al. 2009). Overall, oxidative stress facilitated by Hb oxidation products is a problem many HBOCs face and aim to prevent.

37.1.2 Recombinant HbA 1.1 (rHb 1.1)

Therefore in order to reduce renal filtration of $\alpha\beta$ dimers, recombinant HbA (rHb 1.1) was developed by Somatogen Inc. (Boulder, CO) (Looker et al. 1992). Mutations to the native structure of HbA include an oxygen affinity lowering asparagine to lysine mutation at the β-108 position, and the insertion of a glycerin residue which covalently links the neighboring α chains together (Looker et al. 1992; Moo-Penn et al. 1978). Linking the α globin subunits together stabilized the structure of rHb 1.1 and inhibited dissociation of the tetramer into $\alpha\beta$ dimers (Looker et al. 1992). Several clinical human trials demonstrated that prevention of rHb 1.1 dissociation into $\alpha\beta$ dimers led to reduced oxidative stress and diminished renal toxicity (Viele et al. 1997; Hayes et al. 2001). Unfortunately, rHb 1.1 also elicited noticeable levels of hypertension in surgery patients (Hayes et al. 2001). In another clinical trial, rHb 1.1 caused lower esophageal sphincter tension in humans (Murray et al. 1995). It was hypothesized that the mechanism of this inhibition is linked to NO scavenging by rHb 1.1 (Murray et al. 1995). Therefore, although rHb 1.1 exhibited reduced oxidative stress and renal toxicity, it still exhibited undesirable vascular side-effects (Viele et al. 1997; Hayes et al. 2001; Murray et al. 1995). In light of these side-effects and after acquiring Somatogen Inc. (Boulder, CO), Baxter International Inc. (Deerfield, IL) subsequently engineered rHb 3011

with a reduced dioxygenation rate constant in order to eliminate vasoconstriction and systemic hypertension (Olson et al. 2004; Varnado et al. 2012; Baxter 2012). However, it has recently been shown that rHb 3011 autoxidizes at a greater rate in vitro compared to the earlier generation rHb 0.1, which could lead to increased oxidative stress on the vasculature (Varnado et al. 2012).

37.1.3 Diaspirin Cross-Linked HbA (DCLHb)

Site specifically cross-linking HbA represents another strategy to eliminate HbA dissociation into $\alpha\beta$ dimers. Hence, diaspirin cross-linked HbA (DCLHb) was developed by the United States (US) Army and commercially as HemAssit™ by Baxter International Inc. (Deerfield, IL) (Baxter 2012; Winslow 2003). DCLHb is composed of HbA, in which the neighboring α globin chains are covalently cross-linked using bis-(3,5-dibromosalicyl) fumarate (Chatterjee et al. 1986). DCLHb consists of 96 % tetrameric HbA and 2–3 % of HbA tetrameric dimers (Yu et al. 1997). In Phase III clinical trials, DCLHb induced hypertension in stroke patients (Saxena et al. 1999). Elevated blood pressure levels were also reported when DCLHb was infused in patients after cardiac surgery (Lamy et al. 2000). When administered to traumatic hemorrhagic shock patients, DCLHb recipients died more often than saline recipients (Sloan et al. 1999). Despite stabilization of DCLHb's tetrameric structure via α globin chain cross-links, DCLHb readily oxidized in vitro when H_2O_2 was present (Cashon and Alayash 1995). Therefore, DCLHb exhibited many of the side-effects associated with transfusion of cell-free Hb and is no longer being pursued by Baxter International Inc. (Winslow 2003; Saxena et al. 1999; Lamy et al. 2000; Sloan et al. 1999; Cashon and Alayash 1995).

37.2 Hb Polymerization as a Strategy to Mitigate Vascular Side-Effects

In order to prevent many of the side-effects commonly associated with early commercial HBOCs, the focus has turned to developing HBOCs which are too large to pass through the endothelial cell–cell junctions of blood vessels (Alayash 2004; Palmer 2006). The rationale behind this approach centers on reducing HBOC extravasation into the tissue space so that the HBOC is not in close proximity to the endothelium (Alayash 2004; Palmer 2006). This should lead to less scavenging of endothelial-derived NO by the HBOC, reduced vasoconstriction and hypertension, as well as reduced oxidative tissue toxicity (Alayash 2004; Palmer 2006). In light of this approach, several companies have developed polymerized Hb (PolyHb) solutions as potential RBC substitutes.

