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Development of "Inside-Out" Pegylated Crosslinked Hemoglobin Polymers: Novel Hemoglobin-Based Oxygen Carriers (HBOC)

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DEVELOPMENT OF "INSIDE-OUT" PEGYLATED CROSSLINKED HEMOGLOBIN POLYMERS: NOVEL HEMOGLOBIN-BASED OXYGEN CARRIERS (HBOC)

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To my family and friends

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CHAPTER I

INTRODUCTION

Overview

The development of a blood substitute is a significant medical need as blood shortages have become more common in this current decade and pathogens, such as HIV, pose risks for blood transfusions, limiting the amount of blood available in some parts of the world. Due to the world's demand for blood, the development of hemoglobin-based oxygen carriers (HBOCs) has been an active area of research the last thirty years. An ideal oxygen carrier would deliver oxygen, be disease free, lack immunosuppressive effects, would not need blood typing, be available at a reasonable cost, and have minimal, if any, side effects (Ness and Cushing 2007; Sakai et al. 2000).

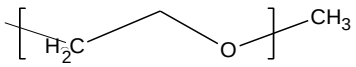
The difficulties in producing an ideal oxygen carrier result from complications affiliated with the effects of HBOCs once administered during clinical trials. During the past decade, approaches to develop a HBOC utilizing polyethylene glycol (PEG) have been frequently used. Conjugation of PEG chains onto proteins has been a growing area of research due to the ability of PEGylated proteins to improve therapeutic potentials by camouflaging the proteins from the immune system. PEG also reduces glomerular filtration of the complexes from the body

(Bailon and Berthold 1998; Harris and Chess 2003; Roberts, Bentley, Harris 2002). PEGylation has been used in HBOCs to counteract some of the unfavorable side effects, such as low retention times of HBOCs *in vivo*, NO scavenging of the Hb, and the vasoconstrictive side-effects due to NO scavenging (Doherty et al. 1998; Muldoon et al. 1996). Several PEG-HBOCs have reached clinical trials, but none have been approved for use in the United States. This review chapter outlines current statuses of the development of PEGylated Hb compounds and suggests some potential modifications that might improve their ability as potential HBOCs.

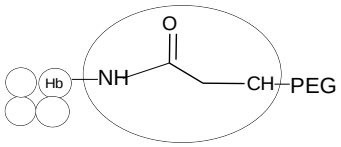
Sites of PEGylation and Variations of PEG

Attaching PEG chains onto a protein can be accomplished by an assortment of reactions. Different linking groups on the PEG molecule can achieve PEGylation at a variety of amino acid residues within the Hb tetramer. The first attempt at the PEGylation of Hb was done via urethane likages (Fig. 1) by Enzon resulting in 10 copies of PEG-5K chains conjugated onto a single bovine Hb tetramer (Acharya et al. 2011). In order to simplify the PEGylation method and produce a more cost effective protocol, Extension Arm Facilitated (EAF) PEGylation was developed at Albert Einstein College of Medicine by Acharya and coworkers (Acharya and and Manjula 2006; Manjula et al. 2005). This form of PEGylation involves the thiolation of the tetramer using a heterobifunctional reagent to produce a thiol functional group that is later used to attach PEG chains onto the desired protein using a complementary functionalized PEG (Acharya et al. 2011; Acharya and and Manjula

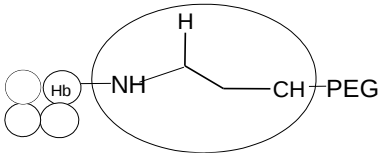
2006; Manjula et al. 2005). EAF-PEG has little impact on the Hb tetramer stability of the PEG-Hb product, in direct contrast from direct PEGylation that destabilizes the quaternary structure of the PEG-Hb tetramer (Manjula et al. 2005). HexaPEGylated Hb's generated by direct PEGylation exist primarily as Hb dimers, and the same was found with EAF-PEGylation (Hu et al. 2007; Li et al. 2008). Various types of EAF-PEGylation techniques have emerged, including the use of propionamide, propylamine, and thiosuccinimido phenyl linkages (Fig 1.) (Li et al. 2008). This list is not exclusive since the production of a thiol functional group can be created in numerous ways, but Figure 1 includes some of the common techniques currently employed for Hb PEGylation.



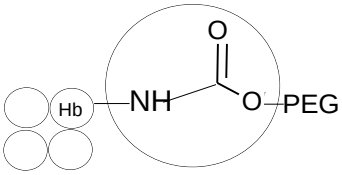
PEG (Polyethylene Glycol)



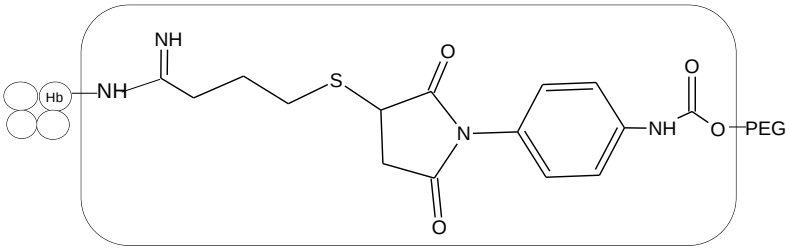
Propionamide Linkage [(Proionly-PEG5K)$_6$-Hb]



Propylamine Linkage [(Propyl-PEG5K)$_6$-Hb]



Urethane Linkage (Enzon DecaPEGylated Hb)



Thiosuccinimido Phenyl Linkage [(SP-PEG5K)$_6$-Hb]

Figure 1. Schematic representation of the linkages in the PEGylated Hbs generated by EAF PEGylation, acylation chemistry and reductive alkylation mediated PEGylation shown by Li et al. The abbreviation "PEG" can be of varying lengths and a general schematic is listed first. For the EAF PEG linkers listed above, the linker type is first followed by its application (listed in parentheses or brackets)(Li et al. 2008).

The common sites of PEGylation on Hb are on various Lys residues modified into thiol groups using Traut's reagent. Li *et al*. determined the frequency of modification (Table 1). The modified lysines were: Lys-8 (β), Lys-11 (α), Lys-56 (α), Lys-90 (α), Lys-95 (β), and Lys-132 (β) (Li et al. 2008). In addition, the free amino groups of the N-terminal Val residues of both chains were also PEGylated after reaction with Traut's reagent (Li et al. 2008). PEGylation at these N-terminal sites destabilized the Hb tetramer (Hu et al. 2012). This may be attributed to the insertion of the PEG into the αβ dimer interface, dissociating the tetramer (Li et al. 2008).

Table 1. Identification of the sites of PEGylation in PEGylated Hb with corresponding P_{50} and Hill Coefficient (n) values as determined by Li et al. (Li et al. 2008).

Residues	Percent Modification in PEGylated Hbs **(P_{50}/n)**		
	HexaPEGylated Hb **(10.8/1.9)**	OctaPEGylated Hb **(8.5/2.1)**	DecaPEGylated Hb **(8.0/2.0)**
Val-1(α)	48	80	100
Val-1(β)	36	48	64
Lys-8(β)	27	33	45
Lys-11(α)	37	46	56
Lys-56(α)	44	56	67
Lys-90(α)	38	42	54
Lys-95(β)	20	31	58
Lys-132(β)	35	44	57

*All PEGylated Hbs were modified via Propionyl-PEG 5kDa

*P_{50} values are reported in mm Hg; n, Hill Coefficient

In addition to the N-terminal Val residues, there are other sites on the Hb tetramer that should be avoided during PEGylation. Conjugation of PEG on Cys-93 (β) has been studied to determine the effect on Hb functionality. Li and coworkers

demonstrated that Cys-93 (β) could be reversibly protected using 4,4-dithiodipyridine (4-PDS) (Li et al. 2009). Consequently, EAF-PEG complexes were made with both protected and unprotected Hb's (Figure 2) (Li et al. 2009; Wang et al. 2014). Compared to native Hb, PEGylation at the Cys-93 (β) residue produced a higher oxygen affinity, a lower Hill coefficient, an increased autoxidation rate, and a greater rate loss of the heme group (Hu et al. 2008; Li et al. 2009; Manjula et al. 2003). Further oxygen binding studies by Li, Acharya, and colleagues, however, determined that avoiding PEGylation of Cys-93 (β) had a limited influence on oxygen affinity if the Hb was previously modified to keep it in a high oxygen affinity state or was unmodified entirely. The increased oxygen affinity observed in EAF-PEG Hb can be a consequence of Hb PEGylation at other sites, rather than modifications at Cys-93 (β). However, modification of the Cys-93 (β) residue does result in a high oxygen affinity hemoglobin (Abraham, Phillips, Kennedy 1983; Li et al. 2009).

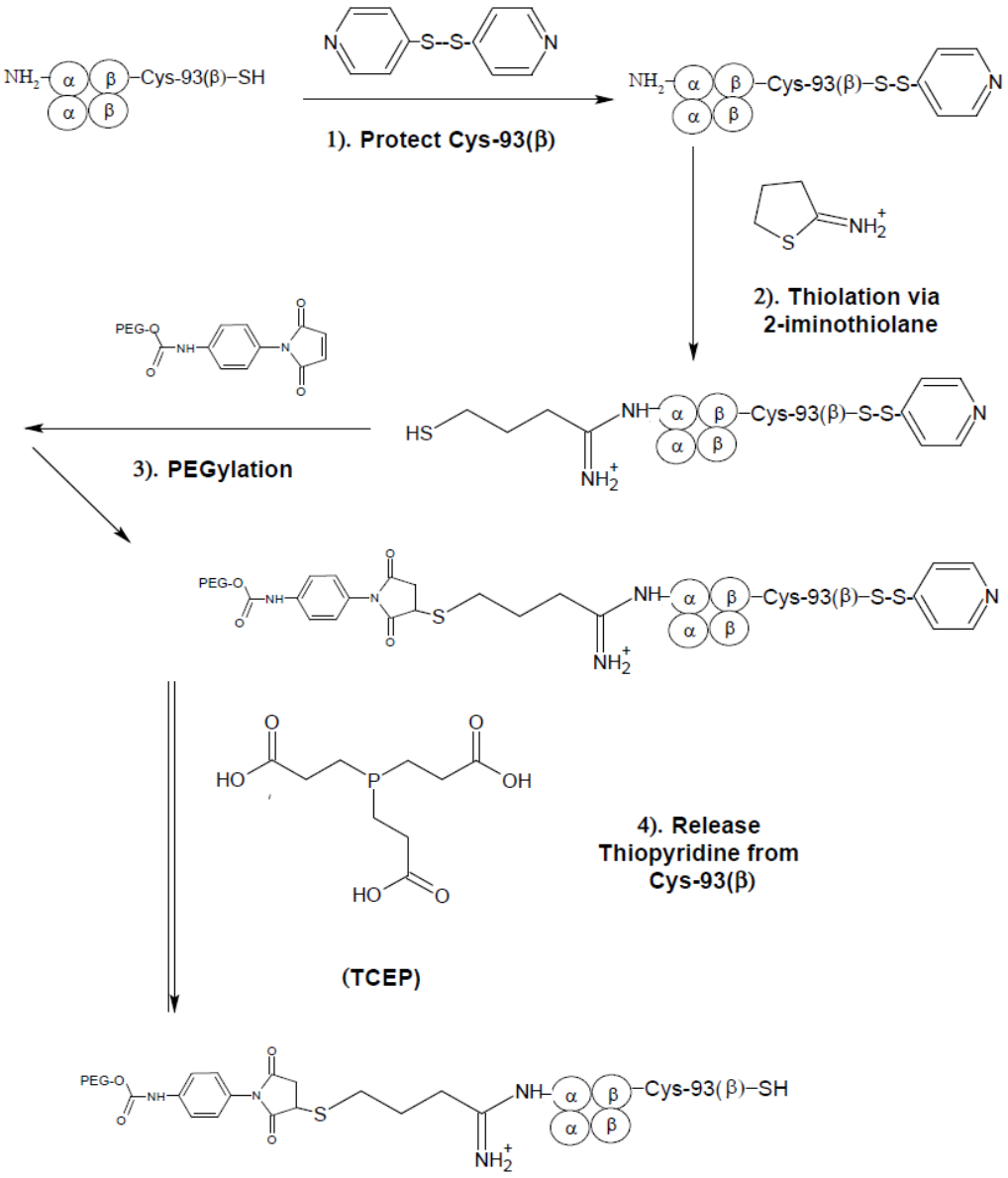


Figure 2. Schematic representation of the extension arm facilitated PEGylation of Hb with reversible protection of Cys-93 (β) performed by Li *et al*. It should be noted that PEGylation is shown on an alpha subunit of Hb but can occur on a subunit with an exposed Lys residue (Hu et al. 2012).

PEGylation on the Cys-93 (β) residues might be advantageous as it reduces the ability of Hb to scavenge NO *in vivo*. Kluger and co-workers found that PEGylated Hb created by this site specific modification produced NO at the heme faster than that of the native hemoglobin or non-PEGylated T state crosslinked hemoglobin (Lui, Dong, Kluger 2008). They proposed that the higher NO production was resultant from an increased stability of R state hemoglobin produced by PEGylation (Lui, Dong, Kluger 2008). The number of PEG chains attached to the hemoglobin tetramer did not appear to correlate with heme-nitrite reactivity so long as one PEG chain is conjugated specifically to the Cys-93 (β) residue. It was also found that heme-nitrite reactions within Hb conjugated with two PEG chains were comparable to Hb modified with six PEG chains (Lui, Dong, Kluger 2008). Lui et al. used a tetra-functional crosslinker to link two hemoglobin tetramers together for stability, followed by PEGylation on the Cys-93 (β) residues on each hemoglobin tetramer. This resulting product was found to be non-hypertensive when administered into mouse models (Lui et al. 2012).

Number of PEG Chains Attached to Hemoglobin and the Resulting Size Modification

The residues on which Hb can be PEGylated have been identified, but this does not imply that all the sites are PEGylated during every procedure. Depending on the reagent used, Hb can be PEGylated between two to ten sites (Li et al. 2008). Acharya and co-workers found that a twenty, forty, or eighty fold excess of

Propionyl PEG to Hb resulted in Hexa-, Octa-, or Deca-PEGylated Hb, respectively (Li et al. 2008). Enzon created this DecaPEGylated bovine Hb (Acharya et al. 2011), which was one of the first attempts at a PEG-Hb nanoparticle. Further studies and the development of thiolation-mediated EAF-PEGylation produced a Hexa-PEGylated oxy-Hb. In doing so, it was found that this was an optimal PEGylation reaction since all of the Hb tetramers were fully PEGylated (Acharya, Manjula, Smith 1996; Manjula et al. 2005). Table 1 shows the percentage of PEGylation at specific residues per Hb tetramer for the Hexa-, Octa-, or Deca-PEGylation species. The P_{50} and n values for each PEGylated Hb (Table 1) show that while it is possible to produce a higher level of PEGylation on Hb, it is not necessary since the oxygen binding properties did not change significantly (Li et al. 2007).

In addition to controlling the quantity of PEG chains attached to the protein, the length of the chains can also vary. Acharya and colleagues investigated PEG chains of weights in the 2-20 kDa range (Acharya et al. 2011; Hu et al. 2008; Li et al. 2008). The most commonly used PEG weight is 5 kDa, but it has not been elucidated that it is more advantageous than the 3 kDa PEG for usage in a blood substitute (Vandegriff and Winslow 2009). PEGylated proteins exhibit an apparent molecular mass that is higher than the molecular weights of the protein and PEG chains combined. This is a result of the PEG chains creating a hydration shell around the protein that increases the hydrodynamic volume (Portoeroe et al. 2008; Vandegriff et al. 2003). The hydrodynamic volumes of various HexaPEGylated-Hbs have been

determined by size exclusion chromatography (SEC) coupled with dynamic light scattering (DLS) (Li et al. 2008). Compared to native Hb (Table 2), PEGylated Hb showed a volume increase from eight to twelve fold, depending on the PEG decoration attached (Li et al. 2008). PEG chains create a shell around the Hb tetramer, increasing the particle volume (Figure 3). This is of relevance to blood substitutes because an increased volume should counteract the NO scavenging and reduce vasoconstriction (Palmer and Intaglietta 2014). Cooper proposed an increased hydrodynamic volume as a protection mechanism against NO scavenging, otherwise made permissible by a HBOC's small size. A smaller total volume HBOC enables the tetramer to move closer to the arterial wall, where NO can be scavenged, leading to vasoconstriction (Cooper 2009). A total PEG mass of around 30 kDa may not suffice to counteract vasoactivity of a PEG-Hb polymer but a total mass of around 50 kDa may be sufficient to decrease NO scavenging (Rohlfs et al. 1998).

Table 2. Influence of intramolecular α crosslinking on hydrodynamic volume, oxygen affinity and Hill Coefficients of PEGylated Hbs (Hu et al. 2007; Li et al. 2008).

Sample	Volume (nm^3)	P_{50} (mm Hg)	Hill Coefficient (n)
HbA	125	13.5	2.8
αα-fumaryl Hb	125	30.5	2.4
$(Propionyl\text{-}PEG5K)_6$-Hb	860	10.0	1.9
$(Propionyl\text{-}PEG5K)_6$- αα-Hb	1375	18.0	1.5
$(Propyl\text{-}PEG5K)_6$-Hb	659	6.3	1.9
$(Propyl\text{-}PEG5K)_6$- αα-Hb	1204	16.0	1.5
$(SP\text{-}PEG5K)_6$-Hb	950	7.0	2.2
$(SP\text{-}PEG5K)_6$- αα-Hb	1047	9.9	1.7

*P_{50} values are reported in mm Hg; n, Hill Coefficient

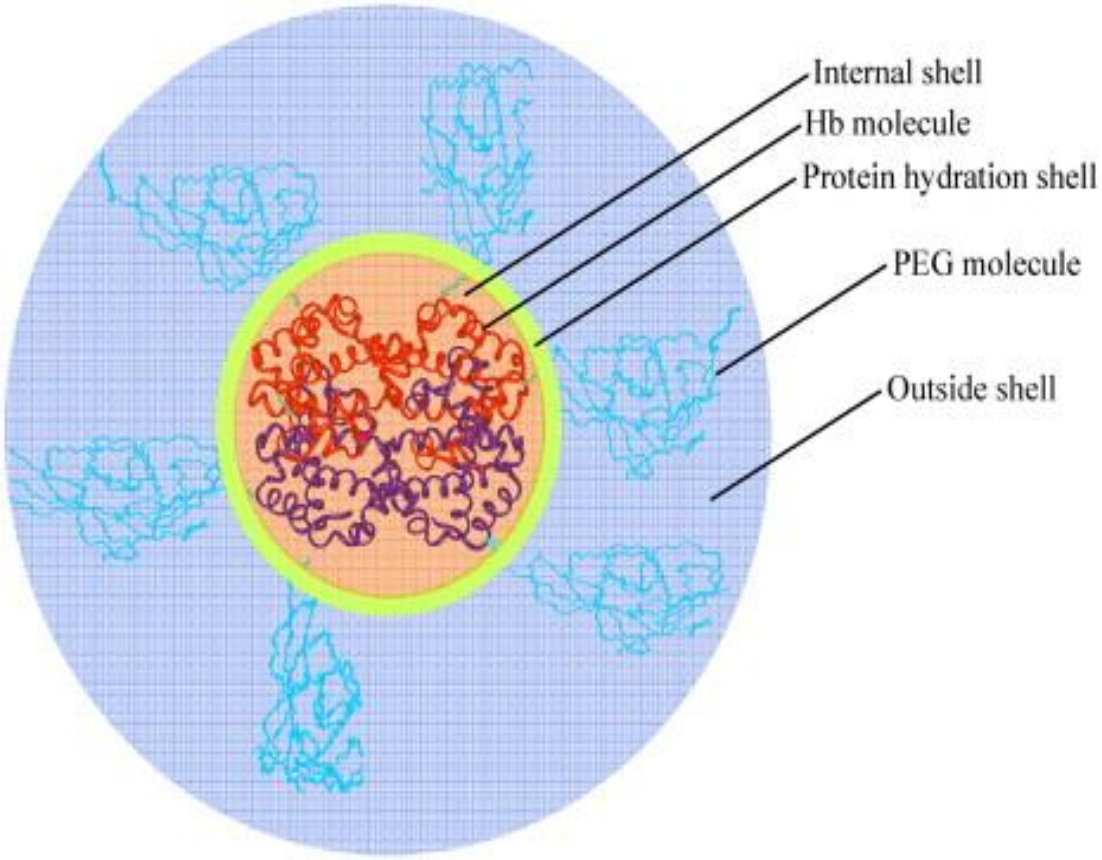


Figure 3. A pictorial representation of hexaPEGylated αα-fumaryl Hb. It has an internal protein shell with a molecular diameter of 6 nm and an outside shell with a molecular diameter of 14 nm. An intermediate protein hydration layer is sandwiched between the two shells. The thickness of this layer and the electrostatic interactions within this layer are expected to be dictated by the chemistry and site selectivity of the PEGylation. The two regions of the molecule differ significantly in the density. The outside PEG shell of medium density is filled loosely with PEG molecules (Li et al. 2008).

Analysis of PEGylated Crosslinked Hemoglobin

One of the first attempts at creating a HBOC was to use stroma free Hb that had been crosslinked in order to maintain the Hb tetramer integrity. Unstable stroma free Hb can dissociate into αβ-dimers that are small enough to be excreted

by the kidney (Bunn, Esham, Bull 1969; Chan et al. 2000). Bis(3,5 dibromosalicyl) fumarate (DBSF) crosslinks ε-amino groups of Lys82β_1 and Lys82β_2 under oxy conditions, producing a Hb with high oxygen affinity and increased stability (Fig. 4) (Walder et al. 1979; Walder, Walder, Arnone 1980; White and Olsen 1987; Yang and Olsen 1991; Zaugg et al. 1980). If this same reaction is done with deoxy-Hb, crosslinking occurs between Lys99α_1 and Lys99α_2 residues. This product exhibits a low oxygen affinity but increased stability (Fig. 4) (Chatterjee et al. 1986; Highsmith et al. 1997; Snyder et al. 1987; Tye et al. 1983; Yang and Olsen 1991).

Crosslinking demonstrates an ability to stabilize the Hb tetramer. When PEGylation procedures were first developed, the stability of the Hb tetramer was a concern. Initially, PEG conjugation, not EAF-PEG conjugation, occupied the center of the Hb tetramer and displaced the water molecules within the central cavity between the Hb αβ-dimers (Caccia et al. 2009; Hu et al. 2007; Hu et al. 2009; Hu et al. 2011). As a result, the Hb became less stable and split into two αβ-dimers. This dissociative effect could be minimized by cross-linking the tetramer prior to PEGylation (White and Olsen 1987).

From a structural point of view, it was suggested that upon crosslinking, stress originally present at the α1β1 interface induced by PEGylation was significantly reduced in comparison to non-crosslinked PEGylated hemoglobin (Hu et al. 2007). It was also noted that the heme environment was slightly perturbed when crosslinking and PEGylation were not used in combination. It is thought that

heme agitation in PEGylated αα-fumaryl crosslinked hemoglobin is significantly reduced as a result of an increased stability between the αβ interface (Hu et al. 2007). Winslow, Acharya, and their respective laboratories found that (propyl-PEG5K)$_6$-αα-Hb compared to (propyl-PEG5K)$_6$-Hb exhibited a significantly enhanced molecular volume and a lower colloidal osmotic pressure (COP) (Hu et al. 2007; Winslow 2003). This lowered COP makes it possible to use a HBOC solution of higher concentrations in clinical trials, which allows for a higher rate of oxygen delivery to tissues in the body that addressed an early limitation of PEGylated hemoglobins (Hu et al. 2007) . It has been found that PEGylation, in conjunction with crosslinking regardless of oxy or deoxy conditions, increases hemoglobin's affinity for oxygen, creating a situation that favors oxygen delivery to hypoxic tissue. In summary, PEGylation and crosslinking should not be mutually exclusive techniques in the production of a HBOC. Rather, they should be used in conjunction to counteract some of the unfavorable side effects produced by the individual techniques.

