Chapter 10
HBOCs from Chemical Modification of Hb

Ronald Kluger and Francine E. Lui

10.1 Fundamentals for Haemoglobin-Based Oxygen Carriers

We have produced several recent reviews related to the technology of chemical stabilization of haemoglobin and a brief view of the future of HBOCs (Kluger 2010; Lui and Kluger 2010). This chapter is intended to provide a broader perspective of the topic of chemical stabilization for the production of HBOCs.

10.1.1 Haemoglobin and Oxygen Binding

Haemoglobins are the oxygen-carrying components of red cella in animals, providing a precisely controlled machine for the acquisition and distribution of atmospheric oxygen within an organism. While human adult haemoglobin (Hb A) is the focus of much of the interest in HBOCs, other sources such as bovine or porcine haemoglobin have been used. However, in this chapter the abbreviation Hb will specifically refer to HbA. Hb is a globular 64 kDa tetrameric assembly of globin subunits consisting of two identical $\alpha\beta$-dimers. Each monomeric subunit is associated with a ferrous heme to which oxygen binds reversibly. The quaternary structure of Hb is complex and there is significant variation between the three-dimensional structure of deoxy-Hb (T-state) and ligated oxy-Hb (R-state), with a 15° shift between the $\alpha\beta$ interfaces of the tetrameric protein. The ability of Hb to bind and release oxygen can be assessed at the level of detail needed to design an HBOC using two parameters: oxygen affinity (characterized by P_{50}), which measures the average energy of binding of the four oxygen molecules to a tetramer

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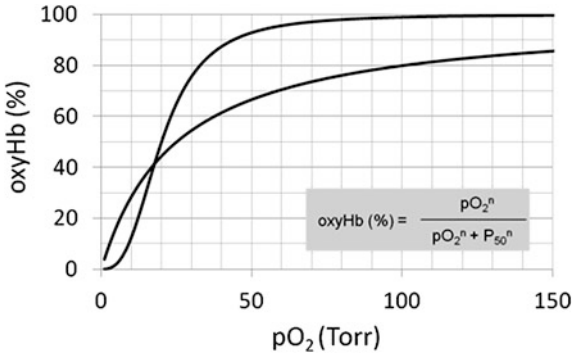

Fig. 10.1 The oxygen dissociation curve of haemoglobin is governed by the oxygen dissociation equation. The sigmodial curve of haemoglobin ($n_{50} = 3$) indicates allosteric cooperativity, and allows for haemoglobin to be more O_2-saturated in the lungs at high oxygen tensions and more effectively deoxygenated at lower oxygen tensions such as in the capillaries. Myoglobin is an oxygen-carrying protein that does not display cooperativity—its oxygen affinity curve is hyperbolic ($n_{50} = 1$)

and the cooperativity of sequential oxygenation (characterized by the Hill coefficient, n_{50}), which measures the extent to which the oxygenation curve is sigmoidal (the result of cooperativity) versus hyperbolic (where there is no cooperativity). A comparative set of cooperative and hyperbolic oxygenation curves is shown in Fig. 10.1.

Oxygen affinity is evaluated from a plot that relates the extent of occupancy of oxygen-binding sites on Hb in solution to the partial pressure of oxygen (P_{O2}) in the environment of the measurement (Fig. 10.1). The inherent oxygen affinity of Hb is quite high and in circulation the tendency would be for the oxygen to be retained by Hb rather than being released in hypoxic environments. However, the affinity is significantly reduced by allosteric effectors in the red cell, including protons,(Perutz 1990) 2,3-disphosphoglycerate [2,3-DPG](Benesch and Benesch 1967), chloride and carbon dioxide. Acellular Hb that has been stripped of 2,3-DPG has a higher oxygen affinity ($P_{50} = 13.3$ torr) compared to Hb in red cells where 2,3-DPG is present at a high concentration, giving $P_{50} = 26$ torr (Riess 2001).

The cooperativity of binding of oxygen to Hb is conveniently measured by the Hill coefficient, where $n_{50} = 3$ for native Hb indicates the presence of substantial sigmoidal character in the oxygenation curve (Bellelli 2010). This positive cooperativity in oxygen binding is manifested as the increased affinity of the remaining set of binding sites for oxygen. A decrease in n_{50} is indicative of decreased cooperativity. The cooperative oxygenation behavior of Hb achieves a highly controlled and effective delivery of oxygen within a small physiological oxygen tension range (100 torr in arterial blood and 30 torr in venous blood).

10.1.2 Interaction with Ligands Other than Oxygen

While the Fe(II) heme center of haemoglobin performs effectively in acquiring and delivering oxygen function, it also binds other diatomic molecules, most notably carbon monoxide and nitric oxide.

10.1.3 Carbon Monoxide

The association between Fe(II)-heme and CO is well-studied and the combination is reversible by introduction of visible light and nitrogen to produce deoxyhaemoglobin (Perrella 1999). CO is also released spontaneously but slowly and while it is present on the heme, it prevents addition of oxygen. The relatively stable bond between CO and Fe(II)-heme has some utility in preparation and administration of an HBOC. The stable bond between CO and Fe(II)-heme keeps the Hb in the ferrous state and eliminates problems from oxidation of the Fe(II)-heme iron to the non-functional Fe(III)-heme state that occurs in the presence of oxygen. (Vandegriff et al. 2008) HBOCs have been tested that are administered in the carbonmonoxy form (Sangart's MP4CO). The amount of CO that is present in one equivalent of the protein is so small that its release is not a problem with respect to the toxic effects of CO, which are associated with blocking the respiratory chain. In general, a CO-derived material is resistant to oxidation and CO will be released in circulation. The CO release may contribute to anti-inflammatory, anti-apoptotic and anti-proliferative effects that can be advantageous (Ryter and Otterbein 2004). The oxy-deoxy cycle of the material will be fully functional as the CO is released. In particular the benefits of utilizing CO-derivatives as HBOCs for clinical trials should have significant advantages in stabilizing the product before administration.