37.2.1 Oxyglobin®

OPK Biotech LLC (Cambridge, MA) developed two HBOCs, which have gone through extensive studies, namely Oxyglobin® and Hemopure® (OPK Biotech 2012). Both Oxyglobin® and Hemopure® consist of glutaraldehyde polymerized bovine Hb (PolybHb) (OPK Biotech 2012). Glutaraldehyde forms intramolecular cross-links within the Hb tetramer and intermolecular cross-links between neighboring Hb tetramers (Chang 1998). Oxyglobin® is composed of PolybHb with an average MW of 200 kDa (Rentko et al. 2006; Day 2003). It is approved for veterinary use in the US (Day 2003). However, Oxyglobin® has been shown to elicit both vasoconstriction and hypertension in vivo (Tsai et al. 2006). Because of its availability, multiple studies have explored the toxicity of Oxyglobin® Butt et al. (2010, 2011). The oxidative stress caused by Oxyglobin® damaged blood brain barrier endothelial cells and elicited cellular apoptosis in vivo (Butt et al. 2011). Iron was found to be deposited in endothelial cells and neurons associated with the blood brain barrier after administration of Oxyglobin® (Butt et al. 2011) In addition, Oxyglobin® administered to guinea pigs and rats has been shown to facilitate iron deposition in renal tissues (Butt et al. 2010). These results indicate extravasation of Oxyglobin® through endothelial cell–cell junctions and its deposition in the tissue space Butt et al. (2010, 2011).

37.2.2 Hemopure®

Hemopure® is a glutaraldehyde PolybHb solution with an average MW of 250 kDa, which was also developed by OPK Biotech LLC (Cambridge, MA) (OPK Biotech 2012; Rentko et al. 2006; Day 2003). Hemopure® is composed of 2 % unpolymerized bovine Hb (bHb) compared to Oxyglobin®, which is composed of 31 % unpolymerized bHb (Rice et al. 2008). A study of resuscitated hemorrhagic shock-induced swine reported that Hemopure® elevated blood pressure less than Oxyglobin®, and this was attributed to the reduced amount of unpolymerized bHb present in Hemopure® compared to Oxyglobin® (Rice et al. 2008). When administered before, during, and after elective aortic surgery, Hemopure® produced hypertension in patients (Kasper et al. 1996; LaMuraglia et al. 2000). Data from another Hemopure® clinical trial highlighted a possible vulnerability in elderly orthopedic surgery patients to negative vascular side-effects (Jahr et al. 2008; Freilich et al. 2009). These findings indicate that despite having less unpolymerized bHb than Oxyglobin®, Hemopure® still presents risks to the vasculature in clinical settings (Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Rice et al. 2008).

37.2.3 Polyheme®

Northfield Laboratories Inc. (Evanston, IL) developed a glutaraldehyde polymerized pyridoxal phosphate cross-linked HbA product known as PolyHeme® (Day 2003; Sehgal et al. 1984). PolyHeme® has an average MW of 150 kDa (Day 2003). The pyridoxilated HbA reduces the oxygen affinity of PolyHeme® Sehgal et al. (1981, 1984). In clinical trials, PolyHeme® was administered to trauma, surgery, and hemorrhagic shock patients, and did not increase blood pressure to unsafe levels (Gould et al. 1998, 2002; Moore et al. 2009). Unfortunately, PolyHeme® has been linked to negative side-effects in various animal studies (Yu et al. 2010; Handrigan et al. 2005). A recent study showed that PolyHeme® induced vasoconstriction in lambs (Yu et al. 2010). PolyHeme® administration also produced organ failure and death in hemorrhaged rats (Handrigan et al. 2005). Negative vascular responses are not surprising considering that Oxyglobin® and Hemopure®, both two larger sized glutaraldehyde PolybHbs, displayed similar negative side-effects in vivo (Rentko et al. 2006; Day 2003; Tsai et al. 2006; Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Yu et al. 2010; Handrigan et al. 2005). PolyHeme® production was shut down after ethical questions were raised over the consent requirements of the final clinical trial (Moore et al. 2009; Chen et al. 2009; Kipnis et al. 2006).