The Effects of PEGylation on Hemoglobin's Oxygen Affinity/P_{50} and Cooperativity

A major challenge in HBOC production has been adjusting the oxygen binding properties. Within a red blood cell, a homeostatic environment allows hemoglobin to maintain its allosteric cooperativity. This allows the RBC to release its bound oxygen efficiently in peripheral tissues. A P_{50} of 26 mm Hg is established by pH

control inside the RBC along with the presence of 2,3-bisphosphoglycerate (2,3-BPG). This results in Hb's characteristic sigmoidal oxygen binding curve. A problem that arises is that most HBOC production utilizes stroma free Hb. Once the protein is extracted from the RBC, the P_{50} value drops from 26 mm Hg to around 13.5 mm Hg (Tables 2 and 3) (Hu et al. 2007; Li et al. 2008; Li et al. 2009; Meng et al. 2014). As a result, variations in crosslinking procedures, in conjunction with variations of EAF-PEG chains conjugated to the protein, have been studied in attempts to circumvent the dramatic decrease in the P_{50} value.

Using DBSF as a crosslinker under oxy-conditions links the β-monomers, favoring the R state conformation (Fig. 4). This reaction at the Lys-82(β) residues results in a Hb with higher oxygen affinity (Table 3). However, crosslinking with the same reagent under deoxy-conditions creates a lower oxygen affinity Hb (Table 3) by linking the α subunits, favoring the T state conformation (Figure 4). PEGylation always increases the oxygen affinity of Hb (Table 3), whether it is stroma free Hb, β crosslinked Hb, or α crosslinked Hb (Hu et al. 2007; Li et al. 2008; Li et al. 2009; Meng et al. 2014). The type of EAF linker used does affect the oxygen affinity. For example, α crosslinked Hb modified with six propionyl PEG5K chains has a P_{50} of 22.8 mm Hg whereas α crosslinked Hb modified with six propyl PEG5K chains has a P_{50} of 16.0 mm Hg (Highsmith et al. 1997; Hu et al. 2007). Protecting the Cys-93(β) residue allows production of a modified Hb tetramer with a lower oxygen affinity (Table 3). However, the effects of PEGylation on the tetramer still produce a higher

oxygen affinity Hb. This is resultant from the Cys-93(β) residue being exposed instead of buried in the Hb tetramer in the R state. Thus, any modifications on this site increase the oxygen affinity of the Hb tetramer by keeping it predominantly in the R state (Abraham, Phillips, Kennedy 1983).

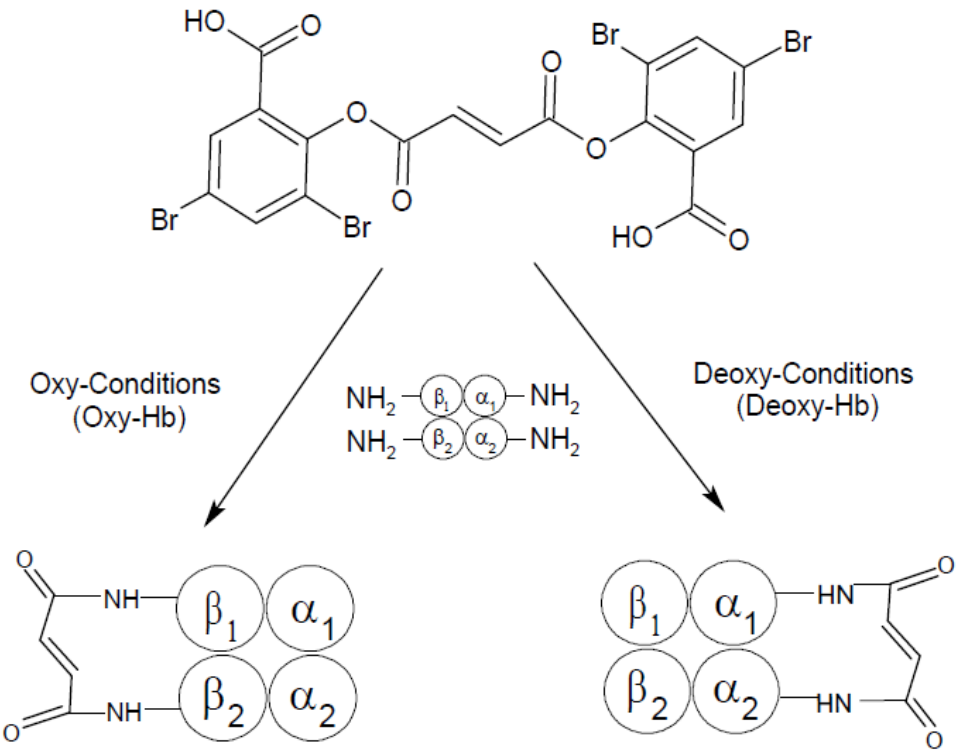


Figure 4. This is a standard crosslinking reaction that utilizes DBSF (a fumaryl crosslinker) to modify Hb. Under oxy-conditions (β crosslinking) crosslinking occurs between the two β99 lysine residues and under deoxy-conditions (α crosslinking) crosslinking occurs between the two α82 lysine residues.

*It should be noted that crosslinking occurs within the central cavity of the Hb tetramer.

Along with altering the P_{50} values, crosslinking and PEGylation play a role in shifting the Hill coefficient (n). Hemoglobin, whether within RBCs or stroma free, has a Hill value around 2.8, but once modified, this value decreases, indicating a decrease in cooperativity (Table 3)(Hu et al. 2007; Li et al. 2008; Li et al. 2009; Meng et al. 2014). Upon PEGylation, the Hill coefficients of all forms of Hb decrease further. Crosslinking and PEGylation restrict the movement of the Hb tetramer, relative to unmodified Hb, decreasing its cooperativity.

Table 3. Influence of intramolecular crosslinking (α, and β) and EAF PEG styles on oxygen affinity and Hill Coefficients (n) (Hu et al. 2007; Li et al. 2008; Meng et al. 2014; Wang et al. 2014).

PEGylated Hb Sample	Oxygen Affinity	
	P_{50} (mm Hg)	Hill Coefficient (n)
HbA	13.5	2.8
αα-Hb	30.5	2.4
ββ-Hb	6.3	2.1
(Propyl-PEG5K)$_6$-Hb	6.3	1.9
(Propyl-PEG5K)$_6$- αα-Hb	16.0	1.5
(Propyl-PEG5K)$_6$- ββ-Hb	5.9	1.9
(Propionyl-PEG5K)$_6$-Hb	10.0	1.8
(Propionyl-PEG5K)$_6$- αα-Hb	22.8	1.8
(Urethane-PEG5K)$_6$-Hb	8.0	2.0
(Urethane-PEG5K)$_6$-αα-Hb	23.3	2.1
EAF-PEG-Hb without free Cys-93(β)	6.9	2.1
EAF-PEG-Hb with free Cys-93(β)	8.5	2.0
EAF-PEG-αα-Hb without free Cys-93(β)	9.9	1.7
EAF-PEG-αα-Hb with free Cys-93(β)	18.5	1.7

*αα-Hb=αα-fumaryl crosslinked Hb; ββ-Hb= ββ-intramolecular succinimidophenyl-PEG2K crosslinked Hb

PEGylation and its Success: Hemospan, Functional Universal Red Blood Cells, and Polynitroxylated PEGylated Hemoglobin

Hemospan, also known as MP4, was created by Sangart via site-specific PEGylation chemistry that attached seven to eight 5 kDa maleimide-activated PEG chains to a single human α crosslinked hemoglobin tetramer (Vandegriff et al. 2008). Hemospan injected into rat and mouse models along with humans was not shown to induce vasoconstriction, but unfortunately, its oxygen delivery was minimal as demonstrated by the reported P_{50} value of 5 mm Hg (Tsai et al. 2003; Vandegriff et al. 2003; Vandegriff and Winslow 2009). This low P_{50} value possesses some beneficial characteristics including a capacity to deliver oxygen to highly hypoxic tissues within humans. However, for overall oxygen delivery *in vivo*, the P_{50} value (5 torr) is too low to use outside of oxygen delivery to dramatically hypoxic tissues Fortunately, MP4 does not induce vasoconstriction (by proxy, hypertension), and there was no gastrointestinal discomfort observed in subjects during Phase I and II clinical studies beginning in 2002 (Bjorkholm et al. 2005; Vandegriff and Winslow 2009). Furthermore, MP4 even progressed to Phase III clinical trials in Europe in attempts to assess its safety in controlled clinical orthopedic surgeries (Vandegriff and Winslow 2009).

In 2007, Acharya and colleagues employed PEGylation for the creation of a universal red blood cell derived from human blood (Nacharaju, Manjula, Acharya 2007). Two PEGylation methods were used. In the first method, free amino groups

on red blood cell (RBC) membrane proteins were converted into exposed thiol groups using Traut's reagent. These thiols were then modified with a maleimide-PEG (Mal-Phe-PEG), with an optional second step of acylation chemistry-mediated PEGylation that attached a succinimidyl propionate-PEG (SPA-PEG) to amino groups on membrane proteins of the RBC (Nacharaju, Manjula, Acharya 2007). The first option for PEGylation yields a product that contains an extension arm produced from intermediate steps while the second option does not produce an extension arm between a membrane protein and the PEG chain. Differences were also seen between these two forms of PEGylated RBCs. It was found that the direct conjugation of PEG chains to RBC membrane proteins via SPA-PEG 5 kDa did not mask the D antigen (responsible for Rh +/- blood group) like the RBC that had EAF-PEG modified proteins (Mal-Phe-PEG 5kD) (Nacharaju et al. 2005; Nacharaju, Manjula, Acharya 2007). Unfortunately, the small molecular size of the 5 kDa PEG chains was unable to mask the Rh system that accompanies blood typing unless an EAF PEGylation method is utilized. It is speculated that this phenomenon is permitted because Rh system antigens are multi-pass-transmembrane proteins with antigentic epitopes that are very close to the cell membrane and that even these small PEG chains are too bulky to react with the Rh antigen protein's amino groups (Avent and Reid 2000; Bradley et al. 2002; Fisher and Armstrong 2002; Nacharaju, Manjula, Acharya 2007). In regards to masking the A antigen, it was found that it could only be achieved by subjecting the RBCs to a two phase PEGylation system by

first attaching 5 kDa PEG chains followed by the attachment of 20,000 kDa PEG chains (Nacharaju et al. 2005). The ABO blood type system antigens are carbohydrates located off membrane lipids and proteins and extend out into the solvent phase. Therefore, in order to mask them, bigger PEG chains were needed (King 1994; Reid and Mohandas 2004). The research of Acharya and coworkers showed that regardless of which antigen A, B, or D was meant to be masked by this two-step PEGylation procedure, it did not matter whether or not EAF PEGylation was utilized because antigen masking was achieved regardless of PEGylation method (Nacharaju et al. 2005; Nacharaju, Manjula, Acharya 2007). Only RBCs PEGylated via thiolation mediated PEGylation exhibited an increased oxygen affinity while those done with SPA-PEG had comparable oxygen affinities to that of unmodified erythrocytes (Nacharaju, Manjula, Acharya 2007). It was concluded that a two-phase PEGylation procedure, Mal-Phe-PEG 5 kDa followed by SPA-PEG 20 kDa, would be most effective for masking A, B, and D antigens while having minimal influence on the RBCs oxygen binding affinity (dropping the P_{50} from 27.5 to 22.5) (Nacharaju, Manjula, Acharya 2007).

SynZyme Technologies created a novel HBOC, incorporating PEGylation and nitroxylation techniques to modify a Hb tetramer, called Polynitroxylated PEGylated Hemoglobin (PNPH) (Carleton, Hsia, Ma 2012). The product is a modified bovine Hb with 12-14 nitroxides and 8-10 PEG conjugations (Carleton, Hsia, Ma 2012; Lewis and Ross 2014; Shellington et al. 2011). The added nitroxides mimic the effects of

superoxide dismutase (SOD), present in a RBC, reducing oxidative stress by quenching reactive oxygen species (ROS) that are toxic to cells (Buehler et al. 2004; Lewis and Ross 2014). PNPH's effects were examined in mice models to assess their neurotoxicity, and ability to attenuate fluid requirements in traumatic brain injury (TBI) with hemorrhagic shock (HS) (Brockman et al. 2013; Lewis and Ross 2014; Shellington et al. 2011). PNPH was found to not be cytotoxic to neurons in areas of the hippocampus and was found post TBI with HS that there was significantly lower brain edema in mice treated with PNPH compared to those treated with lactated Ringer's (LR) solution (Brockman et al. 2013; Shellington et al. 2011). Unfortunately, PNPH has a high oxygen affinity (P_{50} 10 mm Hg), indicating it will mainly deliver oxygen to severely hypoxic tissue (Lewis and Ross 2014; Shellington et al. 2011). The SOD-mimetic abilities of PNPH make it unique from all other previous PEG based HBOCs, however the low P_{50} is a major concern for delivering oxygen during HS or trauma situations.

With these HBOCs, a clear disadvantage is that MP4 and universal RBC's utilize human hemoglobin (or human RBCs) to create the final product, while MP4 and PNPH have a very high oxygen affinity. While this may provide useful compounds that have potentially beneficial applications, all of these products do not address the need for a blood substitute independent of human blood donations. In addition, PEG RBCs would have the same storage problems as normal RBCs and while it is universal donor it is not a stable product.

Conclusions

An effective HBOC would be useful in trauma and other medical situations. Blood transfusions require disease free and properly type matched blood. This problem is exacerbated when blood donations are low, coupled with the short shelf life of donated blood. To counteract these complications, a PEGylated HBOC should be derived from non-human hemoglobin sources, universal for all types of blood, have a longer shelf life compared to donated whole blood, and be disease free. Review of the literature suggests that a larger molecular size HBOC, comparatively to the size of stroma free hemoglobin, is required to be successful *in vivo*. In addition, the HBOC should not dissociate into dimers, possess no nephrotoxicity, and should not scavenge NO from the endothelial tissue of the cardio vascular system. PEGylated hemoglobin has a larger hydrodynamic volume than native Hb and has shown to produce NO instead of removing it from living systems depending on the site of conjugation the PEG chains. Ideally, a PEGylated HBOC will be able to be lyophilized for longer storage. It has already been shown that PEGylation improves the stability of a PEGylated protein when it is reconstituted in liquid post lyophilization (Heller, Carpenter, Randolph 1999). In conclusion, a successful PEG based HBOC will have an increased size, contain extension arm facilitated PEG (EAF-PEG) to not disrupt the Hb tetramer interface, be crosslinked to avoid Hb tetramer dissociation, and be derived from a non-human source.

CHAPTER II

STATEMENT OF RESEARCH

Blood transfusions require disease free and properly type matched blood. This becomes a problem when blood donations are at an all-time low coupled with the fact that the shelf life of donated blood is relatively short lived. Furthermore, there are areas of the world where clean blood is difficult to come by. To counteract these complications a blood substitute should be derived from non-human hemoglobin sources that is universal for all types of blood, have a longer shelf life compared to donated whole blood, and be disease free. If this problem were solved, it would relieve a myriad of problems within the current medical community and blood donation system. Review of the literature suggests that a larger molecular size HBOC, comparative to the size of stroma-free hemoglobin, is required to be successful *in vivo*. In conjunction with the aforementioned characteristics, a HBOC needs to not dissociate into hemoglobin dimers, possess no nephrotoxicity, and it should not be able to scavenge NO from the endothelial tissue of the cardiovascular system. PEGylated hemoglobin has a larger hydrodynamic volume and is immunogenic compared to native hemoglobin. In the end, a successful PEG based HBOC will have an increased size, crosslinked to avoid tetramer dissociation, be unable to scavenge NO, and be able to deliver oxygen to tissues.

The first part of this novel research crosslinks (XL) bovine hemoglobin in a high oxygen affinity state (ββ XL-Hb). After crosslinking, the XL-Hb is covalently attached to an eight arm maleimide PEG backbone via the Cys-93 (β) residue. This methodology covers a PEG back bone in multiple Hb tetramers. This methodology is the reverse of traditional PEGyaltion and thus has been called "inside-out" PEGylation. The hemoglobin is extracted from whole bovine blood, so there is no need for human donations, and the crosslinking procedure will prevent the hemoglobin from dissociating into dimers *in vivo*. In addition, the site of conjugation on the Cys-93 (β) has been found to be favorable in reducing NO scavenging from endothelial tissue making it an ideal site for conjugation of the PEG backbone. The second part of the research crosslinks bovine Hb in a lower oxygen affinity state (αα XL-Hb). After, the XL-Hb is activated with an alkyne by an NHS ester. The alkyne is able to react with an eight arm azide PEG by strain promoted azide-alkyne click chemistry. This form of inside-out PEGylation yields a lower oxygen affinity Hb polymer compared to the previously mentioned methodology. Finally the large size of the polymers should assist in reducing its ability to scavenge NO.

A universal HBOC for every medical situation does not have to exist. Variations amongst HBOCs oxygen affinity would allow medical providers to prescribe a proper HBOC for a given circumstance. Based on these considerations I have produced a high and a low affinity PEG XL-Hb polymer that are intended to be used in lieu of blood transfusions.

CHAPTER III

"INSIDE-OUT" PEGYLATION OF BOVINE β-CROSSLINKED HEMOGLOBIN

Chapter Introduction

Human blood transfusions are the only viable way to rapidly replace lost blood in a patient while delivering oxygen to tissues. Transfusions have limitations due to risks affiliated with blood borne pathogens, the limited shelf life of donated blood, immunogenic responses, hemolytic transfusion reactions, and a dependence on human donation (Jahr et al. 2011). Stroma-free hemoglobin (Hb) is not a viable replacement because of tetramer dissociation in circulation, leading to rapid filtration by the kidneys and subsequent nephrotoxicity. It is also capable of scavenging nitric oxide which induces vasoconstriction leading to increased peripheral vascular resistance and subsequent organ dysfunction (Doherty et al. 1998; Muldoon et al. 1996; Reid 2003). These problems have shown the need for a viable alternative. An ideal hemoglobin-based oxygen carrier (HBOC) would efficiently deliver oxygen to tissues while being pathogen free, universal for all blood types, non-immunogenic, and available at a minimal cost while possessing a long shelf life with minimal refrigeration (Estep et al. 2008; Ness and Cushing 2007; Reid 2003; Sakai et al. 2000). While crosslinking (XL) the Hb tetramer prevents dissociation, tetramers solely modified by crosslinking failed as HBOCs due to

damage to the vascular tissue and NO scavenging (Ness and Cushing 2007; Reid 2003; Sakai et al. 2000).

To counteract these problems previous HBOC design has produced bis-tetrameric Hb polymers, liposome encapsulated Hb nanoparticles, Hb microparticles, and polyethylene glycol (PEG) Hb conjugates (Agashe and Awasthi 2009; Bradley et al. 2002; Lui et al. 2012; Singh et al. 2015; Xiong et al. 2012). Recent reviews have shown that conjugation of PEG to Hb has been one of the more successful methods thus far (Jahr, Akha, Holtby 2012; Palmer and Intaglietta 2014). Protein PEGylation has been shown to improve therapeutic potential by camouflaging the proteins from the immune system and reduce glomerular filtration from the body (Bailon and Berthold 1998; Harris and Chess 2003; Roberts, Bentley, Harris 2002). Several approaches exist to PEGylate Hb. Variations have been made in the size of the PEG chains, the number of PEG chains conjugated to a single Hb tetramer, the sites of PEGylation, the methods of conjugating PEG chains to Hb, and the crosslink status of the Hb (Hu et al. 2007; Li et al. 2008; Li et al. 2009). Acharya and coworkers found that when six PEG chains were conjugated to a single Hb tetramer, the oxygen affinity of the Hb polymer increased in comparison to native Hb (Li et al. 2007). It was previously proposed by Winslow and co-workers (Rohlfs et al. 1998) that a total PEG mass around 50 kDa to one Hb molecule would be sufficient to counteract vasoactivity of Hb in the bloodstream. Moreover, other larger hemoglobin complexes, such as the zero-length crosslinked product by Bucci and co-workers (Oxy Vita) (Matheson et al. 2002), are not vasoactive (Harrington

and Wollocko 2011; Martini et al. 2006; Palmer and Intaglietta 2014). From these studies one concludes that a way to counteract vasoactivity is to produce a polymer with a large size or hydrodynamic volume (Palmer 2006; Palmer and Intaglietta 2014). A polymer of such a size can be produced by conjugating PEG onto the Hb tetramer and will stay in the center of the blood flow preventing deleterious endothelial NO scavenging (Alayash 2004; Palmer 2006; Palmer and Intaglietta 2014).

While most PEG chains have been attached to exposed Lys residues on the Hb tetramer through NHS-Ester amidation, it is also possible to conjugate PEG chains onto the Cys β93 residue (Li et al. 2008). This modification increased the oxygen affinity of hemoglobin but allowed for oxygen deliver to ischemic tissues (Abraham, Phillips, Kennedy 1983; Li et al. 2009). In addition, multiple PEG chains generate a hydration shell around a single Hb tetramer in current PEGylated HBOCs, increasing the hydrodynamic volume. In this chapter the reverse approach has been utilized with a PEG polymer to which multiple Hb tetramers are attached by click chemistry. Click chemistry is defined as a reaction that has a large thermodynamic driving force that favors a single product. The click chemistry used to produce the large Hb polymers is the thiol-maleimide reaction. A PEG backbone with eight arms, each containing a terminal maleimide functional group (Mal-PEG), is capable of reacting with Hb tetramers via the Cys β93 residues. Therefore, the maximum number of Hb tetramers that may be conjugated to a single PEG backbone is eight. However, it is more likely that once the Mal-PEG reacts with one Cys β93, it will then

react with the other exposed Cys β93 in the same tetramer. Thus, one would expect a lower maximum ratio of four, rather than eight, Hb bound to PEG if there is one hundred percent reactivity. This method utilizes thiol-maleimide click chemistry to produce a PEG Hb polymer distinguishable from all other PEG Hb products reported thus far. As a large PEG backbone is covered by multiple proteins, the reverse of traditional PEGylation schemes, we have called this method inside-out PEGylation. Here we show that this form of PEGylation can create a PEG βXL-Hb polymer via site specific chemistry that has the potential to be a viable HBOC for highly hypoxic tissues.