10.1.4 Nitric Oxide

Nitric oxide (NO) is an endogenous vasodilator that plays a critical role in smooth muscle relaxation (Bian and Murad 2003). NO binds to the same heme sites as does oxygen and it does so with a very high affinity. Once bound, it can oxidize the heme to produce metHb (Doherty et al. 1998). The process reduces the bioavailability of endothelium-derived NO and can lead to vasoconstriction and hypertension. Hb within red cells has limited interaction with NO because the unstirred layer surrounding the erythrocyte membrane forms a diffusional barrier between NO and Hb. Moreover, the intravascular laminar flow creates an RBC-free zone that consists only of plasma flowing along the endothelium (Liao et al. 1999). Outside the red cell, acellular Hb scavenges endothelial NO, primarily through its ability extravasate through the endothelium (Matheson et al. 2002;

Sampei et al. 2005; Cabrales et al. 2009). Had this been known early in the quest for an effective HBOC, designs that would avoid this problem would have been an essential feature of every approach, since many potential HBOCs were similar to extracellular Hb. Eventually, the observation of vasoactivity of potential HBOCs in clinical trials led to the ending of most trials (Natanson et al. 2008). The tight binding of NO to Fe(II) heme and its ability to oxidize the ferrous heme to the ferric state was seen as the key factor in understanding the vasoactivity of the HBOC candidates and the critical factor to be overcome in developing a safe and effective product. While oxygenation characteristics of any HBOC are certainly important for efficacy, interactions with NO can cause serious enough problems with safety that they must be overcome in any product. With respect to dealing with NO issues, the possibilities are (Kluger 2010) an Hb derivative that binds O_2 but not NO, (Lui and Kluger 2010) an Hb derivative that generates NO from other species in circulation (Perutz 1990), an Hb derivative that does not extravasate through the endothelium.

10.1.5 HBOCs from Chemical Modification of Haemoglobin

Early attempts at converting Hb to an HBOC started as standard exercises in protein stabilization. These involved using established chemical cross-linking reagents to produce linkages between protein side chains that prevent dissociation of the tetramer (Keipert et al. 1982). It is thus instructive to review design criteria that were considered prior to extensive clinical testing. Ideally, the reagent that is used to convert Hb into an HBOC should introduce chemical linkages only where they would be likely to prevent dissociation of the functional tetrameric ($\alpha\beta\alpha\beta$) protein into its nonfunctional constituent $\alpha\beta$-dimers, without introducing additional modifications that make the result heterogeneous. Logically, the resulting candidate for HBOC status should have oxygen binding properties that meet theoretical requirements that are based on known physiological parameters for circulating red cells. Initially, it was logically assumed that this could simply be based on an analogy to the oxygenation properties of whole blood ($p_{50} = 26$). As well, it is desirable to have analytical methods that enable the sites at which the protein had been modified to be identified. This would relate chemical modifications to critical functional properties.

Where a material has suitable stability and oxygenation properties, production on a large scale is necessary for *in vivo* pre-clinical testing of toxicity, metabolism, safety, and efficacy. Where these tests give desirable results, the reactions must be developed to be run on a larger scale in order to provide sufficient material for clinical testing. In addition, dealing appropriately with NO binding has to be achieved.

10.2 The Need for Chemical Modification

10.2.1 Preservation of Tetrameric Form and Function

As long as Hb is retained within the red cell it remains effective as an oxygen carrier. Outside the red cell native Hb is converted into its components by the liver. Its long-term effectiveness in circulation is due to its location *within* the red cell. The tetrameric form ($\alpha_2\beta_2$) is in equilibrium with its constituent $\alpha\beta$-dimers. The high concentration of the protein within the cell causes Hb to be maintained in the functional tetrameric state. As mentioned earlier, the presence of allosteric effectors within the cell also controls the extent of oxygenation. Most notably, 2,3-DPG enhances the ability of oxygenated Hb to release oxygen by preserving the low-affinity T-state structure of the protein (Benesch et al. 1972). In addition, enzymes (catalase and superoxide dismutase) within the cell catalyze the destruction of reactive oxygen species, such as peroxide and superoxide, that arise from the interaction of the ferrous heme and bound oxygen (D'Agnillo and Chang 1998). The inevitable auto-oxidation of the ferrous heme to the nonfunctional ferric state is reversed by metHb reductase (Huennekens et al. 1957).

In circulation outside a cell, Hb quickly becomes ineffective as an oxygen carrier for a variety of reasons: irreversible dissociation of the tetramer into $\alpha\beta$-dimers (Ackers and Halvorson 1974), oxidation to ferric metHb (metHb), and the absence of effectors. The lack of protection that is available from the cell membrane of red blood cells and the helper enzymes within causes the Hb-dimers not only to be ineffective as oxygen carriers, the smaller species eventually become a source of physiological stress and organ damage. The haemoglobin subunit dimers are taken up through circulation within the liver, forming a complex with haptoglobin (Ship et al. 2005; Chow et al. 2008; Boretti et al. 2009). The complex is processed in the hepatosomes where the components of the protein are prepared to be recycled. If large amounts of free Hb (tetramers or dimers) are present, the hepatosomes become fully occupied and the liver is bypassed. The dimers are then excreted through the glomerulus of the kidneys (Keipert et al. 1982; Feola et al. 1988). This leads to haemoglobinuria and renal injury. Thus, at the very least, a successful HBOC would have to retain the tetrameric state of Hb. This serves to minimized the release of dimers and the harmful effects in the kidney (Winslow 2006). This can be achieved either by encapsulating Hb into a lipid membrane (Chap. 11), or by chemical modifications to the protein that maintain the functional tetramer-like structure.

10.2.2 Alterations to Oxygen-Binding Properties

Modifications that maintain Hb in a tetrameric form alter the physical properties of the protein as well as, the functional properties. The most obvious functional changes are in the oxygen binding properties (P_{50} and n_{50}) of the modified protein.

Thus, each modified Hb binds and releases oxygen differently. These changes to oxygen binding are readily assessed by measurements of oxygenation curves of pure components against oxygen tension (pO_2) while stability is followed in the visible absorption peaks of oxy- and deoxy-heme proteins as well as CD spectra.

A higher value of P_{50} for the modified protein compared to unmodified Hb indicates that the modification lowers the energy of binding to oxygen (by favoring the *T*-state to a greater extent), while a lowered P_{50} indicates a higher oxygen affinity (favoring the *R*-state). We can think of these effects as being the result of cross-linking that creates points of resistance to conformational change necessary for cooperativity in oxygen binding. The effect on affinity (P_{50}) is due to the linker preventing relaxation of the chains to a lower energy R-state (Kluger et al. 1996), while reduced Hill coefficients (n_{50}), result from disruption of the overall environment of the solution around the protein. Since the designated function of an HBOC is oxygen delivery, the efficiency of delivery will be the key factor in determining how much material is needed to replace red cells in their functional capacity. As the established use of banked red cells is to replace lost red cells, it is logical to do a straight replacement of what has been lost. On the other hand, Hb within an HBOC can be more or less effective compared to the same amount of Hb within a red cell and the amount to be used is more appropriately based on the efficacy of oxygen transport of the material as determined by the oxygenation parameters.