37.2.4 Hemolink™

Hemosol Inc. (Toronto, Canada) developed the *O*-raffinose cross-linked HbA product Hemolink™ (Day 2003; Adamson and Moore 1998). Hemolink™ has a wide range of MWs which consists of less than or equal to 5 % 32 kDa, 33 % 64 kDa, 63 % 128–600 kDa, and less than or equal to 3 % greater than 600 kDa species (Adamson and Moore 1998). In Phase II and Phase III clinical trials, Hemolink™ administration lead to hypertension in coronary artery surgery patients (Cheng et al. 2004; Greenburg and Kim 2004; Hill et al. 2002). Hemolink™ exhibited diminished renal toxicity in vivo and NO reactions in vitro compared to HbA (Lieberthal et al. 1999). In this study, Hemolink™ also induced hypertension in rats (Lieberthal et al. 1999). Thus despite Hemolink™ having a low affinity for NO in vitro, there was still evidence of systemic hypertension (Lieberthal et al. 1999). Hemolink™ production was discontinued due to negative side-effects observed in clinical trials (Chen et al. 2009).

37.2.5 OxyVita™

Of all the aforementioned PolyHbs which have reported average MWs, all of them ranged between 150 and 250 kDa (Rentko et al. 2006; Day 2003). All four of the PolyHbs exhibited some form of vasoconstriction, hypertension, and/or oxidative

stress (Tsai et al. 2006; Butt et al. 2010, 2011; Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Yu et al. 2010; Handrigan et al. 2005; Cheng et al. 2004; Greenburg and Kim 2004; Hill et al. 2002; Lieberthal et al. 1999). The presence of vasoconstriction and systemic hypertension make it reasonable to assume that these commercial HBOCs are still able to extravasate through the endothelium and scavenge NO despite their larger size compared to HbA (Rentko et al. 2006; Day 2003; Adamson and Moore 1998; Tsai et al. 2006; Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Yu et al. 2010; Handrigan et al. 2005; Cheng et al. 2004; Greenburg and Kim 2004; Hill et al. 2002; Lieberthal et al. 1999). Therefore, it is possible that since Oxyglobin® induces oxidative stress, similarly sized HBOCs may possess similar potential for oxidation Butt et al. (2010, 2011). To avoid vasoconstriction, hypertension, and oxidative stress, some groups have developed ultrahigh MW PolyHbs.

OXYVITA Inc. (New Windsor, NY) developed a high MW RBC substitute known as OxyVita™ (OXYVITA Inc. 2012; Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009). Also known as zero-link polymerized bHb, the ultrahigh MW of Oxyvita™ has been reported to be 25, 33 and 42 MDa in separate studies (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009). OxyVita™ is synthesized by cross-linking the β globin chains of bHb with bis(3,5-dibromosalicy)-adipoate (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009; Kwansa et al. 2000). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide is then used to polymerize the bHb tetramers (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009). OxyVita™ has been shown to produce low levels of vasoconstriction, which did not lead to hypertension in animal studies (Matheson et al. 2002; Bucci et al. 2007). These benefits have been attributed to the large size of the PolybHb, which prevents its extravasation into the tissue space and thus its inability to scavenge NO (Matheson et al. 2002; Bucci et al. 2007). Therefore, the zero-link cross-linking process appears to be able to prevent vascular side-effects common to many commercial HBOCs (Matheson et al. 2002; Bucci et al. 2007). Despite this major advantage, OxyVita™ possesses a high oxygen affinity (Matheson et al. 2002; Jia and Alayash 2009). The high oxygen affinity makes it unclear how well OxyVita™ will deliver oxygen under physiological conditions compared to other commercial HBOCs (Matheson et al. 2002; Jia and Alayash 2009). OXYVITA Inc. is presently raising resources to pursue further trials of OxyVita™ (OXYVITA Inc. 2012).