Materials

Untreated bovine blood was obtained from Animal Technologies (Tyler, TX). Eight Arm Maleimide-PEG (Mal-PEG) was purchased from Creative PEGWorks (Winston Salem, NC). Sephadex chromatography media were purchased from GE Healthcare Biosciences (Pittsburg, PA). Micro separatory centrifuge filters (300 kDa) were purchased from Sartorius Stedim (Bohemia, NY). Hemox Antifoam and the HEMOX oxygen analyzer were purchased from TCS Scientific (New Hope, PA). DBSF was made by the method of Zaugg et al (Zaugg, King, Klotz 1975). All other chemicals and gel filtration markers kit for protein molecular weights 29 kDa - 700 kDa were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Methods

Bis(3,5-dibromosalicyl) Fumarate (DBSF) Synthesis and Conformation

Bis(3,5-dibromosalicyl) fumarate (DBSF) was prepared by a procedure similar to that for the succinate diester done by Walder et al. (Walder et al. 1979) 1.35 mL (0.0125 moles) of fumaryl chloride was mixed with 100 mL of benzene (toluene can be substituted for benzene). Following that, 7.55 grams (0.0255 moles) of 3,5-dibromosalicylic acid and 6.38 mL (0.05 moles or 6.06 g) of N,N-dimethylaniline were added to the previous mixture. The slurry was allowed to mix at room temperature for three hours. After which, thirty mL of ice-cold deionized water were added and the mixture was acidified with a few drops of 12 M HCl and allowed to stir for another 20 minutes. The liquid was decanted off, and the solid was washed three times with benzene/toluene via vacuum filtration. The sample was then allowed to dry and it was scraped into a beaker in which just enough acetone was added to dissolve the DBSF (the solution became clear not cloudy) and an equal amount of water was added. If the DBSF came out of solution slightly, more acetone was added to make the mixture clear. The sample was then put through a vacuum filtration system to remove any solid particles that did not dissolve in the acetone/water and the liquid was collected. Following this, 6 M HCl was added to the mixture until the DBSF was seen to precipitate out of solution, and turned cloudy again. If the pH was lowered, and the DBSF did not come out of solution, water was added to help the DBSF precipitate out. Figure five shows the scheme by which DBSF was synthesized. The mixture was once again run though the filtration

system, and the solid DBSF was collected. The product, $C_{18}H_8O_8Br_4 \bullet C_2H_6O$, had a melting point of 223-225°C.

Around seven mg of the produced DBSF, Sigma Aldrich's DBSF, and LKT Laboratories DBSF were taken and separately suspended in deuterated acetone (Acetone-d_6). NMR spectra were taken of each sample on a Gemini-300. The solvent was not ideal to produce a reliable NMR spectrum, so the procedure was repeated the same as before but deuterated acetone was substituted for deuterated DMSO (DMSO-d_6).

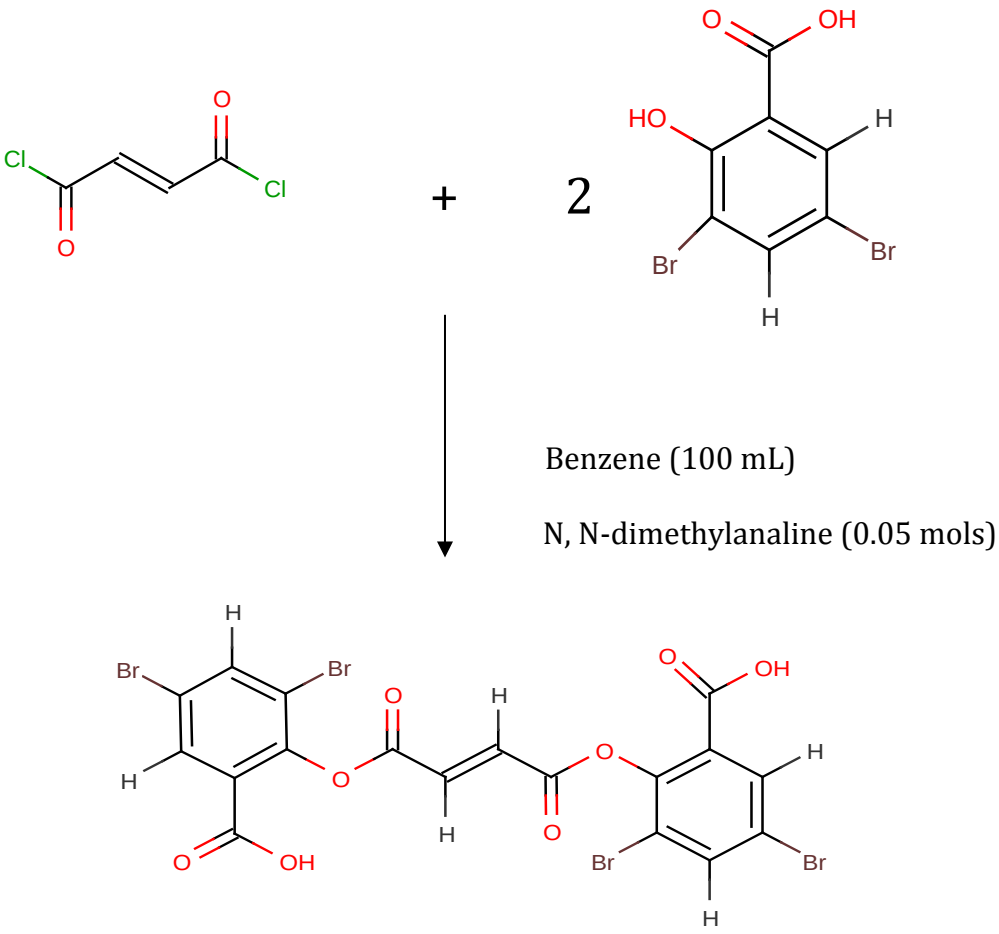


Figure 5. Synthesis of DBSF.

Crosslinked Hemoglobin Synthesis

Methods described by Hanash and Shapiro (Hanash and Shapiro 1981) and Dozy et al. (Dozy, Kleihauer, Huisman 1968) were modified for the purification of Hb from bovine blood. After ammonium sulfate fractionation and dialysis, the impure Hb solution was added to Sephadex A-50 gel in 50 mM Tris, 1 mM NaCN, pH 8.5 buffer in centrifuge tubes. Unbound proteins were separated by centrifugation and then the bound Hb was released from the gel upon the addition of Tris buffer at pH 7.2. The procedure for crosslinking oxy-hemoglobin was carried out as outlined by Walder et al. (Walder et al. 1979). DBSF was reacted with oxy-hemoglobin at a molar ratio of 1 Hb: 1.2 DBSF at room temperature for four hours before dialysis in 50 mM Tris, 1 mM NaCN pH 8.5 buffer. Separation of unmodified Hb from βXL-Hb was accomplished by a pH gradient of 8.5 to 6.8 on a Kontes Chromaflex column using Sephadex A-50 gel in 50 mM Tris, 1 mM NaCN buffer. SDS-PAGE procedures, outlined by Schmidt et al. (Schmidt and Brosious 1979), showed equal intensity bands at 14.4 kDa and 30 kDa, verifying crosslinking.

Reduction of Methemoglobin to Oxyhemoglobin

The procedure used was a revised version of the Dixon and McIntosh method (Dixon and McIntosh 1967). A 25 cm x 1 cm Kontes column was loaded with Sephadex G-25 gel in 5 mM phosphate buffered saline solution (PBS) pH 7.0, 1 mM EDTA buffer. Following equilibration, 0.5 mL of a 0.1 g/mL solution of sodium dithionite was added to the column followed by addition of 0.3 mL of the PBS buffer. A 5 mL sample was then passed through the gel and reduced by the dithionite.

PEGylation of Crosslinked Hemoglobin

βXL-Hb was dialyzed in 50 mM MOPS, 10 mM EDTA, pH 7.4 buffer. The sample was then passed through a 0.2 micron filter to remove any particulates. βXL-Hb was then reacted with a 40 kDa, 8-Arm Mal-PEG at a molar ratio 80 βXL-Hb: 1 Mal-PEG at room temperature for one week. Unreacted Hb was removed using a 300 kDa micro separatory centrifuge filter spun at 2950 g. The retained complex was collected for further purification.

Purification of PEGylated Crosslinked Hemoglobin via Size Exclusion Chromatography

A pre-packed Sephacryl S-300 column was equilibrated with 50 mM MOPS, 0.15 M NaCl, pH 7.4 buffer. Concentrated samples retained by the 300 kDa filter were then applied, and the large molecular weight PEG βXL-Hb fractions were collected and concentrated. The concentrated samples were then ran on a pre-packed Sephacryl S-500 column that was previously equilibrated with 50 mM MOPS, .15 M NaCl, pH 7.4 buffer. Known protein standards were ran to determine a standard curve.

SDS-PAGE of PEGylated Hemoglobin

Confirmation of large molecular weight species produced by PEGylation of βXL-Hb was obtained using a PhastGel SDS-PAGE system (Schmidt and Brosious 1979).

Determination of Molecular Weight via Analytical Ultracentrifugation (AUC)

AUC was carried out in a Beckman Coulter ProteomeLab XL-A analytical ultracentrifuge using standard 2 sector cells with quartz windows in 4- or 8-hole

rotors. All sedimentation velocity experiments were at 50,000 rpm with a preset scan number of 100 per cell. The reference sector was filled with 450 μL of buffer (50 mM MOPS, 10 mM EDTA, pH 7.4) and the sample sector was filled with 430 μL of sample with an absorbance at 412 nm (1 cm path length) between 0.75 and 1.1. Buffer density (1.00718 g/mL) was measured at 20.0° C in a Mettler/Paar Calculating Density Meter DMA 55A and the buffer viscosity (0.010543 Poise) was measured in an Anton Parr AMVn Automated Microviscometer. A partial specific volume of 0.746 mL/g for bovine hemoglobin was used in calculating molecular weight (DeMoll et al. 2007). Data were analyzed using the program Sedfit (www.sedfit.com). Molecular weights and sedimentation coefficients were determined using the continuous c(s) mode in the sedfit program. Native tetrameric hemoglobin yielded an $s_{20,w}$ value of 4.25S which agrees with the accepted value (Field and O'Brien 1955). The c(s) plots were deconvoluted using nonlinear least-squares fitting algorithms available in the Origin (v. 9.0) software package (OriginLab Corporation, Northapmton, MA). Data were fit to multiple Gaussian components, increasing the number of components until residual plots (data-fit) were random and the chi square value for the fit was minimal. Six components were needed to adequately describe the c(s) distribution.

Secondary Structure Characterization of PEG βXL-Hb via Circular Dichroism

CD spectra were obtained on an OLIS DSM 20 CD Spectrophotometer. Water was used to collect baseline data and as the dilution solvent for the Hb samples. The final Hb concentrations in the cuvette for the three samples were 0.01420 mg/mL

PEG βXL-Hb, 0.02301 mg/mL Hb, and 0.02238 mg/mL βXL-Hb. For each sample a wavelength range from 190-260 nm was scanned. The spectra were smoothed using Solvitsky Golay 7 point method (Whitmore and Wallace 2004; Whitmore and Wallace 2008).

Thermal Denaturation Procedure

Experiments followed the procedure according to Olsen (Olsen 1994). Experiments were conducted in 10 mM MOPS, 0.9 M guanidine HCl pH 7.0. Samples were prepared so that the absorbance at 409 nm was between 1 and 1.2. Samples were monitored every minute on an Agilent 8453 spectrophotometer. A temperature increase of 0.3°C/ min was controlled by Neslab Endocal, Temperature Programmer ETP-3, and Digital Controller/Readout DCR-4. Absorbance versus temperature was plotted and the fraction denatured was determined by the equation $F_D = (A-A_N) / (A_D-A_N)$ where A is experimental absorbance, A_N is absorbance of native protein, and A_D is absorbance of denatured protein. A_N and A_D were determined by fitting a linear line to the portion of the absorbance versus temperature plot that corresponded to the effect of temperature on the native or denatured spectrum respectively. The curve of F_D versus temperature was smoothed in Microsoft Excel with an exponential smoothing function.

Autoxidation Experiments

Autoxidation of native, crosslinked, and PEG crosslinked hemoglobin was carried out according to Yang and Olsen (Yang and Olsen 1989). Hemoglobin samples were fully reduced and dialyzed in 10 mM MOPS, pH 7.0. Sodium cyanide

was added to each sample and allowed to equilibrate at room temperature for five minutes prior to data collection. The experiments were conducted at 37°C and allowed to equilibrate before spectra were recorded from 500-700 nm on an Agilent 8453 spectrophotometer.

Oxygen Binding Studies For Determination of P_{50} and Hill Coefficients

The samples, fully reduced with dithionite, were added to 4.0 mL of 10 mM MOPS, pH 7.4 and 10 μL of Hemox Antifoam. Oxygen binding was determined using a HEMOX Analyzer for native bovine Hb, native Hb with inositol hexaphosphate (IHP), βXL-Hb, βXL-Hb with IHP, and PEG βXL-Hb. Hill coefficients (n) and oxygen affinity (P_{50}) were obtained from Hill plots.

Results

Bis(3,5-dibromosalicyl) Fumarate (DBSF) Synthesis and Conformation

As seen in Figures 6-8 (self-synthesized, Sigma, and LKT Laboratories DBSF) via the NMR spectra that there are three noteworthy peaks between 7.2 ppm and 8.6 ppm. The integration of these peaks in each of the H-NMR were calculated to be δ 8.35(2Hd, J=2.1 Hz), 8.10(2Hd, J=2.1 Hz), 7.35(2Hs), δ 8.35(2Hd, J=2.4 Hz), 8.10(2Hd, J=2.4 Hz), 7.35(2Hs), and δ 8.35(2Hd, J=2.7 Hz), 8.10(2Hd, J=2.4 Hz), 7.35(2Hs) for self-synthesized, Sigma, and LKT Laboratories DBSF respectively. The two peaks between 8.0-8.4 ppm corresponds to the aromatic hydrogens, while peak at 7.35 corresponds to the aliphatic hydrogens. This verified a successful DBSF synthesis in relation to Sigma and LKT Laboratories purchased DBSF by the nearly identical H-NMR spectra.

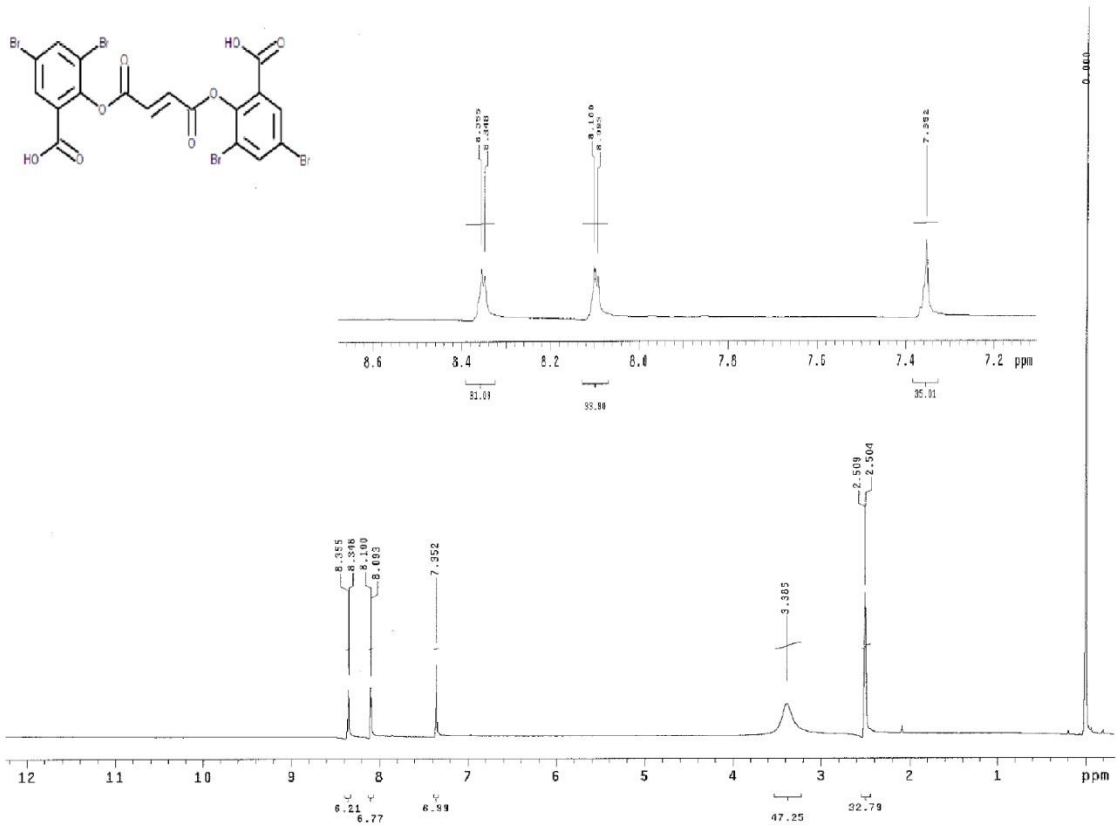


Figure 6. Self-synthesized DBSF H-NMR spectrum in DMSO-d6 solvent with an expanded spectrum above showcasing the range 7.2 ppm to 8.6 ppm. The peaks of interest and their integration can be seen at δ 8.35(2Hd, J=2.1 Hz), 8.10(2Hd, J=2.1 Hz), 7.35(2Hs).

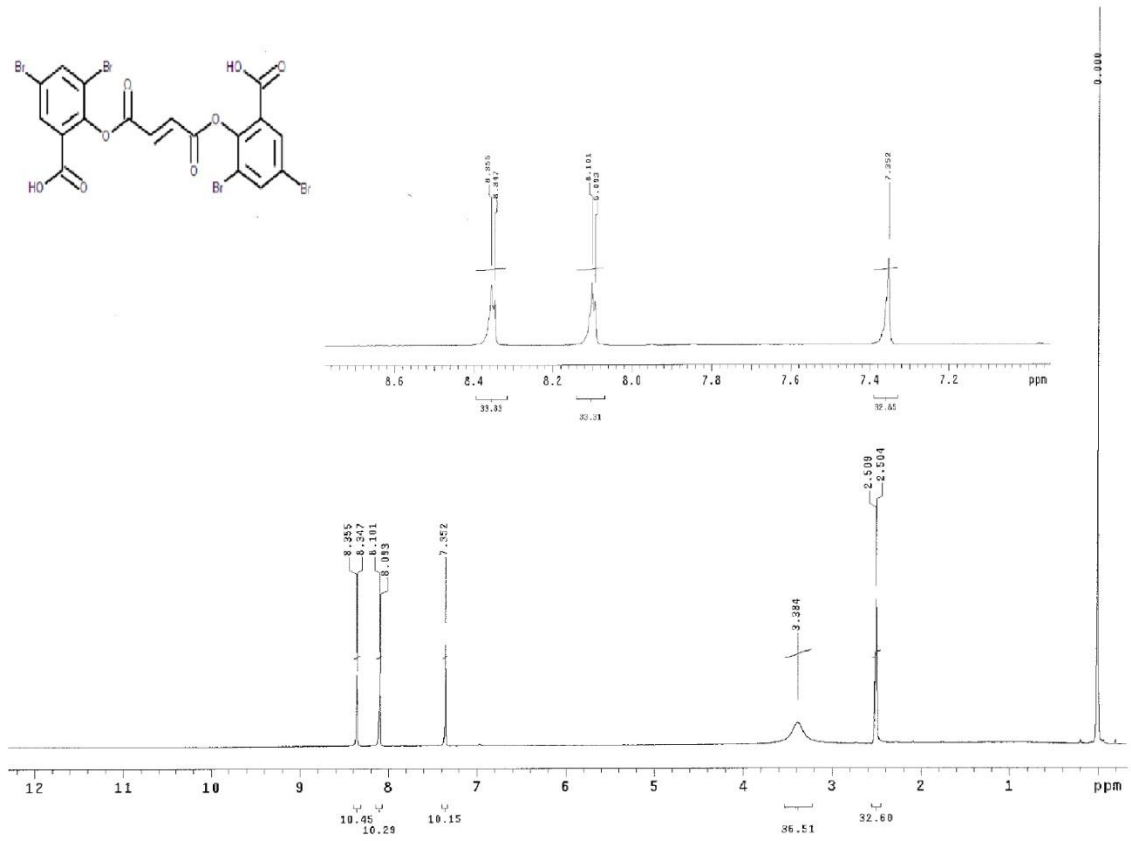


Figure 7. Sigma purchased DBSF H-NMR spectrum in DMSO-d6 solvent with an expanded spectrum above showcasing the range 7.2 ppm to 8.6 ppm. The peaks of interest and their integration can be seen at δ 8.35(2Hd, J=2.4 Hz), 8.10(2Hd, J=2.4 Hz), 7.35(2Hs).

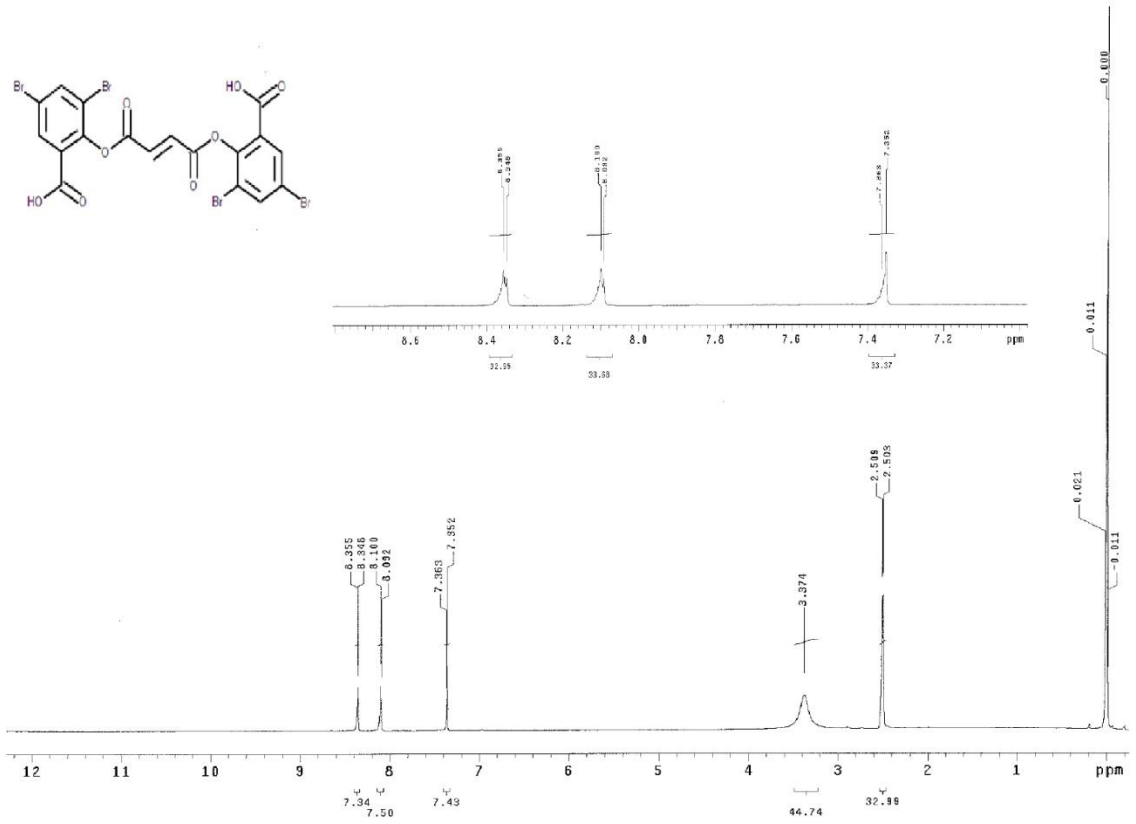


Figure 8. LKT Laboratories purchased DBSF H-NMR spectrum in DMSO-d6 solvent with an expanded spectrum above showcasing the range 7.2 ppm to 8.6 ppm. The peaks of interest and their integration can be seen at δ 8.35(2Hd, J=2.7 Hz), 8.10(2Hd, J=2.4 Hz), 7.35(2Hs).