10.3 Approaches to Hb Modification for Use as HBOCs

A common set of reagents used for modification of Hb in general includes electrophiles that react with nucleophilic functional groups on side chains of amino acids (Jones et al. 1993; Kluger 1994; Kluger et al. 1994; Wodzinska and Kluger 2008) (Fig. 10.2). These side chain groups react with synthetically prepared electrophilic reagents that can be modified for specific modification of the protein.

10.3.1 Regiospecificity, Homogeneity/Heterogeneity

A reagent that is specific for a particular functional group on the protein will react usually give multiple products because the functional group will be at more than one site on each subunit. Reaction with such a reagents produces a highly heterogeneous mixture of altered proteins. In contrast, a reagent can be designed to react with limited subsets of a reactive functional group based on differences in local environments. With this added specificity, certain cross-linked Hb derivatives will be formed preferentially, creating at outcome that is potentially homogeneous or one that contains very few components. Another complication of a less

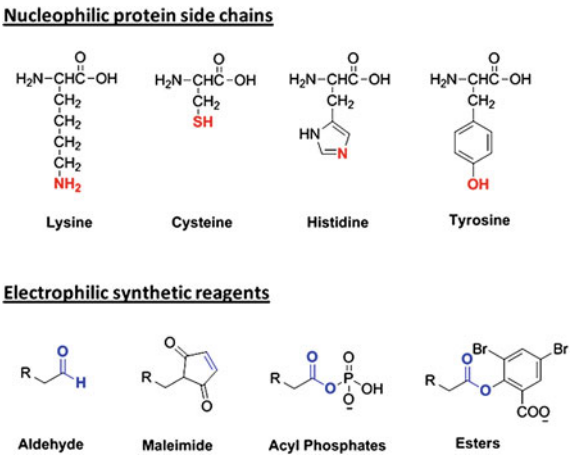

Fig. 10.2 The nucleophilic site of protein side chains (in *red*) react with electrophilic synthetic reagents (in *blue*) to create stable covalent bonds. Electrophilic synthetic reagents can either be general reagents that react with most protein side chains, or they can be chemically modified to include certain groups that contribute to site specificity

specific reagent is that reaction can continue and lead to uncontrolled polymeric derivatives (see Fig. 10.3).

The source of regiospecificity in chemical reactions is normally the result of the inherent chemical properties of the functional group that reacts and the functionality of nearest neighbors. In contrast, regiospecificity of a reaction within a protein is the result of chemical properties within the regional environment of the potential reaction sites (Jones et al. 1993; Keipert and Chang 1988; Martinek and Torchilin 1988). In this sense, a reagent that is regiospecific within a protein is described as being "site-directed" or "site-selective". In dealing with cross-linking, these reagents are typically bi-functionally reactive (reactive groups on both ends). In that case, regiospecificity is essential because two different parts of the reagent are reacting. If one end is site-selective and the other is not, either end may react first, limiting the possibilities for the second step.

10.3.2 Intra- and Inter-Molecular Linking

Cross-linking reagents are normally designed for *intra*-molecular linking (within Hb tetrameric subunits). Linking reagents are less common and are designed for *inter*-molecular linking (between multiple Hb tetramers). When Hb is modified selectively to give intra-molecular cross-linking the process requires a very selective reagent. The resulting chemically stabilized Hb tetramers ($\sim$64 kDa) are referred to as "cross-linked Hbs".

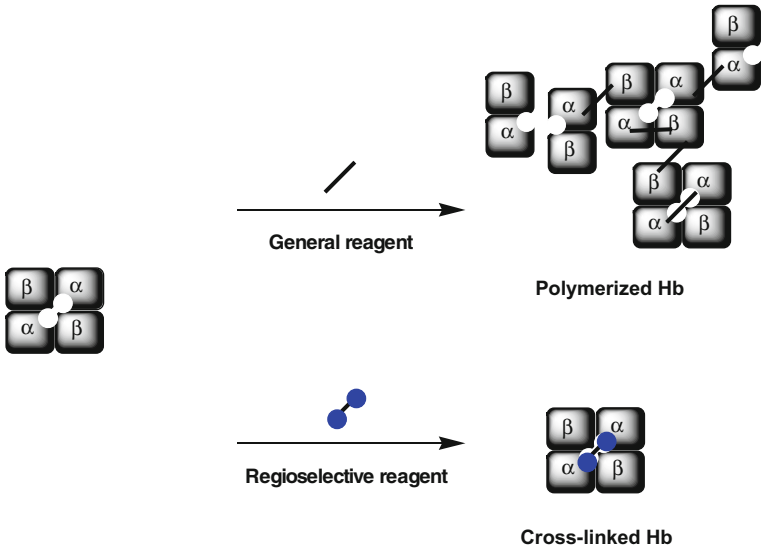

Fig. 10.3 A general chemical reagent does not discriminate between sites and will react with any surface accessible residue to create non-specific cross-links. Many polymerized Hbs are produced with general reagents. A regioselective reagent has chemical groups (depicted in *blue*) that can direct the cross-linker to a specific site, such as through an electrostatic interaction

10.4 Intra-Molecular Cross-Linking

10.4.1 The DPG-Binding Site and Chemical Reagents

One of the sites within Hb that has been targeted for site-selective reaction is the pocket within the Hb tetramer that binds the endogenous allosteric effector: 2,3-diphosphoglycerate (DPG) (Fig. 10.4). The negatively charged penta-anionic DPG is attracted electrostatically to the polycationic DPG-binding site on Hb, which contains multiple protonated amino groups derived from the side chains of lysine residues. The protons on the amino groups in the DPG-binding site are in dynamic equilibrium between the amino groups so that the amino groups are also available as reaction sites (Benesch and Benesch 1967; Benesch et al. 1972). Thus, the DPG-site-selectivity of anions can be used as the basis of an approach to making a cross-linking reagent site-specific. Since reagents commonly react as electrophiles, the cross-linking "war head" should be an anionic electrophile. Since anions are nucleophiles the two characteristics must be contained in adjacent functionalities. The electrostatic forces that direct DPG to its cationic binding site will then direct an anionic cross-linker to the DPG binding site. This ensures that the reagent reacts at a known site, rather than at multiple surface available sites.