37.2.6 Overview of Commercial PolyHbs

Many of the commercial PolyHbs discussed above failed Phase III clinical trials, before their potential for negative side-effects were fully understood (Jahr et al. 2008; Freilich et al. 2009; Moore et al. 2009; Greenburg and Kim 2004; Chen et al. 2009). It is reasonable to expect that if a systematic study of these PolyHbs had been performed they would not have progressed as far without serious design

alterations. Investigations into the gaseous ligand binding/release kinetics, ability to induce vasoconstriction, systemic hypertension, and oxidative stress-inducing potential of these PolyHbs could have foreshadowed the side-effects observed during Phase III clinical trials (Jahr et al. 2008; Freilich et al. 2009; Greenburg and Kim 2004). In the future, PolyHb development should include systematic preliminary studies to prevent a reoccurrence of these common side-effects. Therefore, the wide difference in MW between the low MW PolyHbs Oxyglobin®, Hemopure®, PolyHeme®, Hemolink™ and the ultrahigh MW PolyHb OxyVita™ highlight the need for a systematic study to investigate the effects of PolyHb MW on its' safety profile (i.e. ability to induce vasoconstriction, systemic hypertension and oxidative toxicity) (Rentko et al. 2006; Day 2003; Adamson and Moore 1998; Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009).

37.3 Systematic Study of PolyHb MW on Safety Profile

In a series of publications, Palmer's group reported the results of a systematic study investigating the biophysical properties and in vivo responses of variable MW glutaraldehyde PolybHbs (Cabrales et al. 2009; Cabrales et al. 2010; Baek et al. 2012; Palmer et al. 2009a; Buehler et al. 2010; Zhou et al. 2011).

Palmer's group first demonstrated that the quaternary state of bHb could be used to control the oxygen affinity of the resultant PolybHb solution Palmer et al. (2009a). This work took advantage of the fact that Hb can be held in either the tense (T) or relaxed (R) quaternary state by simply fully deoxygenating or fully oxygenating the bHb solution, respectively (Palmer et al. 2009a). Hence, T-state and R-state PolybHbs were synthesized by polymerizing T-state and R-state bHb with glutaraldehyde, respectively (Palmer et al. 2009a). In this work, T- and R-state PolybHbs were synthesized at the following glutaraldehyde to bHb molar ratios (i.e. cross-link densities): 10:1, 20:1, 30:1 and 40:1 (Palmer et al. 2009a). It was observed that T-state PolybHb displayed a higher P_{50} compared to R-state PolybHb (Palmer et al. 2009a). In addition, the cooperativity coefficient of both T- and R-state PolybHb decreased with increasing cross-link density (Palmer et al. 2009a). This result is consistent with increasing cross-linking density, thereby reducing the mobility of the globin chains in the Hb tetramer (Palmer et al. 2009a).

A subsequent study evaluated the effect of T-state PolybHb MW on the extent of vasoconstriction and systemic hypertension (Cabrales et al. 2009). In this study, T-state PolybHbs with glutaraldehyde to bHb molar ratios of 20:1, 30:1, 40:1, and 50:1 were synthesized in the lab (Cabrales et al. 2009). Ultrafiltration was then used to fractionate each PolybHb solution into a fraction greater than 500 kDa in MW and a fraction less than 500 kDa in MW (Cabrales et al. 2009). All of the PolybHb solutions with MW less than 500 kDa were shown to induce vasoconstriction and hypertension in top-loaded hamsters outfitted with the window chamber, and this was likely due to extravasation of low MW PolyHb through the endothelium and subsequent PolybHb scavenging of endothelium-derived NO

(Cabrales et al. 2009). Negative vascular effects were less prevalent for the PolybHbs with MW greater than 500 kDa. (Cabrales et al. 2009). Most notably, no vasoconstriction and only minor hypertension were present with the 40:1 and 50:1 PolybHbs (Cabrales et al. 2009). The diminished vascular side-effects exhibited by the PolybHbs greater than 500 kDa were similar to those observed for the ultra-high MW PolybHb OxyVita^{TM} (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009; Cabrales et al. 2009). An important distinction between OxyVita^{TM} and the high MW T-state PolybHbs is the high P_{50} of the T-state PolybHbs and the low P_{50} of OxyVita^{TM} (Matheson et al. 2002; Jia and Alayash 2009; Cabrales et al. 2009). Previous work has shown that high P_{50} HBOCs deliver oxygen to tissues more readily versus low P_{50} HBOCs (Sakai et al. 2005).