XL-Hb Reaction Verification

Figure nine depicts the crosslinked Hb tetramer under oxy-conditions in which the fumarate from the DBSF molecule links the Lys82β1 and Lys82β2. This oxyhemoglobin crosslinking occurs on the β-subunit end of the central cavity in the Hb tetramer. Because it stabilizes the R state of Hb, the βXL-Hb already shows a higher affinity for oxygen than standard Hb within a RBC or even uncrosslinked Hb. Verification of this XL-Hb product was seen via SDS (Figure 10) with distinct bands around both 14.4 kDa and 30.0 kDa, ideally of equal intensity. Each Hb monomer is around 15 kDa, so the band at 14.4 kDa is representative of the alpha subunits disociation, where the band around 30.0 kDa indicates that the beta subunits were in fact linked together.

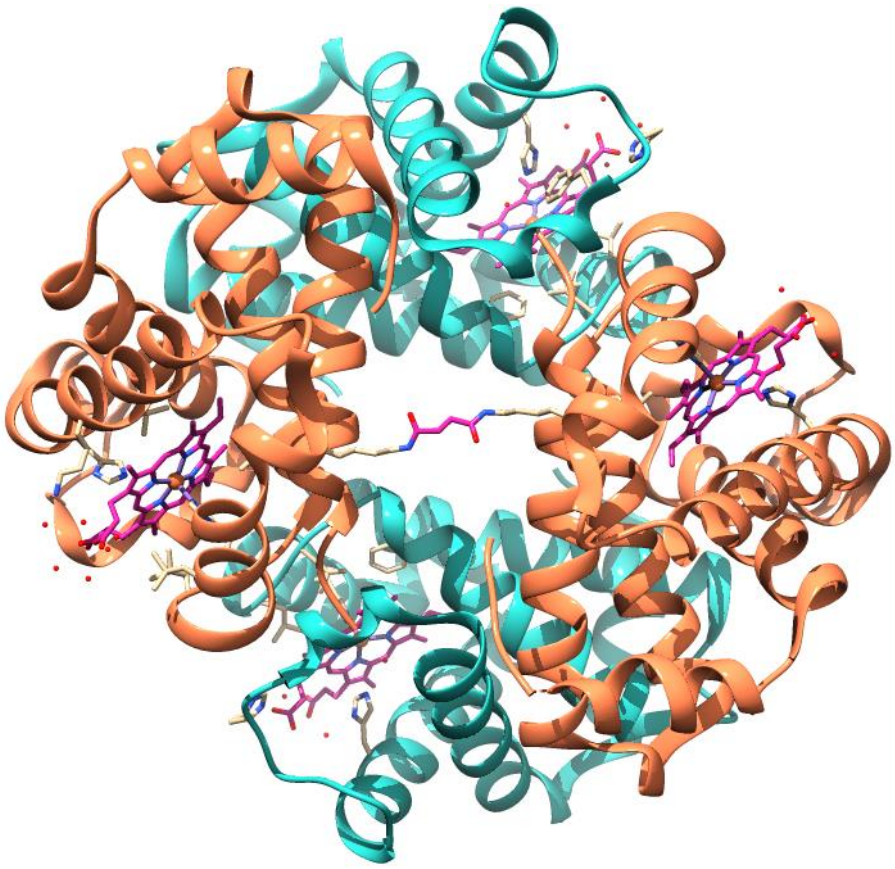


Figure 9. Model of oxy-XL Hb. DBSF crosslinks within the central cavity at the 2,3-BPG binding site.

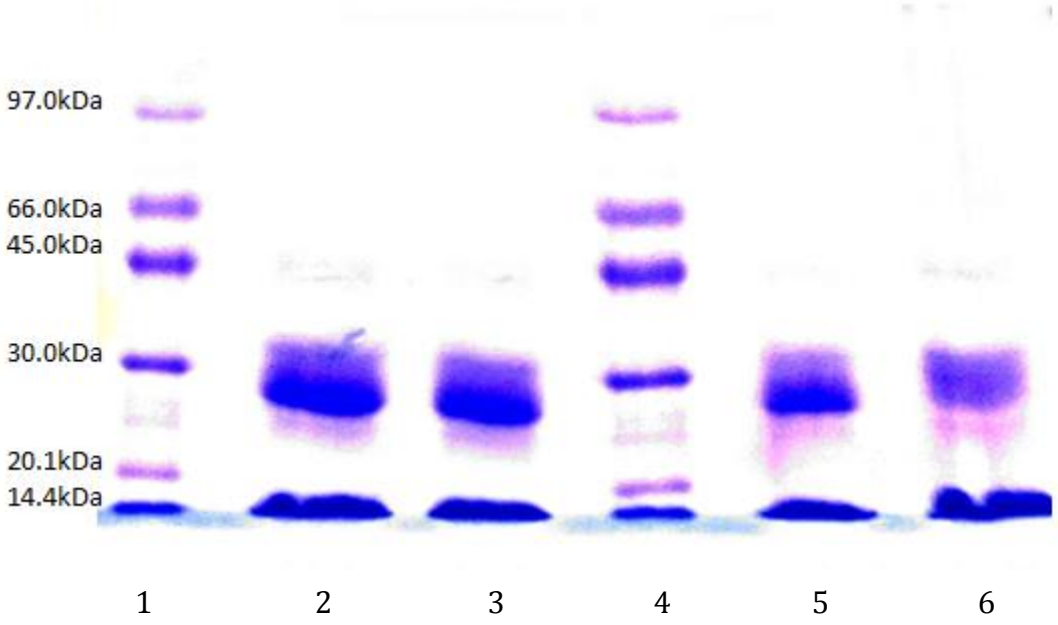


Figure 10. SDS confirmation of the crosslinked Hb Product. Lanes 1 and 4 are the markers while 2, 3, 5, and 6 are samples of XL Hb. The band at 14.4 kDa is a result from the alpha subunit separation from the Hb tetramer, while the band around 30.0 kDa is a result of the XL beta subunits.

XL-Hb Purification Verification

The separation of uncrosslinked Hb, βXL-Hb, overly crosslinked Hb and MetHb by Sephadex A-50 gel is possible due to the removal of two positive charged residues (Lys82β1 and Lys82β2) on the overall protein. This means that the less positive XL-Hb will bind to the gel more tightly then the more positive uncrosslinked Hb. As the pH of the buffer is lowered, the various Hb species will gain positive charges and will be released from the gel. The species of Hb elute from the column in the order: native Hb, βXl-Hb, overly Xl-Hb, and finally (with a drastic pH drop) MetHb. Overly XL-Hb will be more negative as other Lys groups (besides

the desired Lys82β1 and Lys82β2) have reacted with DBSF. Figure eleven shows the absorbance at 415 nm via UV-Vis in relation to the fraction number coming off the column. By looking at the data in Figure 11, it is evident that there were at least three major peaks as expected. The fractions were pooled together for each peak and SDS was run to determine which pools contained the purest samples of βXL-Hb. Figure 12 shows two SDS gels side-by-side that represents the eight pools. Figure 12, reading left to right, lanes 3, 5, 10, and 12 are markers while the left most lane (1) is the pool collected from the fractions that were last to come off the column and lane eleven is the pool collected from the beginning fractions. It can be seen reading left to right that the 30.0 kDa band becomes fainter until it disappears in the sample in lanes nine and eleven. This agrees with the fact that uncrosslinked Hb elutes off the column first, followed by XL-Hb as is seen by the darkening presence of the 30.0 kDa band indicating Lys82β1 Lys82β2 crosslinking.

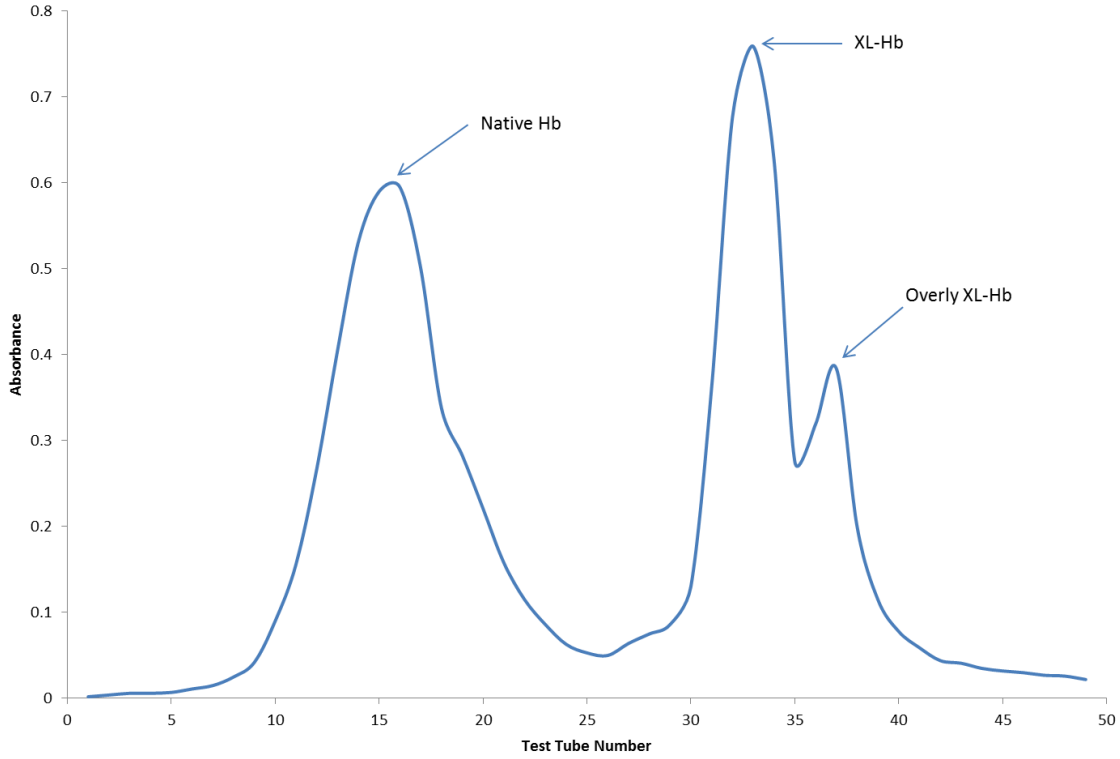


Figure 11. Graph of UV-Vis Spec Samples from Ion Exchange Column Fractions. The wavelength at 415 nm was used because at this wavelength the absorbance is specific for the heme group within the Hb. Wavelengths at 542 nm, and 578 nm are also for the heme groups but were not used to generate the chromatogram. It is apparent that there are 3 distinct peaks which correlate to un-crosslinked Hb, XL-Hb, and finally overly XL-Hb.

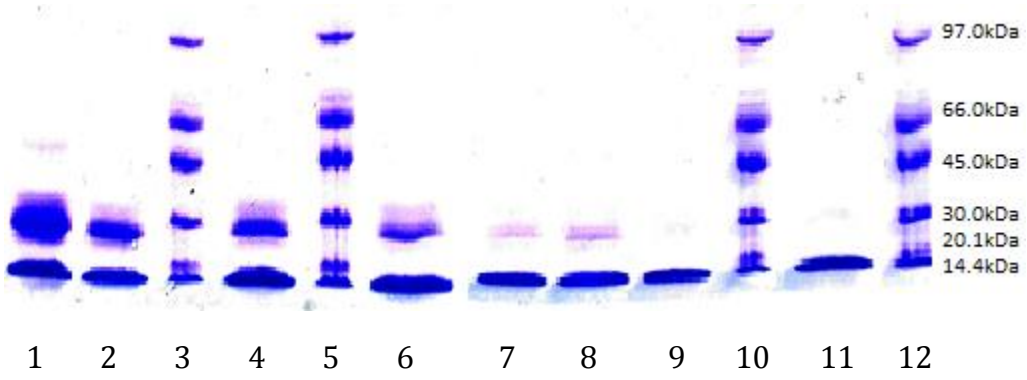


Figure 12. SDS from Ion Exchange Column Pulls. Lanes 3, 5, 10, 12 are the marker while the other lanes are from the 8 pulls created from the fractions shown in figure 11. Reading from right to left, the pulls correlate from early fractions that were collected to the last fractions collected. As seen in lanes 9 and 11 there is only a band present at 14.4 kDa correlating to complete Hb dissociation of the four monomers. Continuing reading right to left the band at 30.0 kDa becomes increasingly darker correlating to a higher production of XL-Hb.

Crosslinked Hemoglobin (βXL-Hb) Production and Confirmation of PEGylation at Cysteine β93 Residue

Verification of βXL-Hb product was obtained with SDS-PAGE (Figure 13) that showed distinct bands of equal intensity around 14.4 kDa and 30.0 kDa. An absence of the 30.0 kDa band with the concomitant presence of a new band at the origin of the gel (Figure 13, lane 3) was present in the PEGylated Hb sample.

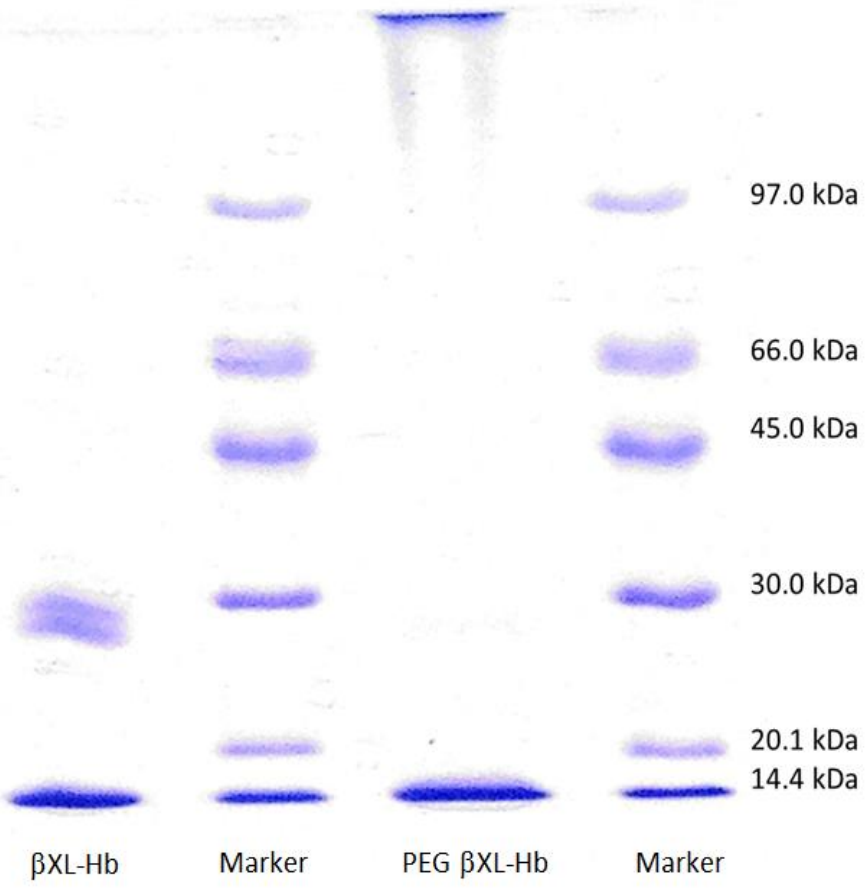


Figure 13. SDS Verification of PEG βXL-Hb. Lanes 2 and 4 are the marker proteins, while Lane 1 is βXL-Hb and Lane 3 the PEG βXL-Hb sample.

Conformation of Large Molecular Weight Species and Purification of PEG βXL-Hb from Unreacted βXL-Hb

Preliminary purification was accomplished by use of a 300 kDa filter, where βXL-Hb passed through the filter while the PEG βXL-Hb compound was retained. The PEGylated sample was further purified with an S-300 column (Figure 14) and the large molecular weight species were then applied to an S-500 column for

additional purification and characterization. It was evident from size exclusion chromatography (SEC) that PEG βXL-Hb and unreacted βXL-Hb Hb can be successfully separated with this two phase SEC protocol. On the S-500 column the characteristic elution peak around 250 mL was βXL-Hb, while the elution peak around 220 mL was the large PEG βXL-Hb sample (Figure 15).

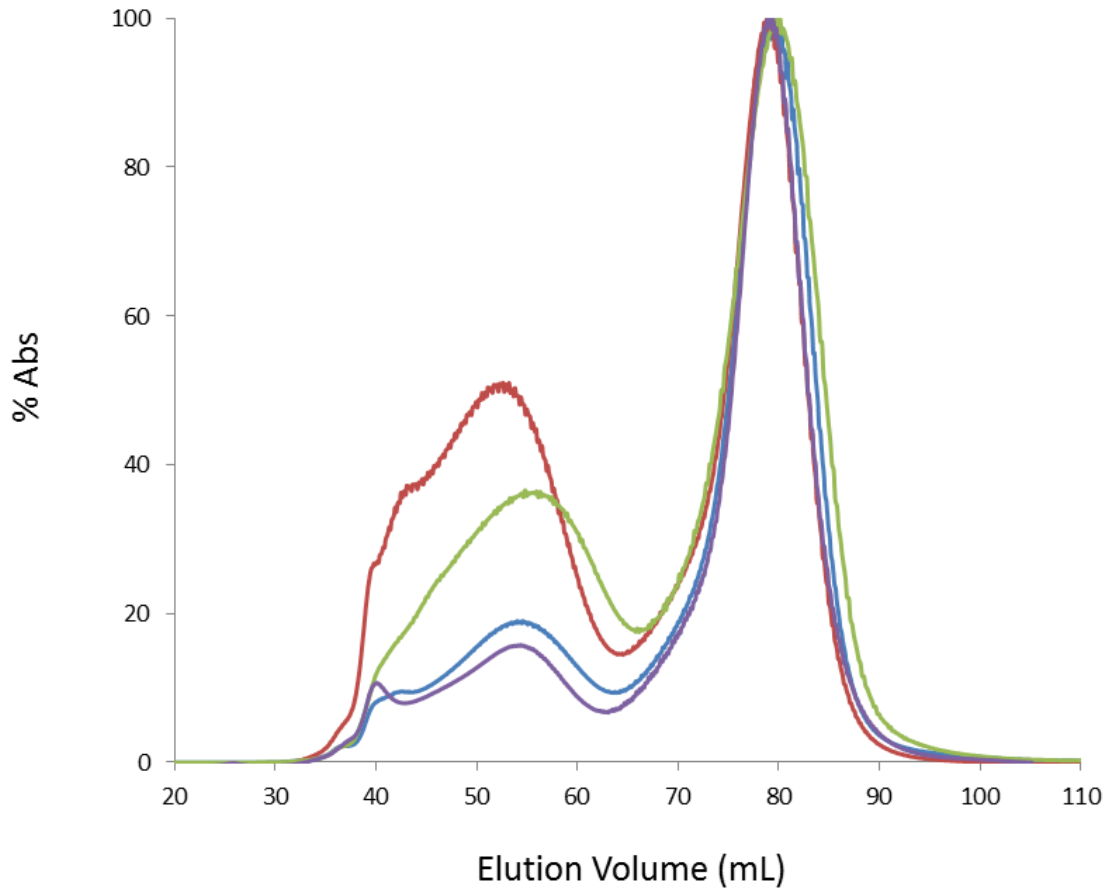


Figure 14. Normalized PEG βXL-Hb SEC chromatogram from the S-300 column. Unreacted βXL-Hb eluted around 80 mL while the larger PEG βXL-Hb product eluted between 40 and 65 mL. All four samples were prepared in the same method on different days.

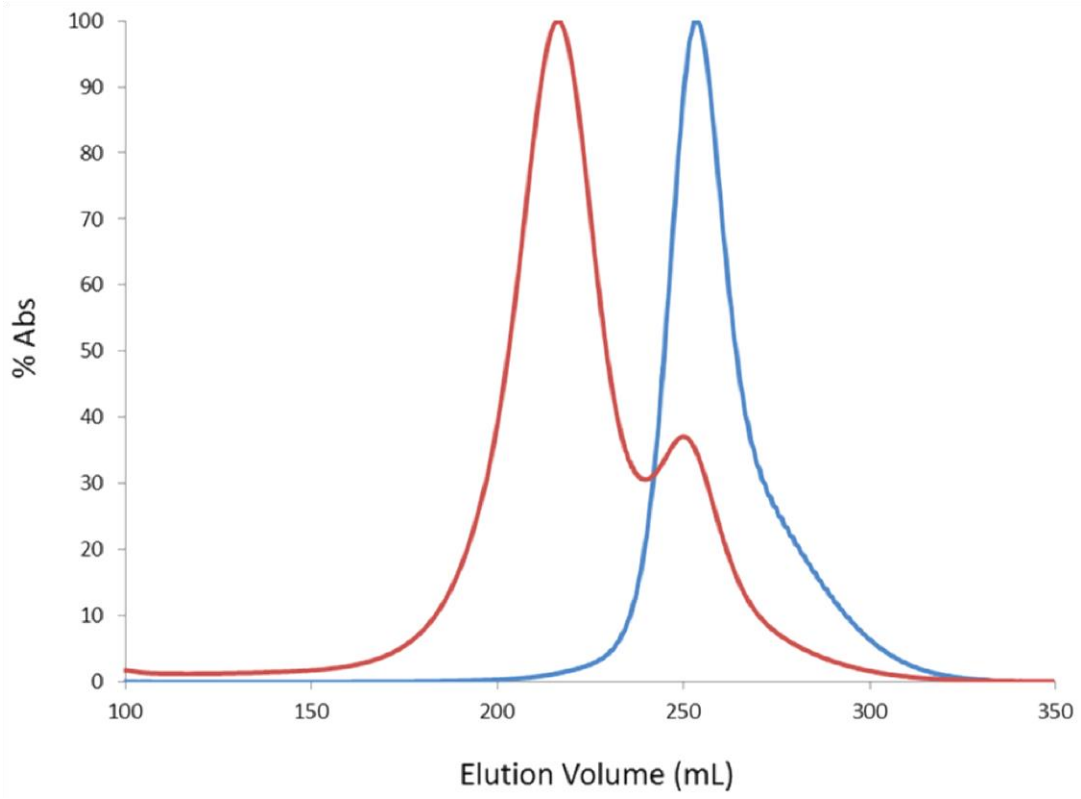


Figure 15. A normalized chromatogram from the S-500 column of a PEG βXL-Hb (Red) sample post S-300 SEC and an un-PEGylated βXL-Hb sample (Blue). Unreacted βXL-Hb elutes around 250 mL while the larger PEG βXL-Hb product elutes around 220 mL.

Size Determination of PEG βXL-Hb Samples via Analytical Ultracentrifugation

Figure 16A shows the Analytical Ultracentrifugation (AUC) sedimentation velocity analysis of the PEG βXL-Hb purified with a S-300 column and subsequently by S-500 column (see Figure 15). Sample heterogeneity was observed by monitoring the amount of product in relation to sedimentation coefficients. Native Hb has a $s_{20,w}$ of 4.25S (Field and O'Brien 1955) indicating that larger species are PEGylated samples. Most (60%) of the species lie in the sample range from 3 Hb : 1 PEG to 8

Hb : 1 PEG. The sample was heterogeneous in composition but there was a consistent pattern of heterogeneity between different samples. Figure 16B shows a deconvolution of a c(s) versus $s_{20,w}$ indicating the predominant HBOC species as 3 Hb : 1 PEG.