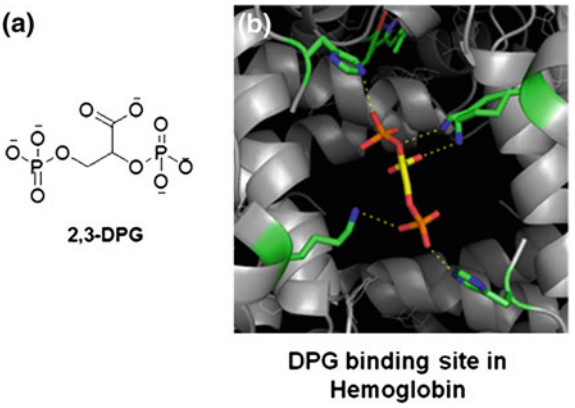

Fig. 10.4 **a** The natural allosteric effector 2,3-diphosphoglycerate (2,3-DPG) is a penta-anionic molecule that binds to **b** the polycationic DPG-binding site within Hb (Figure obtained using PyMOL v1.5 and PDB code 1B86)

10.4.2 αα-Cross-Linked Hb (DCLHb)

An early and impressive example of a site-specific modification being used to produce a pure cross-linked tetramer was based on observations by Klotz and coworkers. They observed that acetyl salicylic acid (ASA, aspirin), which is an anion and an electrophile in neutral solution, reacts only with amino groups in the DPG binding site of sickle Hb, explaining the anti-sickling effects of that drug (Klotz and Tam 1973) (Fig. 10.5). Further evaluation revealed that this selectivity by anionic aspirin derivatives for the DPG site in all Hbs is best achieved by using 3,5-dibromosalicylate (DBS) esters. Klotz and Walder showed in particular that the bifunctional 3,5-dibromosalicylate ester derivative of fumaric acid (bis(3,5-dibromosalicyl) fumarate, DBSF), can be a highly efficient and selective cross-linker (Snyder et al. 1987; Walder et al. 1979).

As noted above, polyanions bind selectively to the polycationic site that binds DPG in the red cell (Benesch et al. 1972). With the site occupied by polyanions, anionic cross-linkers are unable to bind and therefore do not react in competition. They are still able to react at the other end of the site with high selectivity for the ε-amino group of each α-99 lysine. Thus, the only product from the reaction of deoxyHb with DBSF in the presence of DPG or IHP has a fumaryl cross-link

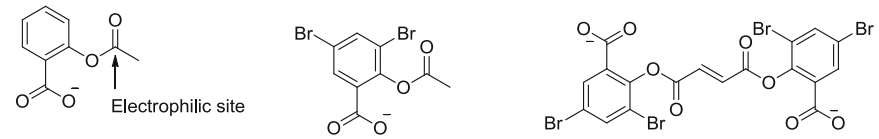

Fig. 10.5 Aspirin and its dibromo (DBS) anionic derivatives target the cationic DPG binding site within the *β*-subunits. The bifunctional reagent bis (3,5-dibromosalicyl fumarate), DBSF creates specific cross-links based on the reactivity of salicylates with Hb

between the α-99 lysine side chains (Snyder et al. 1987). This cross-linked Hb is αα-fumaryl Hb. A product from Baxter that has this structure was give the trade name DCLHb, an acronym for "Diaspirin cross-linked Hb". The similar publicly disclosed species was called ααHb and was studied extensively by Winslow (Vandegriff et al. 1988; Winslow 1989; Keipert et al. 1994). The cross-linked tetrame has a molecular weight of about 64 kDa, with $P_{50} = 32$ mmHg and $n_{50} = 2.6$. DCLHB was the product evaluated as an HBOC by Baxter laboratories (Winslow et al. 1988; Estep et al. 2008).

In order to understand the specificity and reactivity of the DBS groups with Hb, a systematic evaluation of chemical derivatives of the 3,5-dibromosalicylate leaving group was carried out by De Stefano and Kluger (Kluger and De Stefano 2000). This was done to understand how DBSF reacts within the DPG-binding site. The correlation of reactivity against the basicity of the leaving group in the reaction with propylamine (a mimic of a protein side chain) showed that 3,5-dibromosalicylates are much more reactive than predicted from their pK_A (approximately 15 times faster) based on other salicylates. In fact, in the reaction with Hb there is a further advantage in the reactions of 3,5-dibromosubstituted esters compared to the unhalogenated analogues. The yield of specifically modified Hb and the minimal level of hydrolysis make them superior to other derivatives that were tested. Examination of the structure of the reagents suggests that it is likely that the size and orientation of the bromine atoms provide a large steric bulk that directs the reaction to an amino group near the surface of the protein at the highly cationic DPG binding site. In addition, the adjacent carboxyl on the leaving group can also assist in promoting the nucleophilic amino group of lysine-82 of the *β*-subunit to become deprotonated to a larger extent by changes to the local polarity.

10.4.3 Cross-Linked Tetramers and Vasoactivity

Two explanations have been proposed for the toxic effects associated with administration of first generation HBOCs. One theory is that HBOCs elicit adverse effects, including vasoconstriction by scavenging nitric oxide (NO), the vasodilator produced by the endothelium. It had been established in the 1990s that the endothelial relaxation factor (ERF), the signal for vasodilation for relaxation of the muscle surrounding blood vessels is nitric oxide (NO) (Furchgott et al. 1992; Bian and Murad 2003). This shares metal-binding properties with oxygen and associates strongly with ferrous atoms within the hemes of Hb. A logical and consistent explanation of the problem is that the tested HBOCs, unlike red cells, are able to permeate the endothelium. The localized depletion of NO would be expected to block relaxation of blood vessels (Sampei et al. 2005). In a critical test of the possible sources of vasoactivity, Zapol and co-workers demonstrated that intravenous infusion of either murine tetrameric Hb or a well-studied HBOC induced prolonged systemic vasoconstriction in wild-type mice but not in mice congenitally deficient in endothelial nitric oxide synthase (NOS3) (Yu et al. 2008, 2009).

Mice that could not produce NO in their endothelium did not succumb to the vasoconstrictive effect of HBOCs. (Yu et al. 2008, 2010 Vaporidi et al. 2010).