Therefore, the ability of T-state and R-state PolybHbs to deliver O_2 was evaluated in vivo to better understand the effect of PolybHb oxygen affinity in influencing tissue oxygenation (Cabrales et al. 2010). Hence, 40:1 R-state and 50:1 T-state PolybHbs were used in this study, and all HBOCs possessed MWs greater than 500 kDa (Cabrales et al. 2010). It was observed that T-state PolybHb delivered more O_2 to tissues in the hamster chamber window model versus R-state PolybHb (Cabrales et al. 2010). As expected, this was largely attributed to the high oxygen affinity of R-state PolybHb (Cabrales et al. 2010). Thus the ability of T-state PolybHbs to adequately transport oxygen in vivo was confirmed in this study (Cabrales et al. 2010).

A thorough investigation into the gaseous ligand binding/release kinetics and autoxidation kinetics of high MW T- and R-state PolybHbs was then conducted to better understand their in vivo oxidation potential and pharmacokinetics (Buehler et al. 2010). Stop flow kinetic measurements were used to determine the O_2 dissociation, CO association, and NO dioxygenation rate constants of 40:1 R-state and 50:1 T-state PolybHbs, all PolybHbs had MWs $>$ 500 kDa (Buehler et al. 2010). The O_2 dissociation rate constant increased for 50:1 T-State PolybHb and decreased for 40:1 R-state PolybHb compared to bHb, indicating that O_2 is released faster from the 50:1 T-state PolybHb compared to 40:1 R-state PolybHb (Buehler et al. 2010). The reported value of the CO association rate constant of 50:1 T-state PolybHb was marginally lower than that of bHb, whereas this rate constant increased for 40:1 R-state PolybHb (Buehler et al. 2010). The NO dioxygenation rate constants of both the 50:1 T-state and 40:1 R-state PolybHbs were shown to be similar to that of bHb (Buehler et al. 2010). This indicates that both PolybHbs and cell-free bHb interact similarly with NO (Buehler et al. 2010). Counter intuitively, an earlier study observed that T-state PolybHbs do not produce vasoconstriction and hypertension, which have been linked to NO scavenging (Cabrales et al. 2009). Thus since PolybHbs are capable of interacting with NO, it is hypothesized that their large size (compared to the size of the endothelial cell–cell junctions lining the blood vessel wall) prevents them from extravasating through the endothelium and scavenging NO (Cabrales et al. 2009; Buehler et al. 2010). In vivo, 50:1 T-state PolybHb underwent autoxidation at a significantly lower rate and remained in circulation longer than 40:1 R-state PolybHb (Buehler et al. 2010). This result is important, since the reduced rate of heme oxidation prolongs T-state PolybHb

oxygen delivery in vivo (Buehler et al. 2010). Therefore, high MW T-state PolybHbs seem to be an ideal HBOC due to their lack of vasoconstriction and hypertension, increased circulatory half-life, and reduced in vivo oxidation compared to R-state PolybHbs (Buehler et al. 2010).