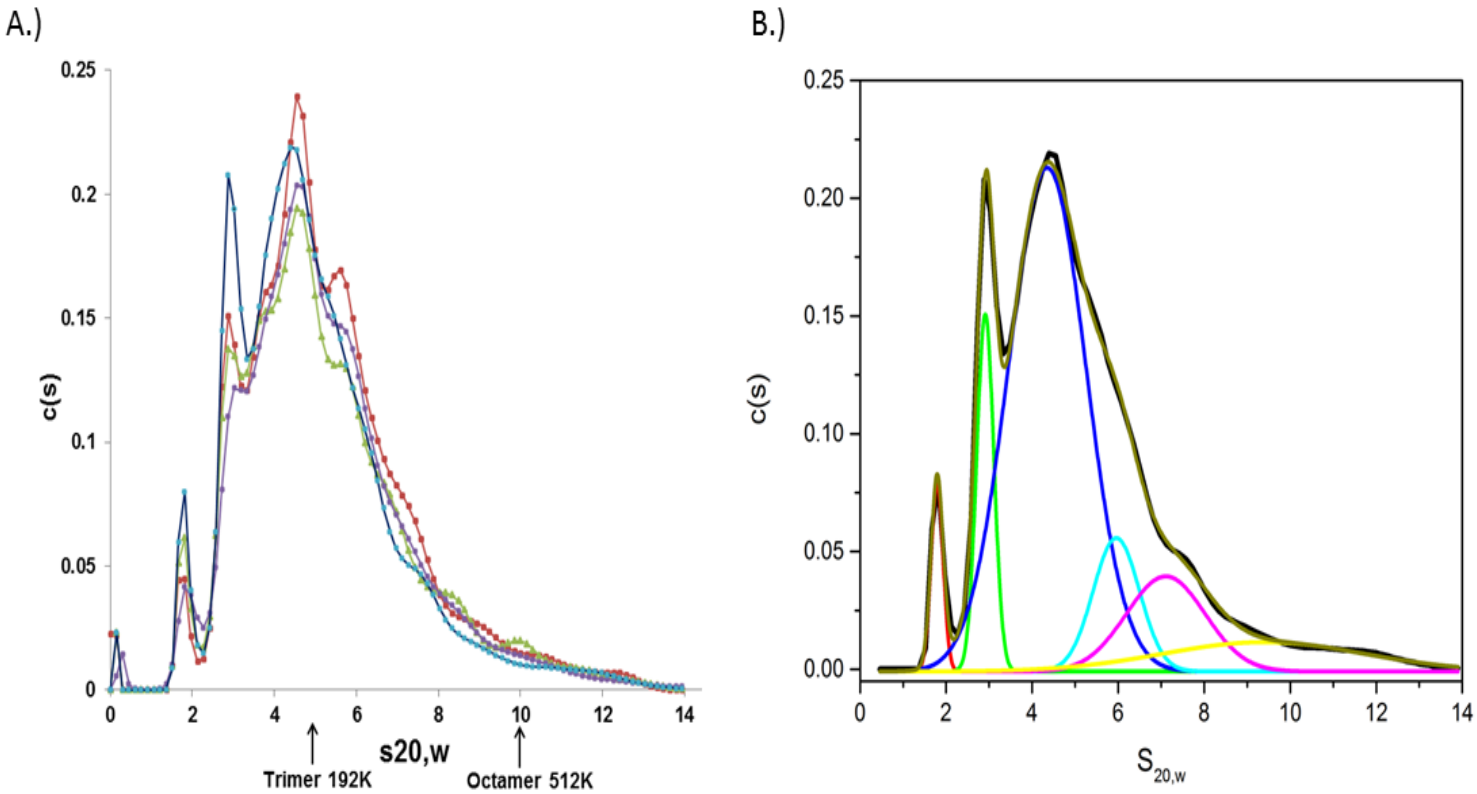


Figure 16. (A) c(s) versus $s_{20,w}$ plot of four different PEG βXL-Hb samples determined by AUC. Approximately 60% of the product's composition ranges from 4.5 S to 10S, indicating three to eight Hb tetramers conjugated onto a single PEG backbone. (B) A deconvoluted c(s) versus $s_{20,w}$ of a sample from Figure 16A showing the major speciation within the PEGylated sample.

Circular Dichroism Analysis of Secondary Structure in PEG βXL-Hb

Circular Dichroism (CD) was used to determine if crosslinking and PEGylation perturbed the secondary structure of Hb. Spectra of native Hb, βXL-Hb, and PEG βXL-Hb showed the characteristic signals for alpha helical structure at 195, 208, and 222 nm (Figure 17). Hemoglobin is 75% alpha helical by nature.

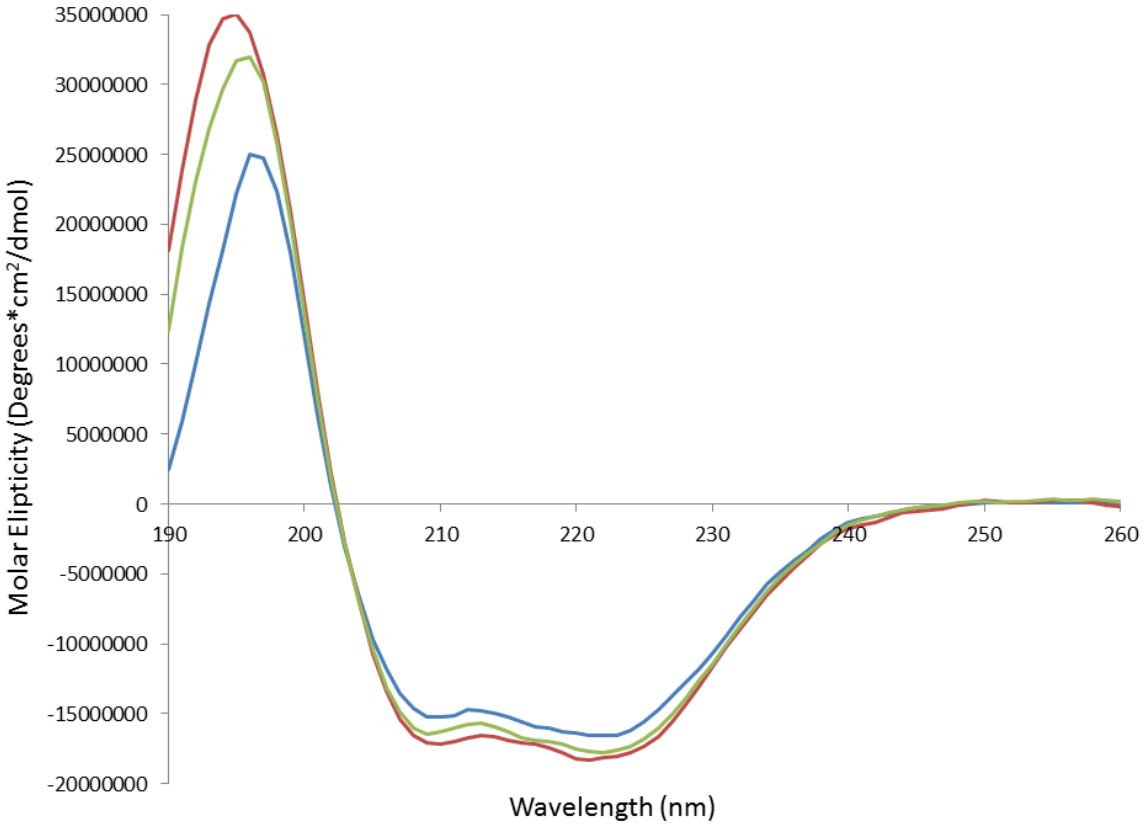


Figure 17. CD spectra of native Hb (green), βXL-Hb (blue), and PEG βXL-Hb (red). All three spectra show the characteristic signals for alpha helical structure at 195 nm, 208 nm, and 222 nm.

Thermal Denaturation of Hb Samples

Thermal denaturation curves for native Hb, βXL-Hb, and PEG βXL-Hb (Figure 18) showed that crosslinking and PEGylation increase the stability of the Hb tetramer compared to native Hb (Figure 18). The melting temperatures (T_m) for the samples can be seen in Table 4. A single factor ANOVA showed that the differences between all the T_m values are statistically significant.

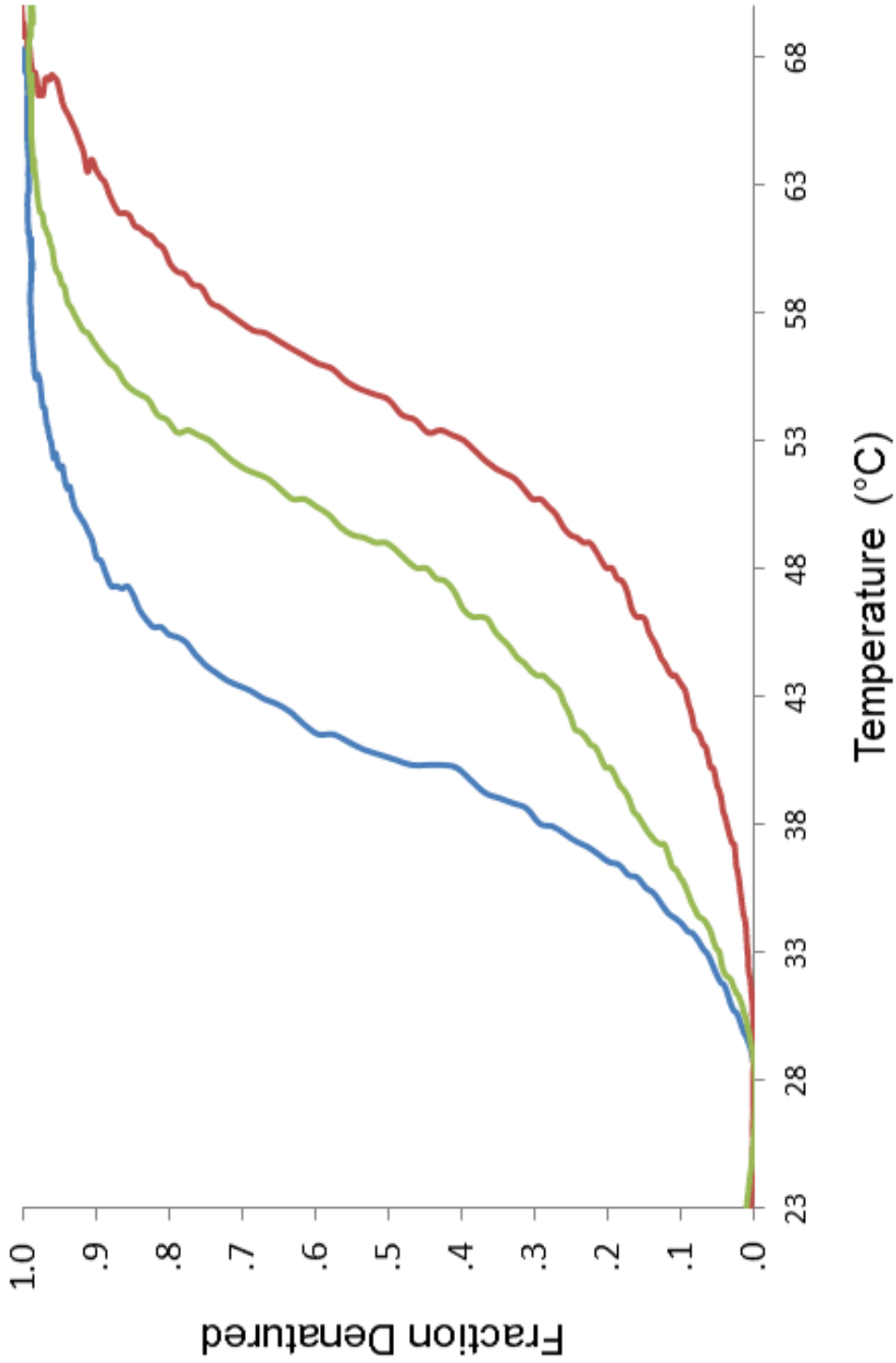


Figure 18. Comparison of native Hb (Blue), βXL-Hb (Red), and PEG βXL-Hb (Green) fraction denatured versus temperature based on the absorbance at 409 nm.

Autoxidation of Hb Samples

Autoxidation experiments were conducted at 37°C to see the effects of crosslinking and PEGylation on the autoxidation rates at physiological temperatures. Table 4 contains the relative rate constants (k_{rel}) of autoxidation. The relative rate constants were determined by dividing the slopes of methemoglobin production for βXL-Hb and PEG βXL-Hb by the slope of native Hb. It was determined that crosslinking increased Hb autoxidation rate by 1.59 times and PEGylation further increased the autoxidative rate to 3.43 times that of native Hb. Figure 19 shows the spectral changes observed in the PEGylated Hb sample. There was a decrease in the oxyhemoglobin absorbance at 540 and 576 nm as the sample autoxidized.

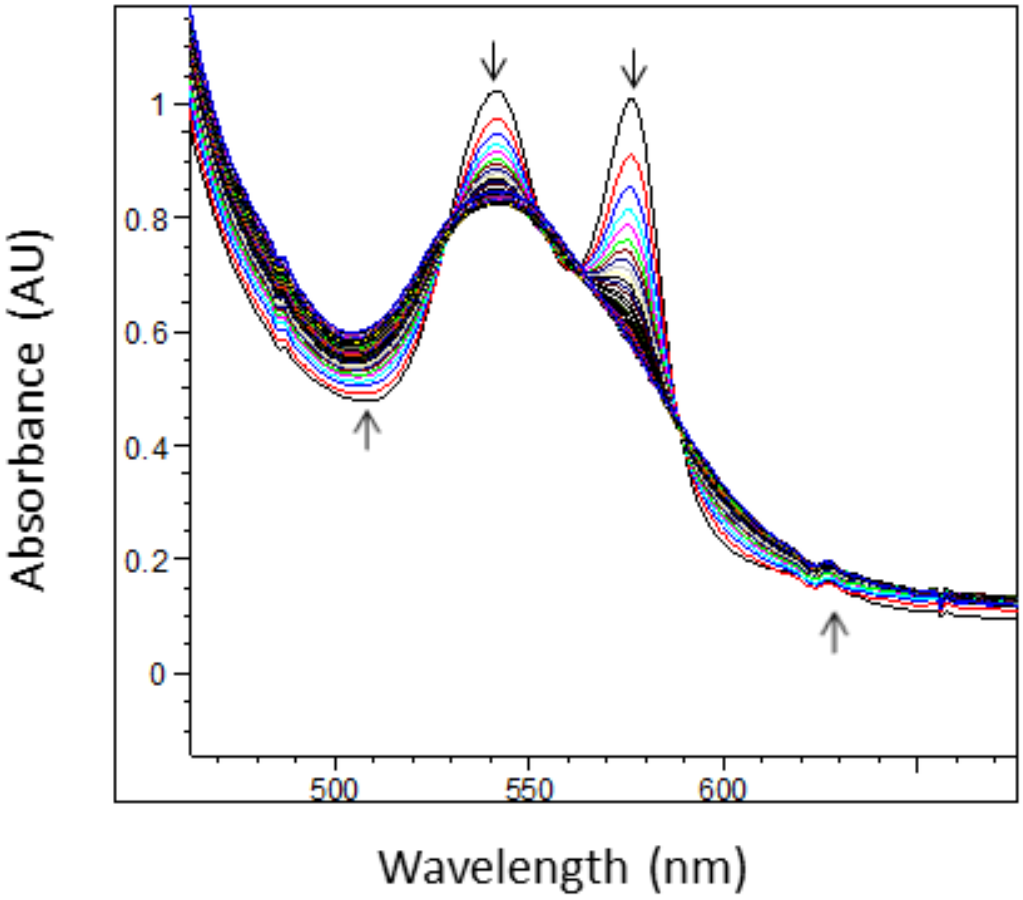


Figure 19. Autoxidation and spectral changes in PEG βXL-Hb sample. The spectra showed a decrease in absorbance at 576 and 540 nm and an increase of absorbance at 630 and 510 nm as the sample was allowed to oxidize.

Oxygen Binding Determination of Hb Samples

Figure 20 shows the experimental determined oxygen binding curves. Table 4 shows the calculated P_{50} and Hill coefficients (n) determined for native Hb, βXL-Hb, and PEG βXL-Hb. The βXL-Hb species had a higher oxygen affinity than native, stroma-free hemoglobin as expected (Walder et al. 1979). The addition of Inositol Hexaphosphate (IHP) to native Hb shifted the oxygen binding curve to the right but

addition of IHP to βXL-Hb and PEG βXL-Hb did not show this shift. The PEG βXL-Hb sample had a similar oxygen affinity to βXL-Hb. The Hill coefficients showed that the cooperativity of Hb decreased (from 2.91 to 1.72) once Hb was crosslinked (stabilizing the R state) and decreased slightly more following PEGylation (1.72 to 1.43).

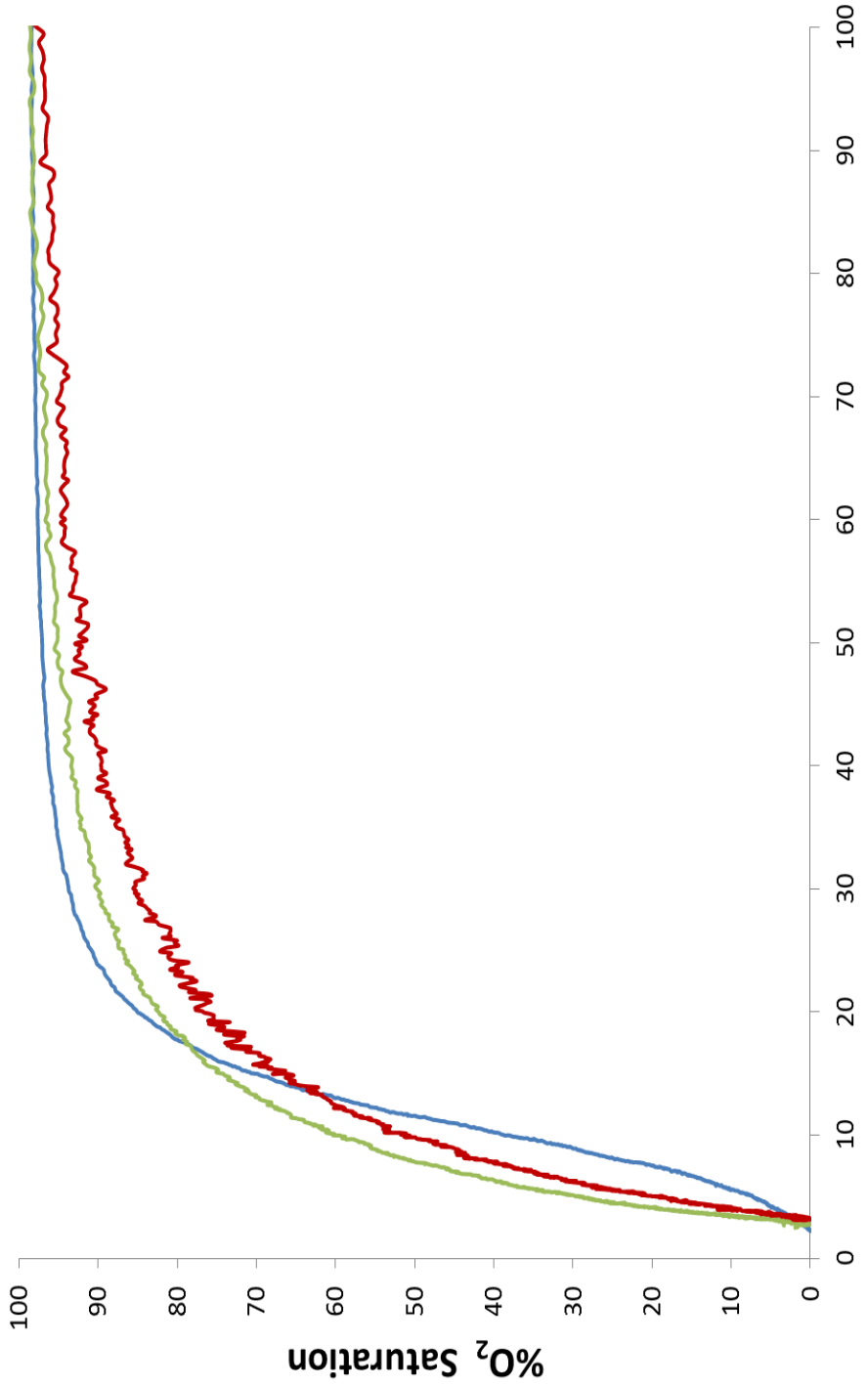


Figure 20. Comparison of native Hb (Blue), βXL-Hb (Green), and PEG βXL-Hb (Red) oxygen binding curves.

Table 4. Experimentally determined T_m (°C) from fraction denatured curves, relative rates of autoxidation at 37°C, and P_{50} (torr) and Hill Coefficients determined at 37°C for native bovine Hb, βXL-Hb, and PEG βXL-Hb.

Sample	T_m (°C)*	k_{rel}	P_{50} (torr)	Hill Coefficient (n)
Native Hb	41.3 ± .9	1	11.25 ± .17	2.91 ± .06
βXL-Hb	54.1 ± .5	1.59 ± .15	9.16 ± .84	1.72 ± .06
PEG βXL-Hb	48.9 ± 1.5	3.43 ± .53	9.67 ± .39	1.43 ± .06

*ANOVA F=80.0, F_{crit}=3.5

Discussion

An inside-out PEG βXL-Hb polymer was successfully produced by reacting the Cys β93 residue of the Hb tetramer with an 8-armed PEG. The SDS-PAGE of βXL-Hb (Fig. 13) showed a band at 30.0 kDa indicative of the β subunits covalently linked together by bis-(3,5-dibromosalicyl)fumarate (DBSF) under oxy-conditions while the band at 14.4 kDa was due to the α monomers. The PEGylated product showed the absence of the 30.0 kDa band, specific for the βXL subunits, and the presence of a new band at the origin suggesting successful PEGylation. The conserved presence of the α subunit band at 14.4 kDa in the PEG βXL-Hb sample indicates a lack of PEGylation on these subunits. The Cys β93 residue is the only cysteine available in bovine Hb for thiol-maleimide click chemistry. Unlike human Hb, bovine Hb has only one Cys residue on each β chain for thiol-maleimide specific click chemistry; there are no Cys residues on the α chains of bovine Hb.

It has been found that increasing the Hb polymer size is one way to counteract vasoactivity (Harrington and Wollocko 2011; Martini et al. 2006; Matheson et al. 2002; Palmer 2006; Palmer and Intaglietta 2014). A larger polymer will move in the center of the blood flow preventing NO scavenging from endothelial tissue (Palmer 2006; Palmer and Intaglietta 2014). Inside-out PEGylation was used to produce a Hb nanoparticle. Polymer size determination and characterization were accomplished by gel filtration and AUC. It was found that the samples were heterogeneous, but reproducible in their pattern of heterogeneity (Figure 16A). The polymeric species could not be resolved with S-500 size exclusion chromatography (Figure 15). The calculated apparent molecular weight of the sample was around 500 kDa based on the standard curve from protein standards. However, the elution volume was nearly identical to that of the apoferritin standard (443 kDa). While these results would indicate a complex of 7 Hb : 1 PEG, the apparent molecular weight determined by this technique does not represent the actual polymer mass due to the large hydrodynamic volume of the PEG. Previous PEGylated HBOCs have been shown to have a large increase in their volume and hydrodynamic radius. The volume can increase over ten times what it was originally while the hydrodynamic radius can more than double when compared to that of native hemoglobin (Hu et al. 2007; Li et al. 2008; Meng et al. 2014; Wang et al. 2014). In addition, appropriate standards for the PEGylated samples are not available. As a result, PEGylation would be expected to yield a larger apparent molecular weight by SEC when compared to

traditional protein standards as seen in the product. It is speculated, however, that the data indicates how the polymer size would appear *in vivo*.