Winslow and coworkers proposed that HBOCs with low oxygen affinity (high P_{50}) can release oxygen in systemic arterioles, an effect that can induce a vasoconstrictive homeostatic response that limits oxidative stress (Tsai et al. 2003). They concluded that an HBOC with high oxygen affinity (low P_{50}) will instead facilitate delivery of oxygen to sites in the capillaries with low oxygen levels. Originally, it was believed that an HBOC with oxygen affinity close to that of RBCs ($P_{50} = 28$ torr) will function effectively. However, it was proposed that the small size of the cross-linked Hb tetramer leads to a mode of oxygen transport that is different from that of RBCs, although the molecule carrying oxygen is essentially the same (Cole et al. 2008). The decreased red cell concentration near vessel walls result I a zone with no oxygen source and thus there is an increased distance for oxygen to diffuse to tissues from RBCs. In contrast, the small size of modified cell-free haemoglobin will allow oxygen to diffuse readily within the lumen, increasing lateral oxygen transport. This is the basis of "facilitated diffusion" that is mediated by small, highly diffusible HBOCs that increase lateral transport by acting as carrier proteins. Designs based on this hypothesis create HBOCs with higher oxygen affinity than red cells to avoid homeostatic responses and to promote movement of oxygen from RBCs to surrounding tissues. The higher affinity carrier attracts oxygen from the red cells and transports it to the endothelial area.

10.4.4 Acyl Phosphate Reagents

The regioselectivity demonstrated by the anionic acylating DBS has been cited by Klotz as the key to the functioning of salicylate derivatives. It follows that other anionic reagents that are negatively charged will share the same specificity for the DPG binding site in Hb. Manning proposed that members of another class of anionic acylation agents, acyl phosphate monoesters, would also be effective antisickling agents (Ueno et al. 1985, 1986; Ueno and Manning 1988). This was based on an earlier report by Tsui and Kluger that methyl acetyl phosphate is an effective anionic acylation agent (Kluger and Tsui 1980, 1986).

Ueno and Manning showed that methyl acetyl phosphate selectively acylates the amino groups of the β subunits within the DPG-binding site of HbA, making the specificity similar to that of the reaction of the 3,5-dibromosalicylates (Ueno et al. 1986) (Fig. 10.6). A method of producing a variety of cross-linkers based on acyl phosphate monoesters was developed from readily available bis-acid chlorides as precursors (Grant et al. 1988; Kluger et al. 1990). This permitted the construction of cross-linking derivatives with a defined site of reaction and defined cross-link spans by straightforward chemical reactions (Kluger et al. 1994, 1996). These reagents are efficient, water soluble, and react with a great degree of specificity with amino groups of the β-subunits in the DPG binding site. The acyl phosphate analogous to DBSF, FBMP, is shown below (Jones et al. 1993). FBMP

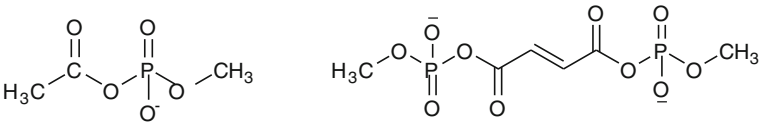

Fig. 10.6 Acyl phosphate groups such as the simple methyl acyl phosphate are also anionic directing groups that can react within the DPG-binding site of HbA. Fumaryl bismethyl phosphate (FBMP) is ab alternative anionic electrophilic reagent that can cross-link residues in the DPG-binding site of Hb, stabilizing its tetrameric state

reacts with deoxygenated Hb to produce cross-links between an α-amino group of β-Val-1 and the ε-amino group of β-Lys-82 as well as between the two β-Lys-82 amino groups.

10.4.5 Effects of Cross-Link on Oxygen Affinity

A surprising discovery was made during the evaluation of the oxygenation properties of the cross-linked products: the oxygen affinity of the resulting cross-linked protein, when the link is between the α-amino group of β-Val-1 and the ε-amino group of β-Lys-82, is directly related to the span of the cross-link. An inverse linear relationship between the free energy of oxygen binding (log P_{50}) and the cross-link span in the product. This was observed where the proteins were cross-linked between β-Val-1 of one chain and the β-Lys-82 of the other chain (Jones et al. 1993). In contrast, where the cross-link is between two β-Lys-82 sites, a positive correlation between P_{50} and bridge length is observed but the variation is small. Crystallographic analysis by Schumacher and Brennan showed that the cross-link prevents full relaxation upon oxygen binding, with longer links leading to more relaxation (Schumacher et al. 1995, 1997). These studies provide chemical knowledge required to design and adapt Hb to a desired P50.

10.4.6 Trifunctional Reagents: The "Spare Tire" Approach to Affinity and Conjugation

The reagents that have been used to react regioselectively with amino groups of the protein are either dibromosalicylate esters or acyl phosphate monoesters. While both react rapidly with amino groups of the protein, they also react with water in the protein environment. This is the main drawback of the ester-based reagents. For a bi-functional cross-linker, a complete protein cross-link requires reaction to occur at two sites on the cross-linker. If one is hydrolyzed by water it is

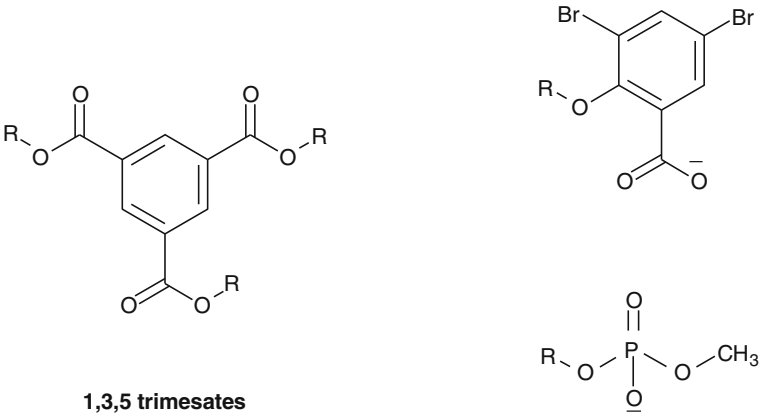

Fig. 10.7 Tri-functional reagents can be activated either with DBS groups or with acyl phosphates. Both reagents can react with haemoglobin, cross-linking the tetramer efficiently, and even leaving a third electrophilic site available for other reaction

no longer reactive; only one site can react and this effectively prevents formation of a cross-link. This leaves a protein in a state that can be readily dissociated into the undesirable dimers.

As a partial solution to the problem, we reasoned that reagents with more than two sites could produce a cross-link even after one hydrolysis has occurred. Our most effective approach utilized derivatives of 1,3,5-trimesic acid. Both the tris-acyl phosphate monoester (Wodzinska et al. 1991) and tris-dibromosalicylate esters of trimesic acid (Kluger et al. 1992) were prepared and both reacted with Hb to produce cross-linked protein with high efficiency (Fig. 10.7). Both materials provide an efficient route to efficient production of cross-linked Hb. In additional, the remaining ester can provide a site for conjugation and coupling (Kluger et al. 1992; Kluger and Song 1994). In fact, the remaining ester can be utilized to couple two Hb tetramers together to produce bis-tetramers.