An evaluation of T- and R-state glutaraldehyde polymerized HbA (PolyhHb) demonstrated that PolybHbs and PolyhHbs share many of the same biophysical characteristics (Buehler et al. 2010; Zhang et al. 2011). In this study, 50:1 and 40:1 T-state PolyhHbs and 30:1 and 20:1 R-state PolyhHbs were synthesized with MWs $>$ 500 kDa (Zhang et al. 2011). Both the P_{50} and oxygen dissociation rate constants of T-state PolyhHbs were higher than those of hHb and R-state PolyhHbs (Zhang et al. 2011). Thus the low oxygen affinity of T-state PolybHbs and high oxygen affinity of R-state PolybHbs are maintained in T- and R-state PolyhHbs, respectively (Buehler et al. 2010; Zhang et al. 2011). Polymerization decreased the CO association binding rate constant of T-state PolyhHbs, while this rate constant increased for R-state PolyhHbs (Zhang et al. 2011). This is similar to the trends observed in T- and R-state PolybHbs (Buehler et al. 2010; Zhang et al. 2011). T- and R-state PolyhHbs also maintained their ability to interact with NO similar to HbA, regardless of Hb quaternary state (Buehler et al. 2010; Zhang et al. 2011). In addition to these kinetic experiments, the ability of PolyhHbs to deliver O_2 was simulated using a mathematical model of a hepatic hollow fiber bioreactor (Zhang et al. 2011). Modeling results indicated that T-state PolyhHbs were more effective at delivering oxygen to hepatocytes housed in the bioreactor compared to R-state PolyhHbs (Zhang et al. 2011). This study showed that PolyhHbs share many of the same biophysical characteristics as PolybHbs, and indicated that T-state PolyhHbs possess the best potential to serve as oxygen transporting RBC substitutes (Buehler et al. 2010; Zhang et al. 2011).

Another study evaluated the ability of PolybHbs to deliver oxygen using the same mathematical model of a hepatic hollow fiber bioreactor used to evaluate PolyhHbs (Zhou et al. 2011; Zhang et al. 2011). In this study, T- and R-state PolybHbs were synthesized at the following glutaraldehyde to bHb molar ratios: 10:1, 20:1, and 30:1, and previous results for 40:1 R-state and 50:1 T-state PolybHbs were incorporated in the hepatic hollow fiber model (Buehler et al. 2010; Zhou et al. 2011). The results of these simulations showed that all T-state PolybHbs were able to more efficiently deliver O_2 to hepatocytes housed in the bioreactor compared to R-state PolybHbs, further solidifying the superior oxygenation potential of T-state PolybHbs as RBC substitutes (Zhou et al. 2011).

In order to evaluate the effect of PolybHb size on oxidative tissue toxicity, T-state PolybHbs were synthesized at the following glutaraldehyde to bHb molar ratios: 10:1, 20:1, 30:1 and 40:1 (Baek et al. 2012). Guinea pigs were then subjected to a 50 % blood for PolybHb solution exchange transfusion and the pharmacokinetics of PolybHb was evaluated along with iron deposition in the spleen, liver and kidneys (Baek et al. 2012). The results of this study showed that the 30:1 PolybHb elicited less in vivo oxidation in the blood compared to the 10:1, 20:1, and 40:1 PolybHbs, that was not a function of PolybHb size (Baek et al. 2012). However, iron deposition in the kidneys decreased as a function of increasing

PolybHb size (Baek et al. 2012). Similarly, the extent of systemic hypertension decreased with increasing PolybHb size, while the circulatory half-life of PolybHb increased as a function of increasing PolybHb size until it reached a maximum for the 30:1 PolybHb (Baek et al. 2012). In light of these results, the 30:1 T-state PolybHb exhibited the best pharmacokinetics with the least iron deposition in the kidneys along with the absence of systemic hypertension upon transfusion (Baek et al. 2012). Additional studies will need to be performed to further assess the clinical safety of this material.

37.4 Conclusions

In summary, Palmer's group has systematically investigated the biophysical properties, and in vivo responses upon transfusion of variable sized T- and R-state PolyHbs (Cabrales et al. 2009, 2010; Baek et al. 2012; Palmer et al. 2009a; Buehler et al. 2010; Zhou et al. 2011; Zhang et al. 2011). The results of these studies have identified high MW T-state PolyHbs as a low oxygen affinity HBOC, which does not elicit vasoconstriction, hypertension, or oxidative tissue toxicity (Cabrales et al. 2009, 2010; Baek et al. 2012). In addition, high MW T-state PolyHbs are able to deliver O_2 in both in vitro and in vivo scenarios (Cabrales et al. 2010; Buehler et al. 2010; Zhou et al. 2011; Zhang et al. 2011). Therefore, these results set the stage for exploring the clinical potential of high MW T-state PolyHbs as RBC substitutes in transfusion medicine.

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