Although Hb is in high molar excess for the reaction, an 8 Hb : 1 PEG is not the major polymer species, as shown by the AUC results (Figure 16). After the reaction of a tetramer to one arm of the PEG the probability of an unreacted arm reacting with the other exposed thiol of the same Hb is greatly increased. As seen in figure 16A, 60% of the polymer species possess three to eight Hb tetramers conjugated onto one Mal-PEG backbone. The deconvolution data (Fig. 16B) indicates that there are four predominant PEG βXL-Hb species being produced. Three of the four species have three to six Hb tetramers conjugated to one Mal-PEG. The peak near 2.5S is unconjugated hemoglobin. The data indicates the major product is 3 Hb : 1 PEG complex, shown diagrammatically in Figure 21. The broadening of the major peak in Figure 16B shows the difficulty in separating the 3 Hb and 4 Hb species. Maleimide-derived click chemistry shows a high specificity for thiol groups but is not restricted to these reactions. Maleimide reactions with Lys residues are much less favorable at pH 7.4 than to Cys residues, yet plausible (Dondoni 2008; Hoyle and Bowman 2010; Khan 1985).

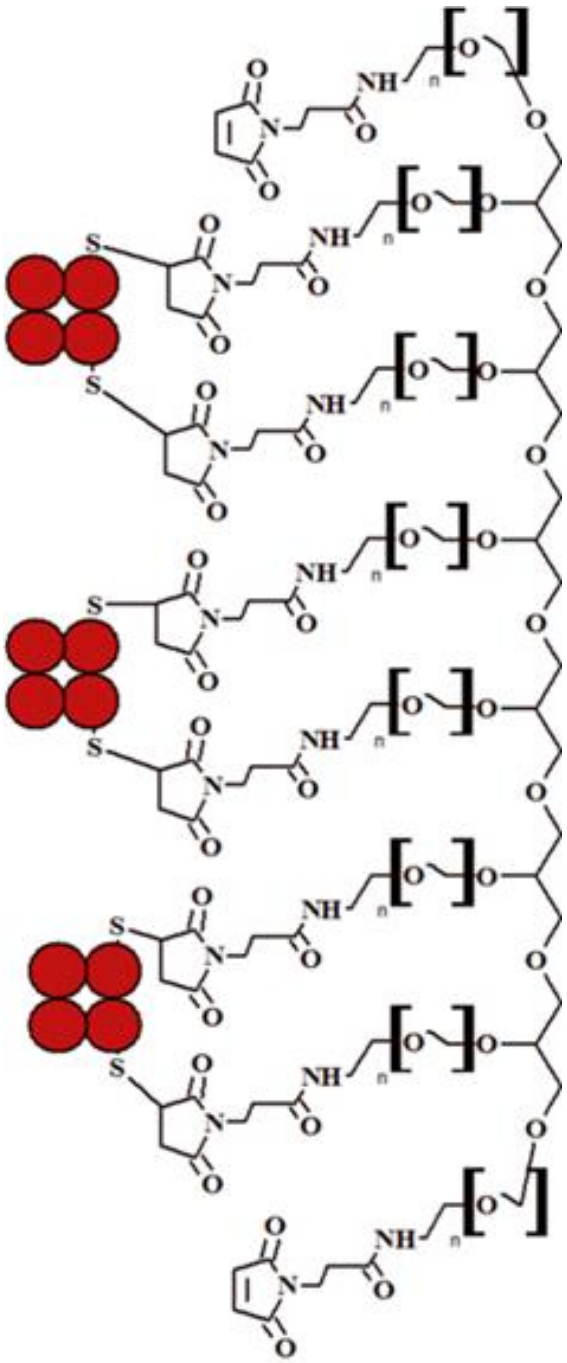


Figure 21. Schematic representation of Inside-out PEGylated crosslinked hemoglobin. While possible to conjugate eight Hb tetramers to one PEG polymer, it is more probable two arms will bind to a single tetramer. The major species produced is depicted above (3 Hb : 1 PEG).

The large size of a 3 Hb: 1 PEG species will likely prevent the HBOC from coming into close proximity with the arterial lining, thus preventing NO scavenging *in vivo* (Palmer 2006; Palmer and Intaglietta 2014). As observed for PEGylated HBOCs of comparable size, the size of inside-out PEGylated Hb should be sufficient to eliminate vasoconstrictive side effects (Bjorkholm et al. 2005; Li et al. 2007; Vandegriff et al. 2003). The increased volume of the PEG βXL-Hb complex will also hinder filtration of the polymer by the kidneys, increasing circulation time *in vivo* (Conover et al. 1997; Vandegriff et al. 2003). Crosslinking of the tetramer prevents its dissociation, reducing concerns of nephrotoxicity. These modifications increase the stability of the Hb tetramer as seen in the fraction denatured results when compared to native Hb (Table 4). Thermal denaturation experiments (Figure 18) showed that crosslinking greatly increases the stability of the tetramer compared to that of native Hb (Native Hb = 41.3°C, βXL-Hb = 54.1°C) (White and Olsen 1987). PEGylation lowers the stability of the tetramer (48.9°C) compared to the βXL-Hb species but the PEGylate species is still more stable than native Hb. This indicates that crosslinking dictates the tetramer stability more than inside-out PEGylation.

While crosslinking and inside-out PEGylation increase the stability of the tetramer, it also increases the autoxidation rate of Hb. It has been previously shown that crosslinking under oxy-conditions with DBSF increases the rate of oxidation (Yang and Olsen 1989). The data presented here agrees with previously reported k_{rel} values and spectral changes affiliated with autoxidation of the heme (see Figure 19 and Table 4). Upon PEGylation, the rate of oxidation increases significantly to

3.43 times that of native Hb. Previous work has shown that PEGylation of Hb increases the rate of autoxidation. Acharya and coworkers showed that PEGylated Hbs exhibit a 2-3 fold increase in oxidation rates (regardless of the methodology used for PEGylation) when compared to native Hb (Hu et al. 2008). It is also known that conjugation of PEG onto Cys β93 shows a faster rate of oxidation when compared to PEGylated Hb species that are modified on Lys residues (Hu et al. 2008; Wang et al. 2014). Cys β93 is adjacent to the proximal His that coordinates with the iron in the heme. PEGylation on this residue perturbs the heme environment allowing a larger number of water molecules to enter and facilitate oxidation. In addition, PEGs also have the unique property to sequester approximately 2-3 water molecules per repeating unit (Hu et al. 2005), increasing the odds for water-mediated oxidation. However, it has been speculated that the rate of autoxidation will be less *in vivo* due to antioxidant and reducing agents present within plasma (Hu et al. 2008; Snyder et al. 1987; Yang and Olsen 1989).

Addition of IHP with no observable shift in oxygen affinity for βXL-Hb verified the success of the oxy-crosslinking procedure between the β82 Lys residues that blocks the allosteric binding site (Walder, Walder, Arnone 1980). The Cys β93 residues are exposed only within the R state tetramer, and conjugation to this site produces a higher oxygen affinity Hb (Abraham, Phillips, Kennedy 1983). Previous studies also demonstrated that an increased oxygen affinity was a byproduct of PEGylation, independent of the conjugation site (Li et al. 2009). The data presented here show that the conjugation of PEG to Cys β93 does not further increase the

oxygen affinity if the Hb is previously crosslinked in a high oxygen affinity state, unlike other PEGylated HBOCs (Table 1). βXL-Hb stabilizes the R state conformation of the tetramer, increasing oxygen affinity in comparison to stroma-free Hb. Negligible changes were found between P_{50} values for βXL-Hb (9.16 torr) and PEG βXL-Hb (9.67 torr). Other PEG-based HBOCs have exhibited large increases in oxygen affinity post crosslinking and PEGylation (P_{50} from 3-6 Torr) (Hu et al. 2007; Li et al. 2008; Li et al. 2009; Lui, Dong, Kluger 2008; Lui et al. 2012). Crosslinking and inside-out PEGylation dramatically decrease the cooperativity of the Hb tetramer (Table 4). There were negligible differences between the three samples CD spectra indicating that crosslinking and PEGylating hemoglobin do not significantly change the secondary structure of the protein (Figure 17) (Sreerama, Venyaminov, Woody 2000; Whitmore and Wallace 2004; Whitmore and Wallace 2008). Thus, the loss of cooperativity is not a result of secondary structure conformational changes, but rather from chemical modifications. Crosslinking of the tetramer restricts the movement needed for cooperativity, while PEGylation may further restrict quaternary structural change. The heightened oxygen affinity ensures that the polymers will be fully oxygenated leaving the lungs and will only deliver it to the most oxygen deficient tissues. This in turn will help prevent tissue necrosis in patients who have lost severe amounts of blood.

The inside-out PEG βXLHb presented here represents both a poly Hb and a dendrimeric structure. Several other poly or dendrimeric hemoglobins have been produced by both genetic and chemical means. Genetically, placements of Cys

mutants at specific positions have led to the production of polymeric dimers, trimers, and octamers of Hb tetramers (Bobofchak et al. 2003; Fablet et al. 2003; Faggiano et al. 2011; Fronticelli et al. 2001; Vasseur-Godbillon et al. 2006). The largest of these was ~500KDa in mass and had a diminished pressor compared to cross-linked tetrameric Hb, presumably due to a decreased extravasation (Bobofchak et al. 2003). Thus, the same result is expected for the PEGβHb reported here. Kluger and Zhang (Kluger and Zhang 2003) have reported the synthesis of a Hb dendrimer made by attaching β cross-linked Hb to generation 4.0 polyamidoamine dendrimer (PAMAM). As expected the product had very high oxygen affinity. Kluger and co-workers (Singh et al. 2015b) also produced bis-tetrameric Hb using double cross-linking and click chemistry. The advantage of these approaches is the specificity of the connections between the Hb tetramers making the polyHb, but the disadvantage is the amount of genetic manipulation or chemical synthesis needed to produce such products. Inside-out PEGylation offers the possibility of making similar sized polyHbs with commercially available reagents. In addition, the methods presented here should be easily applicable to many other proteins.

Conclusions

We have successful produced an inside-out PEGylated XL-Hb complex. Inside-out PEGylation has been shown to maintain secondary structure, increase protein stability, without altering the P_{50} value compared to the native protein. Unlike previous PEGylated HBOCs, inside-out PEGylation does not dictate protein

function, specifically the oxygen affinity. The functional properties of the polymer are controlled by the method under which Hb was crosslinked. This form of protein modification has applications in many areas of biochemistry beyond HBOC development, such as PEGylation of drug delivery agents and applications with other protein therapeutics. In the past there have been a few dendrimeric Hb particles produced (Fablet et al. 2003; Fronticelli et al. 2001; Kluger and Zhang 2003). However, none of these have utilized multi-arm PEGs in their production. Until now multi-arm PEGs have primarily been utilized in the creation of hydrogels and biofilms (Guebeli et al. 2012; Meyerbroeker, Kriesche, Zharnikov 2013). The results reported here introduce applications of multi-arm inside-out PEGylation that can be used in protein chemistry and enzymology. Applications of inside-out PEGylation could be expanded to the production of multi-enzyme complexes. Inside-out PEGylation may be efficacious in biomacromolecular production and in bioconjugate chemistry.

CHAPTER IV

"INSIDE-OUT" PEGYLATION OF BOVINE α-CROSSLINKED HB VIA STRAIN PROMOTED AZIDE-ALKYNE CYCLOADDITION

Chapter Introduction

A viable alternative to current blood transfusions has been actively pursued for several decades with varying degrees of success. A limited supply of blood, dependent on human donors, has necessitated the creation of a hemoglobin-based oxygen carrier (HBOC) derived from non-human sources. Such a product would be responsible for carrying oxygen to hypoxic tissues and might be useful as a response to hemorrhagic trauma. However, a HBOC has a utility that exceeds applications in hemorrhagic shock. It may also be used to treat carbon monoxide poisoning, acute anemia, organ ischemia, and cardioplegia—all of which induce oxygen deprivation (Jahr, Akha, Holtby 2012). The value of such a product would be augmented if it could be minimally refrigerated, non-immunogenic, universal for all blood types, and available at a minimal cost whilst possessing a long shelf life (Estep et al. 2008; Ness and Cushing 2007; Reid 2003; Sakai et al. 2000). All hemoglobin-based oxygen carriers must begin with source of hemoglobin. However, application of stroma-free unmodified hemoglobin (Hb) to a living system results in complications. The Hb tetramer dissociates into two αβ dimers whose small size enables

them to pass into the kidneys where nephrotoxicity is observed. The short circulation time associated with an unmodified Hb tetramer does not mitigate the impact of another harmful effect—vasoconstriction induced by the scavenging of nitric oxide, a natural vasodilator found in the endothelial walls (Doherty et al. 1998; Muldoon et al. 1996; Reid 2003). The avoidance of vasoconstriction and tetramer dissociation while maintaining the ability to deliver oxygen efficiently has become the basis of all HBOC development.

Crosslinking of extracted hemoglobin via the alpha subunits is one approach at preventing tetramer dissociation *in vivo*. This may be accomplished by the use of a crosslinking reagent, such as bis-(3,5-dibromosalicyl)fumarate (DBSF), under deoxy- conditions (Tye et al. 1983; Zaugg, King, Klotz 1975). Crosslinking of the Hb tetramer under oxy- conditions using this same reagent likewise prevents dissociation of the tetramer. However, these two modifications have opposite effects on the affinity with which the HBOC binds oxygen (White and Olsen 1987; Yang and Olsen 1991). HBOC polymer production methodologies have ranged from dentrimeric Hb, liposomal encapsulated Hb, bis-tetrameric Hb, and PEGylated Hb particle creation (Agashe and Awasthi 2009; Bradley et al. 2002; Fablet et al. 2003; Fronticelli et al. 2001; Kluger and Zhang 2003; Lui et al. 2012; Xiong et al. 2012). Production of a HBOC with a large molecular weight—by polymerization of Hb or conjugation of Hb to a polyethylene glycol (PEG) backbone—has been shown to attenuate vasoconstriction as seen with Hemospan produced by Winslow and colleagues (Bjorkholm et al. 2005; Vandegriff et al. 2003; Vandegriff and Winslow

2009). It has been found that a large polymer that cannot get close enough to the endothelial lining, thus preventing harmful NO scavenging (Martini et al. 2006; Palmer 2006; Palmer and Intaglietta 2014). Furthermore, polymers of large molecular weight, such as the Oxy Vita product developed by Bucci and colleagues (Matheson et al. 2002), have been shown non-vasoactive *in vivo* (Harrington and Wollocko 2011; Matheson et al. 2002). One method to generate a polymer with a large molecular volume is through PEGylation. PEG has been shown to sequester two to three water molecules per repeating unit, which can dramatically increase the hydrodynamic volume and polymer radius (Alayash 2004; Hu et al. 2005). General PEGylation of proteins has also increased the therapeutic value of the product by masking the protein from the immune system and preventing filtration through the kidneys (Bailon and Berthold 1998; Harris and Chess 2003; Roberts, Bentley, Harris 2002). Varying the size of the PEG chains, the number of chains conjugated to a protein, and the sites and methods of conjugation of PEG to Hb modulate the effects of PEGylation of a protein to different degrees (Hu et al. 2007; Li et al. 2008; Li et al. 2009). For example, conjugation of six PEG chains to a single Hb tetramer increases the oxygen affinity (Li et al. 2007). It has also been hypothesized that conjugation of Hb to a PEG mass greater than 50 kDa may counteract the vasoactivity of stroma free Hb *in vivo* (Rohlfs et al. 1998).

This paper offers a modification of the unique scheme of "inside-out" PEGylation presented previously in Chapter 3 of this dissertation. The basic

stratagem of conjugating several bovine cross-linked Hb tetramers to a single eight-arm 40 kDa PEG backbone remains unchanged. However, the PEG is conjugated to the Lysine residues of both the alpha and beta subunits of the tetramer. Such a conjugation necessitates two steps. Firstly, the Lysine ε-amino groups must be activated by reaction with dibenzocyclooctyne-*N*-hydroxysuccinimidyl ester (DIBO-NHS) via an NHS-ester mediated amidation. The alkyne of the subsequent product is then reacted with the azide group conjugated to one arm of the PEG backbone (N_3-PEG). In the past, copper (Cu^+) mediated azide-alkyne cycloaddition (CuAAC) has been previously utilized to produce a bis-tetrameric hemoglobin oxygen carrier (Foot, Lui, Kluger 2009; Singh et al. 2015a; Wang and Kluger 2014; Yang and Kluger 2010). These previous CuAAC have been efficacious in Hb polymer creation. However, the reaction presented here is not done via traditional copper mediated azide-alkyne click chemistry but rather by strain promotion found within the alkyne (Campbell-Verduyn et al. 2011; Gold et al. 2012). This removes any possible contamination of the final product by copper, which may have adverse medical effects (Campbell-Verduyn et al. 2011; Gold et al. 2012). Figure 22A shows the activation of a Lys on Hb, while Figure 22B shows the strain promoted azide-alkyne cycloaddition to achieve inside-out PEGylation. We believe this product will be a successful HBOC candidate to deliver oxygen nondiscriminatory to tissue rather than just to hypoxic tissue.

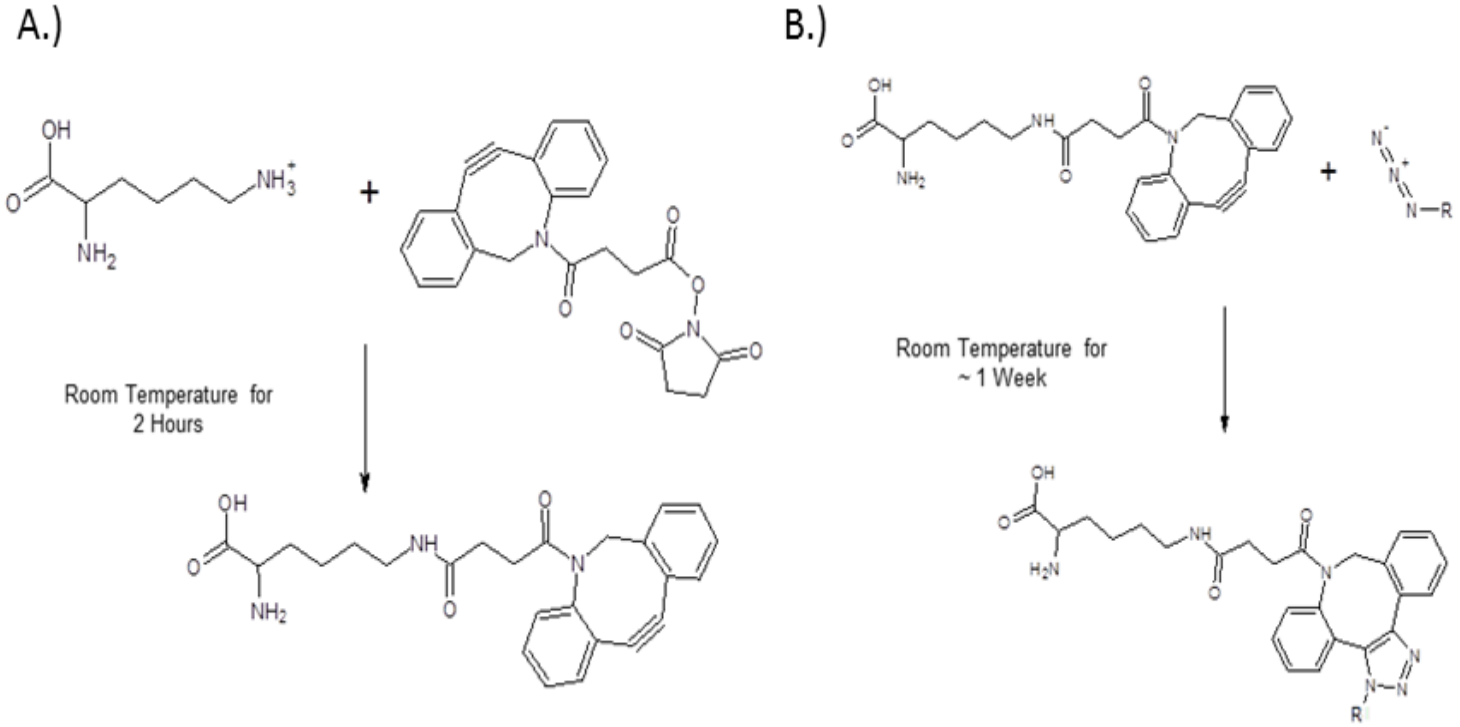


Figure 22. (A) Activation of an exposed Lys residue on Hb with DIBO-NHS by NHS-ester mediated amidation. (B) PEGylation via strain promoted cycloaddition of activated Hb Lys residue with the azide PEG where R is the PEG chain/backbone.

Materials

Untreated bovine blood was obtained from Animal Technologies (Tyler, TX). Eight arm N_3-PEG was purchased from Creative PEGWorks (Winston Salem, NC). Sephadex chromatography media were purchased from GE Healthcare Biosciences (Pittsburg, PA). Micro separatory centrifuge filters (300 kDa) were purchased from Sartorius Stedim (Bohemia, NY). Hemox Antifoam and the HEMOX oxygen analyzer were purchased from TCS Scientific (New Hope, PA). DBSF was made by the method of Zaugg *et al (*Zaugg, King, Klotz 1975). DIBO-NHS, all other chemicals, and gel filtration markers kit for protein molecular weights 29 kDa - 700 kDa were purchased from Sigma-Alrdrich Co. (St. Louis, MO).

Methods

Crosslinked Hemoglobin Synthesis

Methods described by Hanash and Shapiro (Hanash and Shapiro 1981) and Dozy et al. (Dozy, Kleihauer, Huisman 1968) were modified for the purification of Hb from bovine blood. Sephadex A-50 gel in 50 mM Tris, 1 mM NaCN, pH 8.5 buffer was added to centrifuge tubes. Hb was mixed thoroughly with the gel and centrifuged. The process was repeated three to five times until the supernatant coming off the gel was still red from Hb. The supernatants were then pooled and stored in the refrigerator until crosslinking. This method allows for rapid purification of large quantities of bovine Hb.

The procedure for crosslinking deoxy-hemoglobin was carried out according to Walder et al. (Walder et al. 1979). Bis-(3,5-dibromosalicyl)fumarate (DBSF) was reacted with deoxy-hemoglobin at a mole ratio of 1 Hb: 1.2 DBSF for four hours under wet nitrogen. The solution was then dialyzed in a 50 mM Tris, 1 mM NaCN, pH 8.5.

Sephadex A-50 was swelled in 50 mM Tris, 1 mM NaCN, pH 8.5 buffer and poured onto a Kontes Chromaflex column using a decreasing pH gradient. The SDS-PAGE protocol was followed according to Schmidt et al. (Schmidt and Brosious 1979) and showed equal intensity bands at 14.4 kDa and 30 kDa.

Reduction of Methemoglobin to Oxyhemoglobin

The procedure used was a modified version of Dixon and McIntosh's method (Dixon and McIntosh 1967). A Kontes column, 25 cm x 1.0 cm, was loaded with G-25 gel purchased from GE. The column was then equilibrated with 5 mM Phosphate Buffered Saline Solution (PBS), pH 7.0, 1 mM EDTA buffer. Following equilibration, 0.2 g of sodium dithionite was added to 2.0 mL of the same buffer, and 0.4-0.6 mL of this solution was then added to the column followed by addition of 0.2-0.4 mL of the aforementioned buffer. A 5 mL sample of Hb was then added to the column and allowed to pass through the gel.