10.5 Inter-Molecular Cross-Linking (Oligomers, Polymers and Bis-Tetramers)

As we noted earlier, the vasoactivity attributed to the circulation of cross-linked Hb tetramers suggested that they are small enough to extravasate through channels in the endothelium where they scavenge NO. Avoiding extravasation would effectively remove the scavenger from the area where NO resides. Since NO is a signal that is amplified by enhancing the reactivity of guanyl cyclase, the muscles surrounding a blood vessel would be sensitive to even small changes in concentration of NO (Murray et al. 1995; Gukovskaya and Pandol 1994). Based on this

proposition, it is reasonable to propose that a safe and effective HBOC must be significantly larger than an Hb tetramer.

Kim-Shapiro, Schechter and Gladwin have considered the localization of NO along the endothelium in connection with the ability of acellular Hb to induce vasoconstriction (Kim-Shapiro et al. 2006). They proposed that the key features that prevent Hb within red cells from scavenging NO are associated with circulatory flow properties that control proximity to the endothelium. The relatively small protein travels in several directions within the overall circulatory flow while cells are subject to central laminar flow. How much larger than an Hb tetramer does an HBOC have to be in order to have flow properties that minimize scavenging of NO? While this cannot be predicted, evidence suggests that species that are only about twice the size of the tetramer can fulfill the criterion.

10.5.1 Glutaraldehyde Polymerized Hb: Chemistry

In general protein modification, a reasonable place to start has been to use simple reagents that are known to react with protein side chains. Aldehydes been used as reaction sites for bi-functional and multi-functional reagents that combine with amino groups of the side chains of proteins (Habeeb 1967; Habeeb and Hiramoto 1968; Eike and Palmer 2004). Aldehydes react reversibly with amino groups, forming carbinolamines. These are unstable and lose water to produce the more stable imine (Schiff's base). In order for the product to be stable in circulation, the imine can be reduced to an amine by addition of an exogenous hydrogenation reagent. The best reducing reagent is chosen so that it does not otherwise affect the protein. Sodium borohydride as well as sodium cyanoborohydride and borane have been successfully implemented as reducing agents (Eike and Palmer 2004). While the reduction step adds complexity to the cross-linking process, it assures the permanent stability of the potential HBOC.

In considering aldehyde-based reagents for chemically stabilizing Hb tetramers, glutaraldehyde has typically been the reagent of choice. Glutaraldehyde had been widely used to keep multi-subunit proteins from dissociating, and once reduction with sodium cyanoborohydride is complete, provides functional products without creating toxic by-products (Habeeb 1967; Habeeb and Hiramoto 1968; Eike and Palmer 2004). Reactions of solutions of Hb with glutaraldehyde and subsequent reduction should produce materials that will not dissociate. In addition, during the cross-linking process, reactive anionic species additives were included in order to alter the P_{50} of Hb to be similar to that of red cells in blood ($P_{50} = 26$ torr). However, one important consideration that makes the use of the reagent less appealing is that the chemical structure of glutaraldehyde is much more complex than the name implies. Glutaraldehyde undergoes oligomerization to an extent that makes a reacting solution heterogeneous (Migneault et al. 2004) (Fig. 10.8). Furthermore, the reactions of the species in the mixture are also not regioselective within the protein.

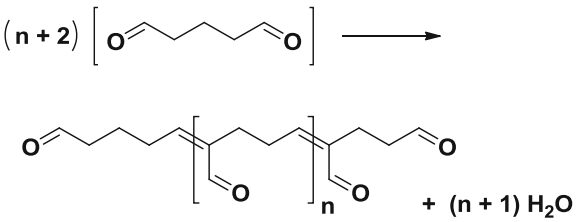

Fig. 10.8 The chemical reagent, glutaraldehyde, polymerizes with itself to produce oligomeric versions of the reagent. This makes the reagent impure, introducing chemical cross-links within the protein that are of different lengths

As a result, solutions of stabilized Hb from reactions with glutaraldehyde are considered heterogeneous (a mixture of different products) and a separation technique such as size-exclusion chromatography is required to remove species whose size is either too low (smaller than tetramers) or too large (oligomers of multiple tetramers). In this type of Hb modification, a mid-sized assembly meets the overall requirements. Hb modified by glutaraldehyde is not only heterogenous in size, the reaction sites are also heterogenous with cross-links between various residues, including lysines, cysteines, histidines, and tyrosines (Habeeb and Hiramoto 1968). An obvious problem with working with such mixtures is that their clinical properties are likely to differ. If there is a positive outcome, the useful components are mixed among others that are probably nonfunctional. In the event of an unacceptable clinical outcome, with such a mixture it would not be possible to know which component caused the difficulty—making improvements impossible from such a mixed product.

10.5.2 Glutaraldehyde Polymerized Hb: Clinical Studies

There were two commercial products previously in development that utilize glutaraldehyde polymerized bovine or human Hb. Both these products have been tested in clinical trials. These Hb polymers have a significant loss of allosteric cooperativity, and the modified Hb's p50 is typically right shifted and depends on the amount of glutaraldehyde used. The products derived from bovine Hb are Hemopure (HBOC-201), developed for human use, and Oxyglobin (HBOC-301) (BioPure, Cambridge, MA), developed for veterinary use. Both products are heterogenous mixtures of variously sized oligomers, ranging from 130 to 500 kDa (Jahr et al. 2008).

Hemopure (HBOC-201) has been extensively studied in multiple animal models and human clinical trials (Chen et al. 2009; Jahr et al. 2007; Pearce and Gawryl 2003). There are conflicting results that indicate that while vasoconstriction has been observed in a large number of studies in both animals and humans (Botzlar et al. 2002), likely resulting from depletion of NO concentrations after Hemopure infusion, other studies failed to observe vasoconstriction or a decrease

in NO concentration (Knudson et al. 2003). Nonetheless, the rapid formation of met-Hb (an indicator for Hb oxidation by interaction with free NO) was also observed in various animal and human settings (Jahr et al. 2008; Chen et al. 2009; Gould et al. 1992). In 2009, after a series of regulatory problems, Biopure filed for bankruptcy and its assets were purchased by OPK Biotech. Neither Hemopure nor Oxyglobin are currently available.

Northfield Laboratories Inc. (Evanston, IL) produced a glutaraldehyde-polymerized human Hb that is produced by reacting the Hb with pyridoxal 5′-phosphate before glutaraldehyde polymerization. This addition of pyridoxal 5′-phosphate, an allosteric analogue of 2,3-DPG, increases the p50 value from 18–22 to 28–30 mm Hg (Gould and Moss 1996; Gould et al. 1992).

10.5.3 O-Raffinose Polymerized Hb

A novel aldehyde-based reagent was developed by Hemosol Inc. for producing their HBOC "HemoLink" that was the subject of extensive clinical trials. The cross-linking agent, "O-Raffinose", is a polyaldehyde that is produced by periodate oxidation of raffinose, a trisaccharide (Hsia et al. 1992; Ali et al. 1997; Eike and Palmer 2004). The reaction of raffinose with periodate is shown in Fig. 10.9. The oxidation produces numerous aldehyde functional sites on the oxidized product (Lieberthal et al. 1999). The multiple reaction sites of the reagent mean that each reaction combination (reagent aldehyde reacting with a protein amino group) would give a unique product, leading to a complex array of products that are stabilized in the Hemosol process by reduction with a borane derivative.

10.5.4 PEG Conjugation

The conjugation of chains of polyethylene glycol to Hb is a straightforward process that leads to a large increase in the volume of the combined species (Nucci et al. 1996; Gombotz and Pettit 2000). The materials, generalized as "PEG-Hb",

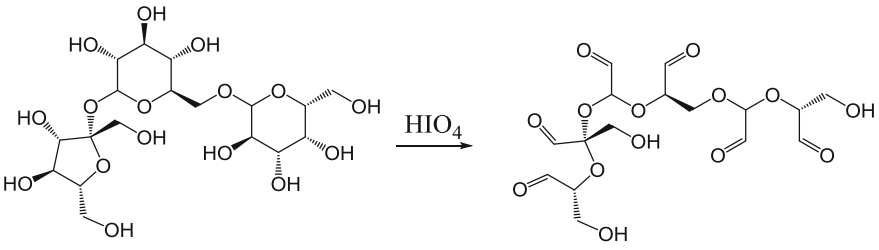

Fig. 10.9 Raffinose is oxidized with periodate to produce a material with a large number of reactive aldehyde groups

have been reported to elicit minimal vasoactivity (Vandegriff et al. 2003). A product with multiple PEG chains conjugated to Hb is the basis of Sangart's MP-4, a development initially led by Winslow and Vandegriff (Vandegriff et al. 2003). Acharya has provided a detailed overview of the preparation and functional properties of PEG-Hb conjugates (Hu et al. 2005). The size and shape necessary to avoid extravasation and scavenging of NO that is accomplished by adding PEG comes with a serious cost—the added weight provides no additional capacity to carry oxygen.

10.5.5 Cross-Linked Bis-Tetramers: Chemistry

The challenge to enlarge the protein while increasing oxygenation capacity in-proportion ideally could be achieved by connecting cross-linked tetramers to one another. The general approach is summarized in the Fig. 10.10.

Our first attempt to develop a reagent that would fit this strategy used a connector that is based on the chemical functionality of PEG, an oligomeric derivative of ethylene glycol. However, reaction of this material with Hb did not give a product that had more than one tetramer linked (Paal et al. 1996; Kluger et al. 1999). Instead, the connecting chain appeared to fold onto itself and all reactions were within the same tetramer. This could obviously be avoided if the connecting chain cannot fold.

Based on the hypothesis that an effective reagent must have a rigid connector between the reactive leaving groups, we prepared a material where the linker is derived from 5-amino-isophthalate as the protein reaction site and terephthalate as the connecting core (Kluger et al. 1999). The linker is then unable to fold and the reaction will produce a material that is a symmetrically connected assembly of two tetramers (Fig. 10.11). We refer to the resulting protein assembly in general as being a "bis-tetramer" to make clear its origins and general structure. Others have referred to such a structure an "octamer".

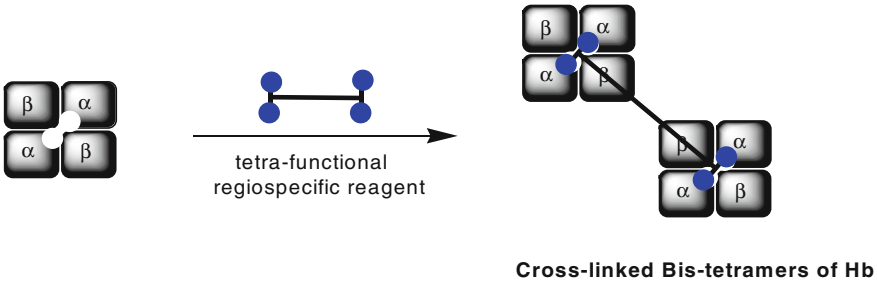

Fig. 10.10 A tetrafunctional cross-linker that is designed with regiospecific directing groups (depicted in *blue*) will react with two separate haemoglobin tetramers at a specific cavity, while tethering the two proteins together. These Hb derivatives are cross-linked bis-tetramers of haemoglobin

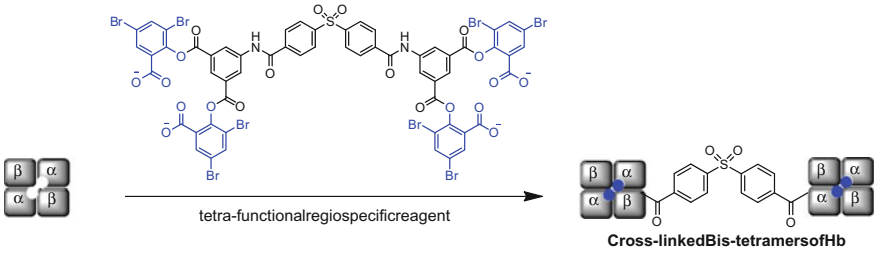

Fig. 10.11 Reaction of haemoglobin with a tetrafunctional cross-linker containing four DBS directing groups produces cross-linked bis-tetramers of haemoglobin

The initial haemoglobin bis-tetramers produced from this reaction proved to be site-specific and pure, but the physical properties were found to be unlikely to be those of a successful product, due to the relatively low coperativity in oxygen binding ($n_{50} \sim 1.8$). Using chemical modelling to visualize the reagent in a 3D space, we realized that replacing one sp^2 atom with an sp^3 along the main bridge would reduce the length of the span between tetramers, forcing interactions between side chains of the tetramers that could be manifested as an increase in cooperativity (Hu and Kluger 2008). The example below meets the structural criteria with $n = 2.7$.