Activation and PEGylation of Alpha Crosslinked Hemoglobin

αXL-Hb was dialyzed in 50 mM MOPS, pH 7.2 buffer. The sample was then passed through a 0.2 micron filter to remove any debris or bacteria. The

concentration of αXL-Hb was determined spectrophotometricaly to activate with Dibenzocyclooctyne-*N*-hydroxysuccinimidyl ester (DIBO-NHS) at a mole ratio of 1 Hb : 1 DIBO-NHS or 1 Hb : 2 DIBO-NHS. The DIBO-NHS was first suspended in dried DMSO equal to 1% of the total reaction volume before being added to the αXL-Hb. The reaction was allowed to proceed for two hours at room temperature. Post activation, the sample was spun in a 30 kDa centrifugal separatory filter (Sartorius Stedim) at 4000 *g* and washed with 50 mM MOPS, pH 7.2 to remove any unreacted DIBO-NHS. The sample was then reacted with the 40 kDa eight arm azide-PEG at a 16 Hb : 1 N_3-PEG mole ratio. The reaction continued for one week either at room temperature or in a refrigerator. Following PEGylation, the PEG αXL-Hb solution was placed in a 300 kDa micro separatory centrifuge filter and spun at 2950 *g*.

Purification and Verification of PEGylation Alpha Crosslinked Hemoglobin

Concentrated samples, post centrifugation, were run on a pre-packed Sephacryl S-300 column equilibrated with 50 mM MOPS, pH 7.2 buffer. The large molecular weight PEGylated αXL-Hb fractions were collected and concentrated on a pre-packed Sephacryl S-500 (GE) for further purification and size determination. A standard curve was produced for the S-500 column using large molecular weight markers.

SDS-PAGE Experiments with PEGylated Hemoglobin

A 10 μL aliquot of PEG αXL-Hb was added to 10 μL of denaturing solution containing bromophenol blue in an Eppendorf tube. The same volume ratio was

used to make up αXL-Hb sample and protein marker. The samples were then placed in a boiling water bath for ten minutes to allow full denaturation. The samples were loaded on a 12.5% SDS homogenous gel. Following the run, the gel(s) was then placed in an auto-developer that stained, de-stained, and preserved the SDS gel (Schmidt and Brosious 1979).

Determination of Molecular Weight via Analytical Ultra Centrifugation (AUC)

AUC was carried out in a Beckman Coulter ProteomeLab XL-A analytical ultracentrifuge using standard 2 sector cells with quartz windows in 4- or 8-hole rotors. All sedimentation velocity experiments were at 50,000 rpm with a preset scan number of 100 per cell. The reference sector was filled with 450 µL of buffer (50 mM MOPS, .15 M NaCl, pH 7.4) and the sample sector was filled with 430 µL of sample with an absorbance at 412 nm (1 cm path length) between 0.75 and 1.1. Buffer density (1.00718 g/mL) was measured at 20.0° C in a Mettler/Paar Calculating Density Meter DMA 55A and buffer viscosity (0.010543 Poise) was measured in an Anton Parr AMVn Automated Microviscometer. A partial specific volume of 0.746 mL/g for bovine hemoglobin was used in calculating molecular weight (DeMoll et al. 2007). Data were analyzed using the program Sedfit (www.sedfit.com). Molecular weights and sedimentation coefficients were determined using the continuous c(s) mode in the sedfit program. Native tetrameric hemoglobin yielded an $s_{20,w}$ value of 4.25 S which agrees with the accepted value (Field and O'Brien 1955). The c(s) plots were deconvoluted using nonlinear least-

squares fitting algorithms available in the Origin (v. 9.0) software package (OriginLab Corporation, Northapmton, MA). Data were fit to multiple Gaussian components, increasing the number of components until residual plots (data-fit) were random and the chi square value for the fit was minimal.

Circular Dichrosim (CD) Secondary Structure characterization of PEG αXL-Hb

Circular Dichroism (CD) experiments were carried out on an OLIS DSM 20 CD Hummingbird Spectrophotometer. Water used to collect base line data was also used as the dilution solvent for the Hb samples. For each sample a wavelength range of 180-280 nm was monitored and one data point per nm was collected while integration time was a function of high volts. The spectra were then smoothed using Solvitsky Golay 11 point (Whitmore and Wallace 2004; Whitmore and Wallace 2008).

Thermal Denaturation Procedure

Experiments were conducted according to Olsen's protocol (Olsen 1994). Experiments were conducted in 10 mM MOPS, 0.9 M guanidine HCl pH 7.0. Samples were prepared so that the absorbance at 409 nm was between 1 and 1.2. Samples were monitored every minute on an Agilent 8453 spectrophotometer. A temperature increase of 0.3°C/ min was controlled by Neslab Endocal, Temperature Programmer ETP-3, and Digital Controller/Readout DCR-4. Absorbance versus temperature was plotted and the fraction denatured was determined by the equation $F_D = (A-A_N) / (A_D-A_N)$ where A is experimental absorbance, A_N is

absorbance of native protein, and A_D is absorbance of denatured protein. A_N and A_D were determined by fitting a linear line to the portion of the absorbance versus temperature plot that corresponded to the effect of temperature on the native or denatured spectrum respectively. The curve of F_D versus temperature was smoothed in Microsoft Excel.

Autoxidation Experiments

Autoxidation of native, crosslinked, and PEG crosslinked hemoglobin was carried out according to Yang and Olsen (Yang and Olsen 1989). Hemoglobin samples were fully reduced and dialyzed in 10 mM MOPS, pH 7.0. Sodium cyanide was added to each sample and allowed to equilibrate at room temperature for five minutes prior to data collection. The experiments were conducted at 37°C and allowed to equilibrate before spectra were recorded from 500-700 nm on an Agilent 8453 spectrophotometer.

Oxygen Binding and Determination of P_{50} and Hill Coefficients

The samples, fully reduced with dithionite, were added to 4.0 mL of 10 mM MOPS, pH 7.4 and 10 μL of Hemox Antifoam and equilibrated to 37 °C. Oxygen binding was determined using a HEMOX Analyzer for native bovine Hb, native Hb with IHP, αXL-Hb, αXL-Hb with IHP, and PEG αXL-Hb. Hill coefficients (n) and oxygen affinity (P_{50}) were obtained from Hill plots.

Results

Crosslinked Hemoglobin (αXL-Hb) Production and Purification

Presence of αXL-Hb product was verified by SDS-PAGE that showed distinct, equal intensity bands around 14.4 kDa and 30.0 kDa (Figure 23). The band at 30.0 kDa was indicative of the α monomers covalently linked together by DBSF under deoxy-conditions while the band at 14.4 kDa was representative of the β monomers.

Confirmation of Large Molecular Weight Species and Separation of PEG αXL-Hb from Unreacted αXL-Hb

Figure 23 is the SDS-PAGE gel of the purified PEG αXL-Hb sample with a new band at the origin of the gel. The presence of this band confirmed large molecular weight species. Figure 24 is a S-300 normalized chromatogram, while Figure 25 is a chromatogram of the first peak of the S-300 column on a S-500 column. This two phase separatory system did not resolve the different PEG αXL-Hb species. There was a noticable separation between the PEGylated and unreacted species. Protein standards were run, and the elution volume of the PEGylated species resembled that of apoferritin (MW= 443 kDa) (Fig. 25). The elution volume of the product was used in tandem with an experimental standard curve that gave a calculated apparent molecular weight of ~ 500 kDa. This value correlates to a product that has 7 Hb : 1 PEG backbone. Regardless of the DIBO : Hb activation ratio, there were no differences in the elution volumes of the product.

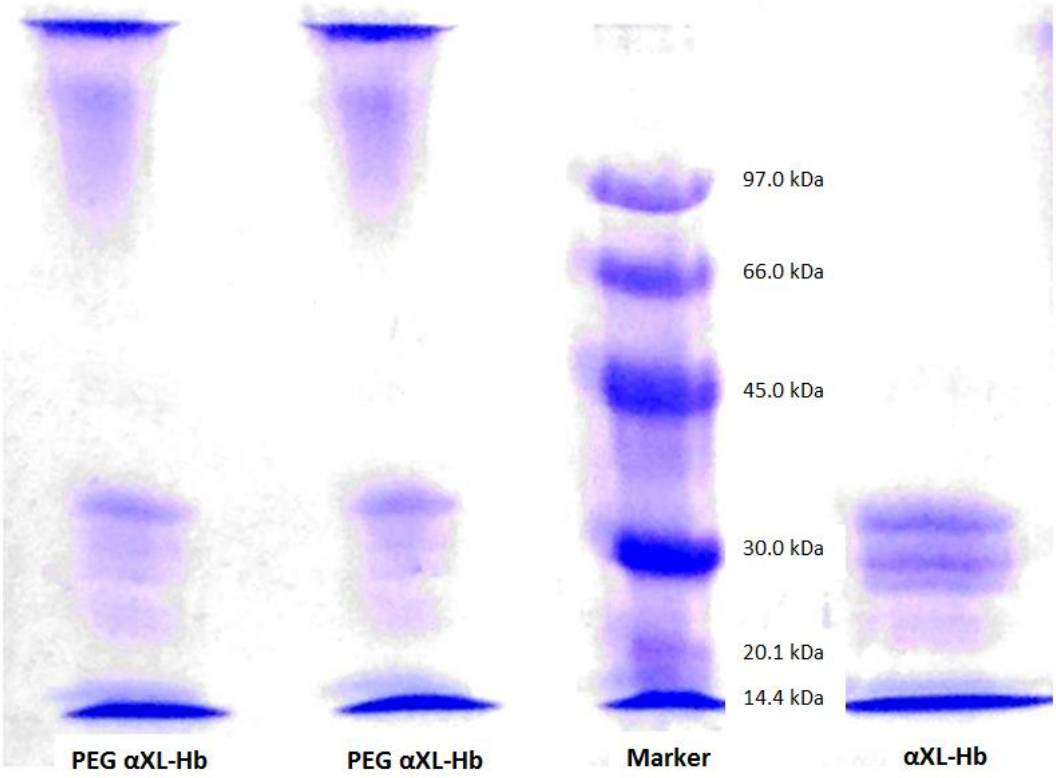


Figure 23. SDS-PAGE results of αXL-Hb and PEG αXL-Hb. The αXL-Hb shows characteristic bands at both 14.4 kDa (β monomers) and 30.0 kDa (XL α monomers). PEG αXL-Hb shows the same characteristic bands along with the presence of a dark band at the origin of the gel. Presence of the 14.4 and 30.0 kDa bands in the PEGylated sample indicate that the activation of Hb via DIBO-NHS and subsequent PEGylation do not show specificity towards either the α or β monomers of Hb, a consequence of the high amount of exposed Lys residues.

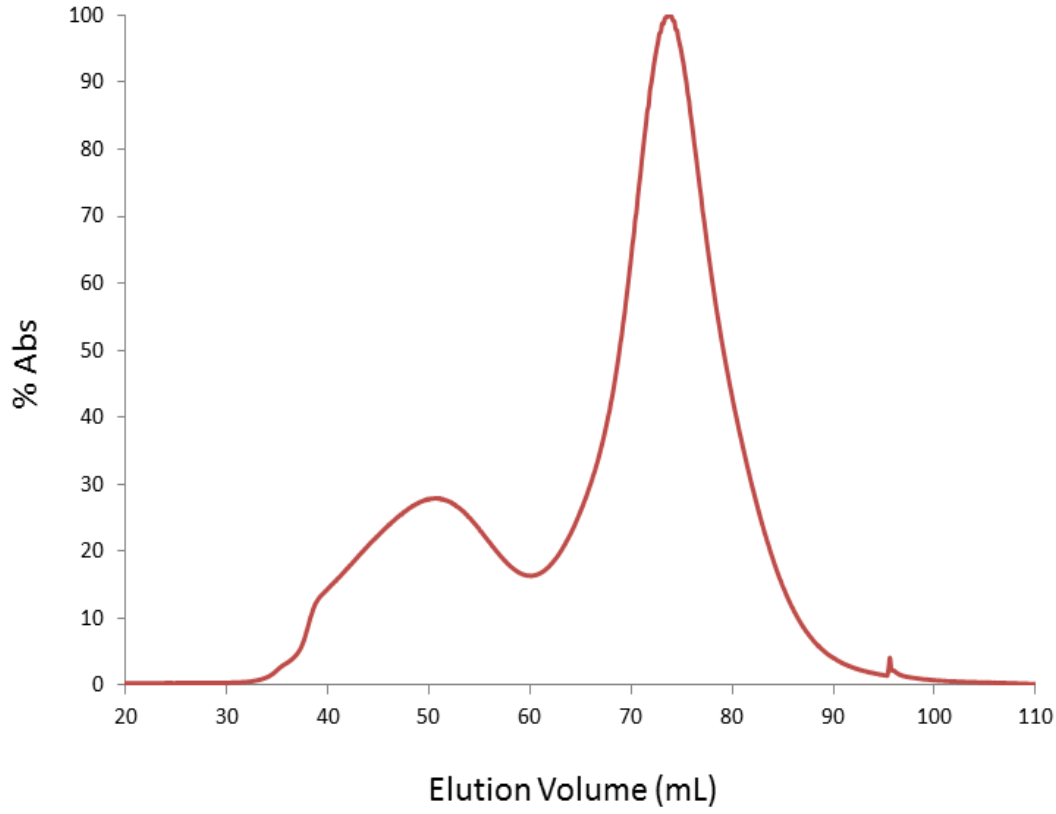


Figure 24. S-300 normalized chromatogram. Unreacted αXL-Hb eluted around 75 mL while the larger PEG αXL-Hb product eluted between 40 and 60 mL.

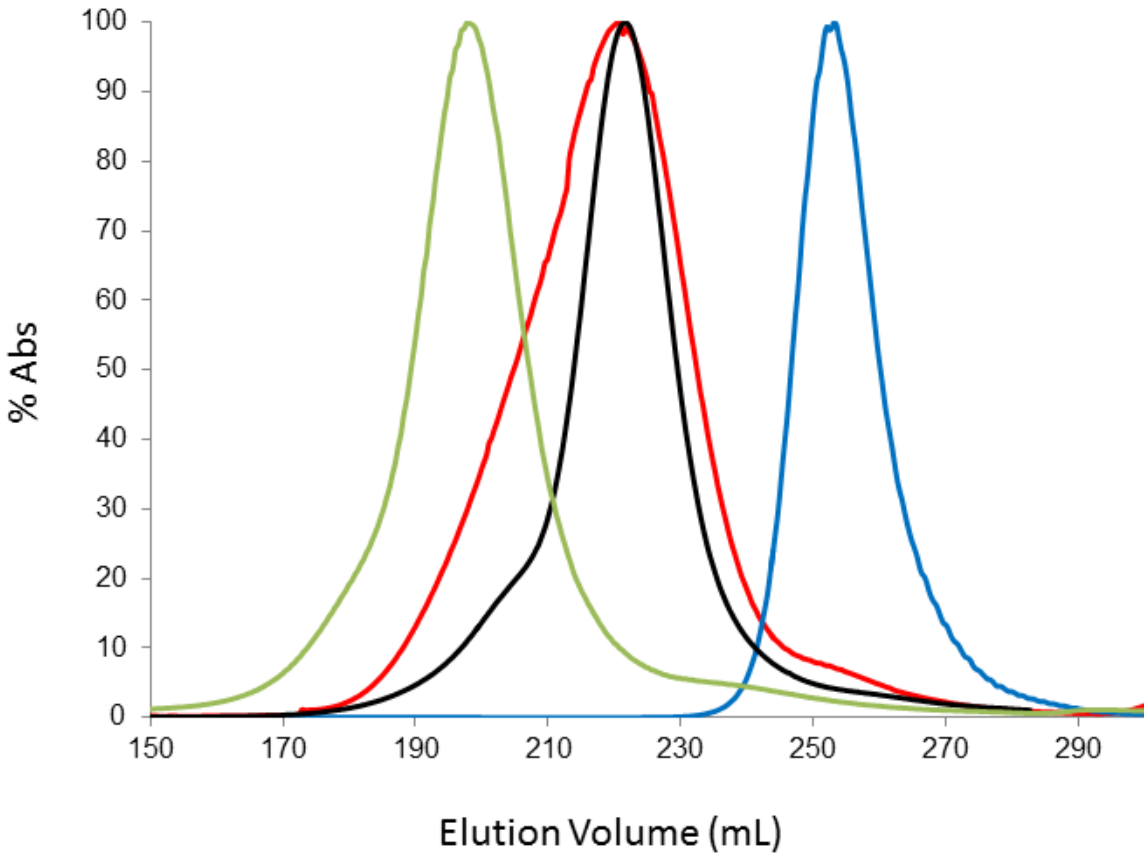


Figure 25. An overlay of the chromatograms from S-500 SEC of PEG αXL-Hb (Red), unreacted αXL-Hb (Blue) and two standards, apoferritin (Black) and thyroglobulin (Green). An elution volume of 220 mL represents the PEG αXL-Hb sample while an elution volume of 250 mL is seen for the αXL-Hb sample. A large amount of the PEGylated product has an apparent molecular weight similar to apoferritin (MW of 443 kDa). However, the broad shouldering on the front side of the column could be resultant of larger MW species whose apparent weight is lower than that of thyroglobulin (MW 669 kDa).

Size Determination of PEG αXL-Hb Samples via Analytical Ultracentrifugation (AUC)

The samples from the S-500 SEC column were analyzed by AUC. Figure 26A shows three separately prepared samples with a 1:1 DIBO to Hb activation ratio while Figure 26B is a deconvolution of one of the data sets. It was found that there were three major high molecular weight PEGylated species. The predominant species appeared to contain 3-4 Hb tetramers per PEG. Figure 27A shows three separate PEG αXL-Hb samples that were prepared with a 2:1 DIBO to Hb activation ratio. The samples were reproducible in their heterogeneity even more so than the 1:1 activation. Figure 27B is a deconvolution of one of the samples. It was found that there were four large molecular weight PEGylated species, with the predominant one being 3-4 αXL-Hb tetramers per PEG.

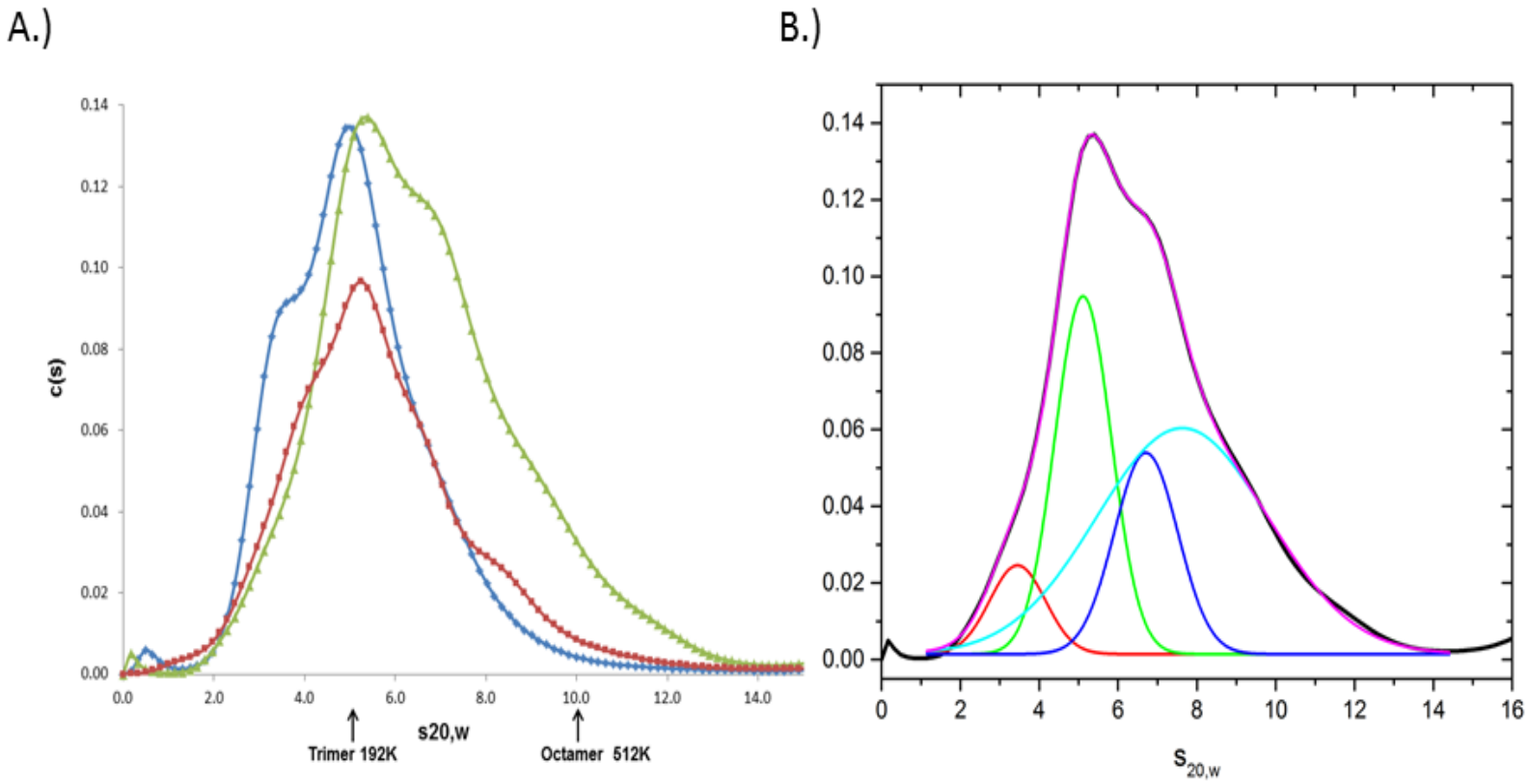


Figure 26. AUC size determination of 1 DIBO : 1 Hb. (A) c(s) versus $s_{20,w}$ plot of three different PEG αXL-Hb. The samples do not show similar heterogeneity, however the major species for all the samples is 3-4 αXL-Hb tetramers per PEG backbone. (B) A deconvoluted c(s) versus $s_{20,w}$ plot from one of the samples in figure 26A. There are three predominant large MW species ranging from 3 to 5 αXL-Hb per PEG backbone.