Further extension of this coupling strategy can produce higher order assemblies of tetramers, essentially making polymers of Hb. The cross-linked ester that results from reaction of Hb with trimesoyl tris(3,5-dibromo salicylate, TTDS) can react with multifunctional nucleophiles to give dendrimeric arrays, giving larger products (Kluger and Zhang 2003). Another approach is to add PEG chains to the bis-tetramer to further increase the size (Lui and Kluger 2009).

10.5.6 Cross-Linked Bis-Tetramers: Pre-clinical Studies

The Zapol group has recently evaluated the potential of an Hb bis-tetramer (BT) and its PEGylated derivative (BT-PEG) as HBOCs (Lui et al. 2012). They studied the effects of administration of both materials on the blood pressure of normal and diabetic mice. The latter have endothelial dysfunction and are particularly sensitive to scavenging endogenous levels of nitric oxide. The diabetic mice thus serve as a sensitized model to evaluate the hemodynamic effects of NO-scavenging by HBOCs. In their report they compare the systemic vasoconstrictor effects of both bis-tetramers (BT) and PEGylated bis-tetramers (BT-PEG) in awake and anesthetized mice and find that systemic vasoconstriction is not produced by these compounds (as compared to injections of murine Hb). Also, infusion into diabetic db/db mice exhibiting endothelial dysfunction demonstrate that infusion of either BT or BT-PEG does not alter systemic blood pressure. Since a major drawback of traditional HBOCs are their vasoconstrictive effects (Ryter and Otterbein 2004;

Jahr et al. 2007; Buehler and Alayash 2004), this pre-clinical study demonstrates that BT-PEG may have many of the necessary qualities required for producing a safer and functional oxygen carrier.

10.5.7 CuAAC Coupling

The preliminary success of Hb bis-tetramers in pre-clinical animal models has prompted us to develop chemical strategies that improve the production yield of Hb bis-tetramers. An alternative strategy to creating Hb bis-tetramers is to combine the cross-linking reaction with a highly efficient bio-orthogonal coupling reaction. A bio-orthogonal reaction utilized chemical groups that are not found in biological systems and must be synthetically prepared. The two bio-orthogonal groups seek each other out within a biological media, and react specifically with each other. In a first attempt we employed the use of the highly effective copper-catalyzed azide-alkyne coupling. First, haemoglobin must be activated with an azide, one of the bio-orthogonal groups. This can be achieved easily by incoporating the azide *into* the chemical cross-linking reagent (Azide-Hb, Fig. 10.12). Haemoglobin is then activated with the azide and cross-linked in a single step (Buehler and Alayash 2004). A bi-functional linker containing two alkyne groups (the complementary bio-orthogonal groups) is then chemically synthesized and added into the reaction system (bis-alkyne, Fig. 10.12). In the presence of Cu(I), the azide reacts in a specific and rapid cycloaddtion reaction with bis-alkyne to form triazole rings that is made up of the sum of the two reactants (Foot et al. 2009). In order to form

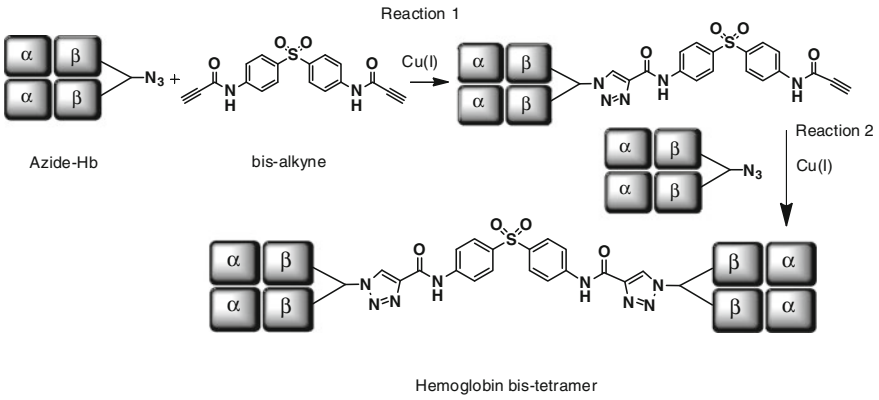

Fig. 10.12 Using bio-orthogonal chemistry to introduce couple two haemoglobin tetramers together. Azide activated cross-linked haemoglobins (Azide-Hb) react with a bifunctional alkyne (bis-alkyne) through a copper catalyzed "click" reaction (Lutz and Zarafshani 2008). The initial coupling (Reaction 1) is slow due to the insolubility of the bis-alkyne. Once the first coupled product is formed, the second coupling step (Reaction 2) is much faster, producing haemoglobin bis-tetramers

a bis-tetramer, cross-linked tetramers containing the azide must react sequentially with the two alkyne groups of a bis alkyne. This can only work if the second reaction (Reaction 2, Fig. 10.12) is faster than the first (Reaction 1, Fig. 10.12). However, one might initially presume that there is no reason for any distinction between the two steps—all the initial azide could react with the bis-alkyne before a second reaction occurs, leaving the final product to be that from Reaction 1.

Instead, since the rate of a reaction depends on the concentrations of the reactants in the same phase, when the reaction is carried out in water, the bis-alkyne has very low solubility and reacts relatively slowly at the interface of the solid with water. In contrast, after the first reaction, the intermediate alkyne is attached to Hb and this alkyne is therefore present at a much higher concentration in the aqueous phase than is the bis-alkyne reagent because of its low solubility. We have now optimized this bio-orthogonal approach to producing Hb bis-tetramers (Foot et al. 2009; Kluger et al. 2010; Yang and Kluger 2010), and shown that it is possible to get Hb bis-tetramers with up to 50 % final yields. Given the benign hemodynamic effects of the first generation Hb bis-tetramers, we are optimistic that continued research in this area of Hb coupling will guide us towards developing new HBOCs that are safe to use.

10.6 Conclusions

Selective and efficient chemical reactions can be used to produce stabilized Hb tetramers as HBOCs in highly pure quantities. However the adverse clinical observations of many of the tested HBOCs indicated that these species will be not be suitable for administration into patients. New chemical procedures of enlarging the protein complexes while retaining specific modifications may be the first step towards developing new HBOCs that are safe and functional. In particular, the addition of PEG chains and the formation of assemblies of tetramers using more complex but efficient reactions has produced materials that have shown promise of being both safe and effective based on studies with animals. The possibility of the long-sought benefits projected for HBOCs should remain a valid prospect as the properties of these and other materials based on expanded knowledge of the complexities of the challenge are revealed and solved.

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