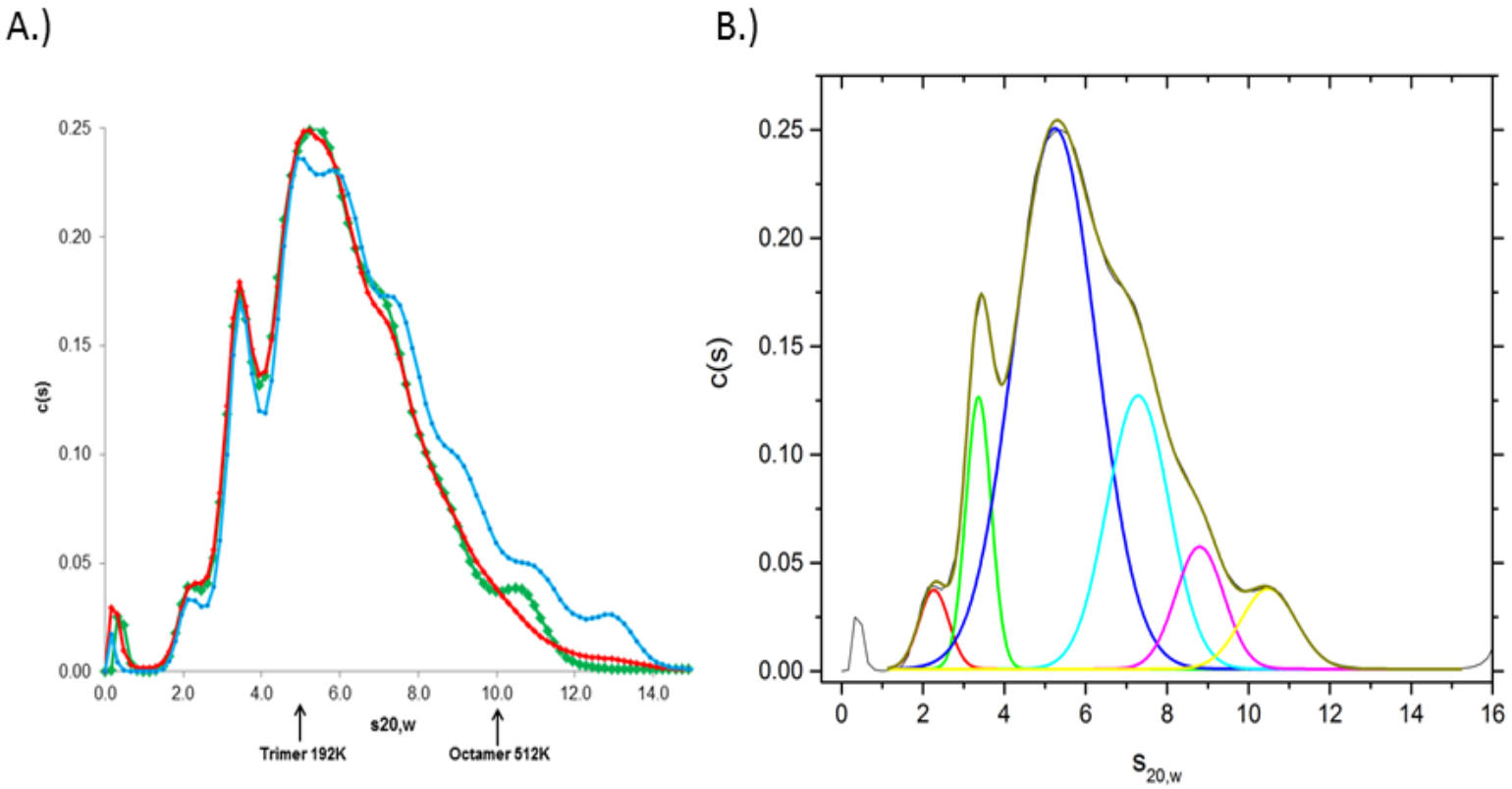


Figure 27. AUC data for 2 DIBO : 1 Hb. (A) c(s) versus $s_{20,w}$ for three individual PEG αXL-Hb samples. The samples are heterogeneous in their speciation but are reproducible in their heterogeneity. (B) A deconvoluted c(s) versus $s_{20,w}$ plot from one of the samples from figure 27A. There are four large molecular weight species ranging from 3 to 8 αXL-Hb tetramers per PEG backbone. The peak near 2.5S is unconjugated hemoglobin.

CD Analysis of Secondary Structure in PEG αXL-Hb

CD analysis of the Hb samples showed characteristic signals for alpha helical secondary structure (Fig 28). The presence of the crosslinker in the central cavity or a PEG backbone attached to the tetramer did not disrupt the secondary structure.

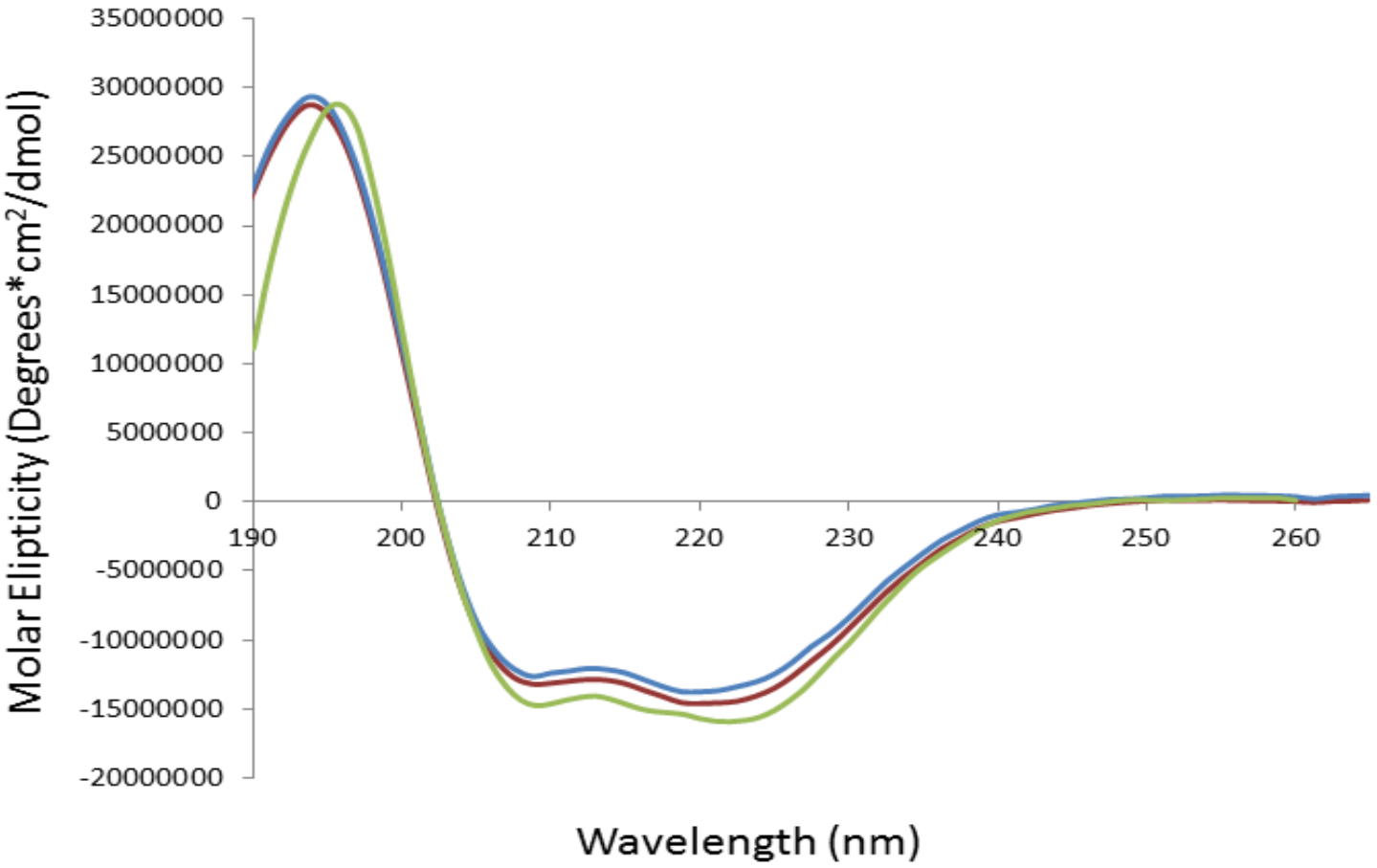


Figure 28. CD spectra for native Hb (green), αXL-Hb (blue), and PEG αXL-Hb (red). All three samples show the characteristic signals at 195 nm, 208 nm, and 222 nm.

Thermal Denaturation of Hb Samples

Comparison of the fraction denatured curves is shown in Figure 29. The melting temperatures (T_m) can be found in Table 5. Crosslinking stabilizes the tetramer compared to native Hb while PEGylation decreases the stability of the crosslinked species. Experimentally determined T_m values were found to be statistically significant in their differences through a single factor ANOVA.

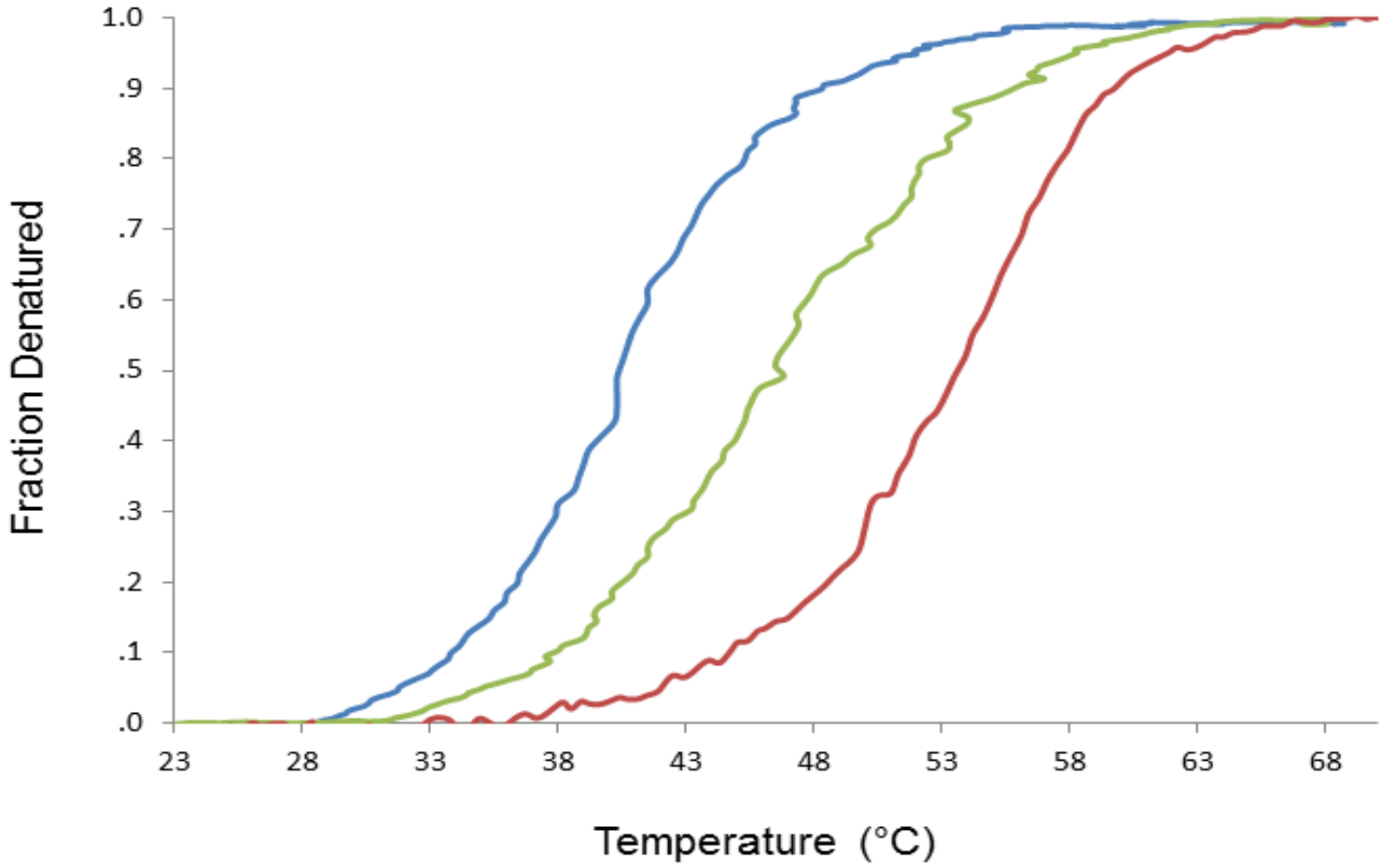


Figure 29. Comparison of fraction denatured curves for native Hb (Blue), αXL-Hb (Red), and PEG αXL-Hb (Green) that were created by following the absorbance at 409 nm.

Autoxidation of Hb Samples

Autoxidation expermients were conducted at 37°C to see the effects of crosslinking and PEGylation on the autoxidation rates at physiological temperatures. Table 5 contains the relative rate constants (k_{rel}) of autoxidation. The relative rate constants were determined by dividing the slopes of methemoglobin production for αXL-Hb and PEG αXL-Hb by the slope of native Hb. It was determined that crosslinking increased Hb autoxidation rate by 1.78 times and PEGylation further increased the autoxidative rate to 2.39 times that of native Hb. Figure 30 shows the spectral changes observed in the PEGylated Hb sample. There was a decrease in the oxyhemoglobin absorbance at 540 and 576 nm as the sample autoxidized.

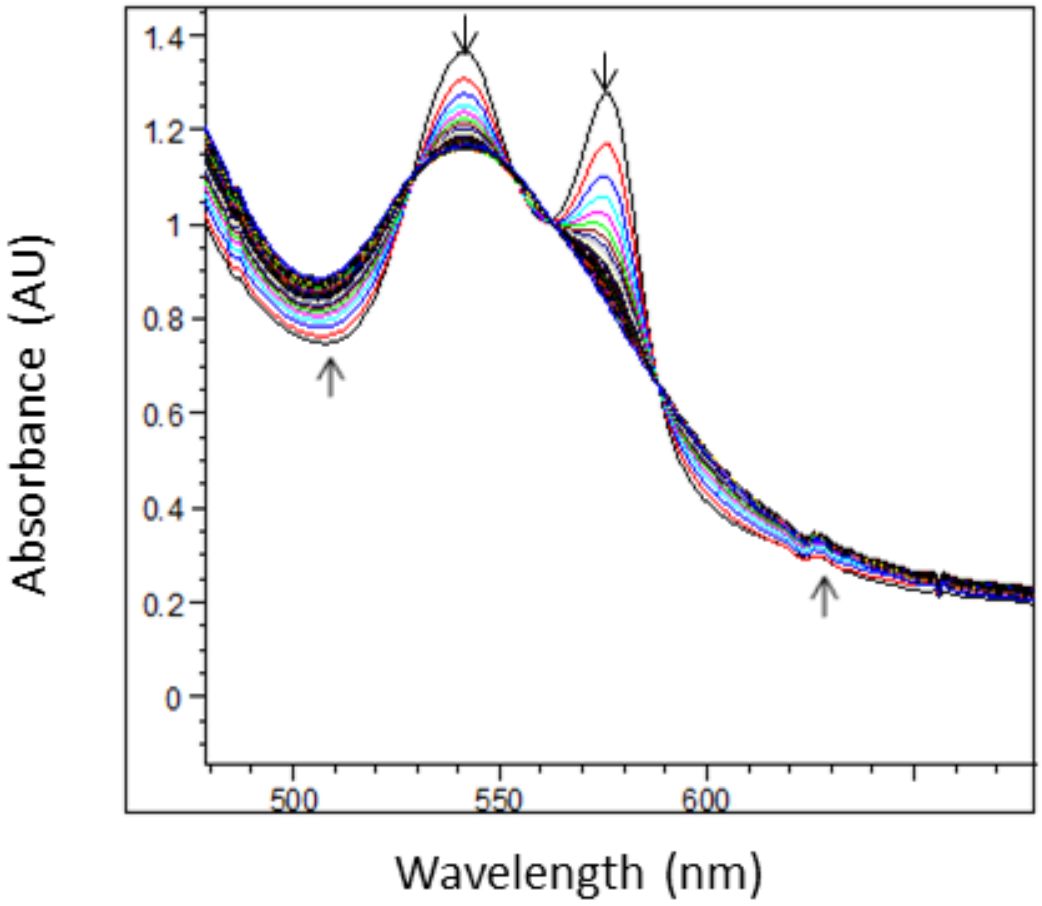


Figure 30. Autoxidation and spectral changes in PEG αXL-Hb sample. The spectra showed a decrease in absorbance at 576 and 540 nm and an increase of absorbance at 630 and 510 nm as the sample was allowed to oxidize.

Oxygen Binding Determination of Hb Samples

Figure 31 shows the oxygen binding curves for the three Hb samples. Table 5 presents the experimental P_{50} values and Hill coefficients (n) that were determined for native Hb, αXL-Hb, and PEG αXL-Hb. It was found that αXL-Hb had a P_{50} similar to that of native Hb within a RBC (P_{50}=27.57). PEG αXL-Hb had a slightly lower

oxygen affinity than αXL-Hb with a P_{50} of 31.97. The difference between the two was negligible suggesting that inside-out PEGylation did not further affect the oxygen affinity of αXL-Hb. Table 5 also shows that upon crosslinking there was a decrease in cooperativity as demonstrated by the reduced Hill coefficient. However, PEGylation did not significantly lower *n* any further.

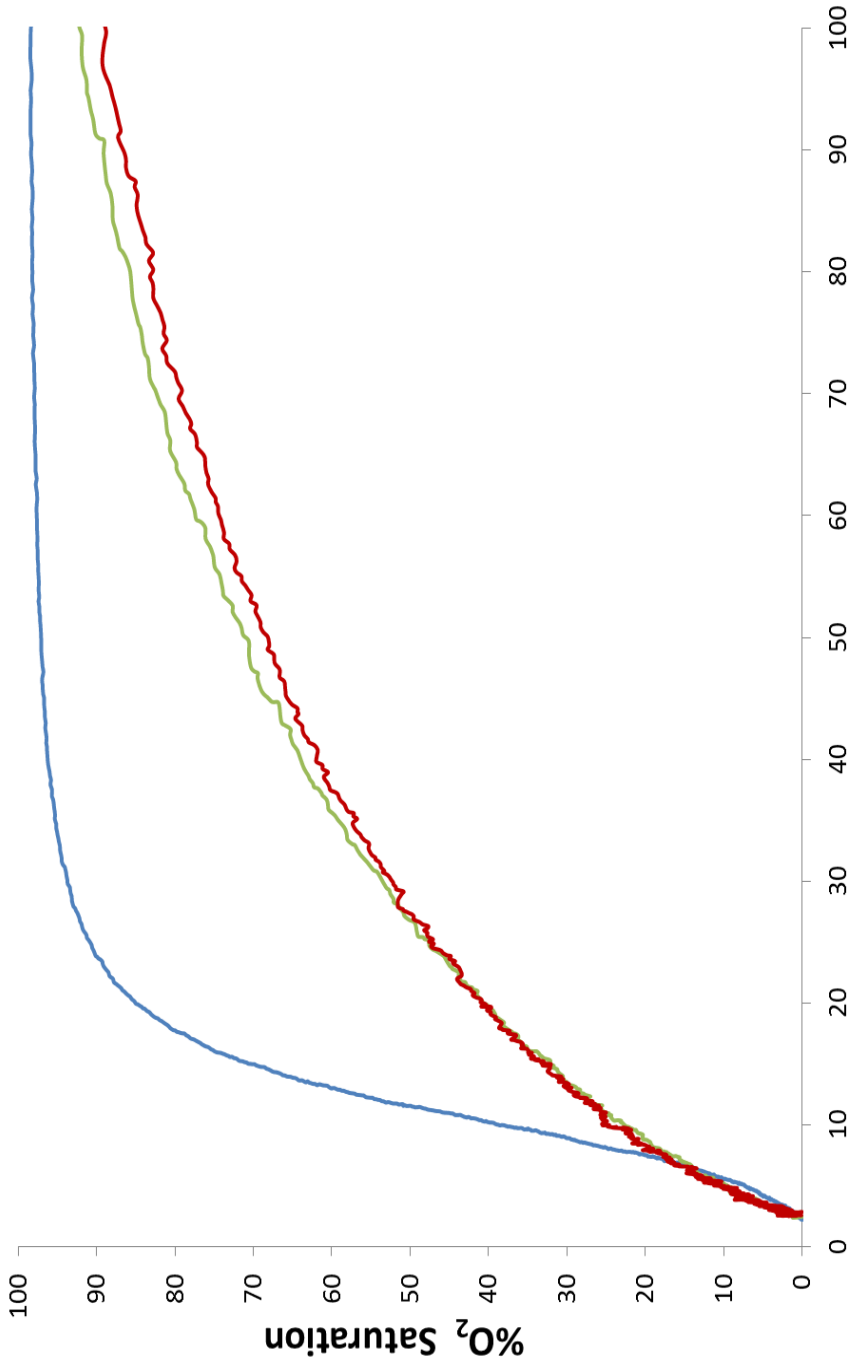


Figure 31. Comparison of native Hb (Blue), αXL-Hb (Green), and PEG αXL-Hb (Red) oxygen binding curves.

Table 5. Experimentally determined T_m (°C) from fraction denatured curves, relative rates of autoxidation at 37°C (k_{rel}), and P_{50} (torr) and Hill Coefficients determined at 37°C for native bovine Hb, αXL-Hb, and PEG αXL-Hb.

Sample	T_m (°C)*	k_{rel}	P_{50} (torr)	Hill Coefficient (n)
Native Hb	41.3 ± .9	1	11.25 ± .17	2.91 ± .06
αXL-Hb	53.5 ± .7	1.78 ± .33	27.57 ± 1.36	1.39 ± .01
PEG αXL-Hb	46.1 ± .7	2.39 ± .34	31.97 ± 4.48	1.26 ± .04

*ANOVA ($F=200.3$, $F_{crit}=5.1$)

Discussion

A large inside-out PEG Hb polymer was successfully produced by modifying exposed Lys residues with a cyclic strained alkyne and PEGylated through copper free azide-alkyne click chemistry. SDS-PAGE verified crosslinking and PEGylation of the Hb species (Figure 23). In the XL-Hb sample, the band at 14.4 kDa is the non-crosslinked β subunits while the 30.0 kDa band is the covalently crosslinked α subunits. These bands were still present in the PEGylated sample with the concurrent presence of a new band at the origin of the gel. This indicates that the activation of Hb via DIBO-NHS and subsequent PEGylation does not occur on only one subunit. This is resultant from the high amount of exposed Lys residues in bovine Hb. However, the α-XL band appears to be lighter in intensity indicating a slightly higher affinity for modification on the α subunits compared to the β subunits. It has been previously reported that there are four Lys residues on the α

and β subunits commonly PEGylated; but by contrast, there is a higher affinity for the α subunits by comparison (Li et al. 2008).

Large Hb polymers have been found to attenuate stroma free Hb's vasoconstrictive properties (Harrington and Wollocko 2011; Martini et al. 2006; Matheson et al. 2002; Palmer 2006; Palmer and Intaglietta 2014). This is achieved through increases in the molecular radius and/or hydrodynamic volume. These size alterations restrict the polymer's ability to come in contact with the endothelial lining, as seen in prior, large, non-vasoactive Hb particles (Matheson et al. 2002). PEG is known to be especially efficacious in achieving these effects. This is resultant from its highly hydrophilic nature and capacity to bind multiple water molecules per repeating PEG unit (Hu et al. 2005). A nine-fold increase in hydrodynamic volume and two-fold increase in molecular radius of PEGylated proteins has been observed and is not uncommon (Meng et al. 2014; Wang et al. 2014). Inside-out PEGylation was used to produce such a Hb polymer. Size determination and characterization were achieved through a two-phase SEC followed by AUC. Separation of XL-Hb and PEG XL-Hb was observed with S-500 SEC but resolution of the polymeric species was not seen (Fig. 25). Regardless of DIBO : Hb activation ratios, there were no differences observed in elution volumes. Furthermore, the elution volume was identical to that of previously reported inside-out PEGylated proteins generated by thiol-maleimide click chemistry (Chapter 3). Figure 25 shows the chromatogram of PEG αXL-Hb and known protein standards. The elution volume of the PEGylated

species resembled that of apoferritin (MW= 443 kDa). According to S-500 SEC, this molecular weight corresponds to a product that has ~7 Hb : 1 PEG backbone. However, this is not an accurate representation of the molecular weight but rather the apparent molecular weight of the polymer in solution. PEG has been shown to increase a proteins hydrodynamic volume up to nine fold as a result of the PEG's ability to retain two to three water molecules per repeating unit (Hu et al. 2005). As expected, PEGylated proteins appear larger than non-PEGylated standards.

AUC was used to characterize the molecular weight and product speciation. It was found that PEG αXL-Hb generated with a 1:1 DIBO-NHS : Hb molecular ratio had three predominant large molecular weight species ranging from 3 to 6 αXL-Hb per PEG backbone (Figure 26). The broad peak in Figure 26B shows the difficulty in delineating between some of the larger polymers. The major product is around 4 Hb : 1 PEG backbone. Production of PEG αXL-Hb with a 2:1 DIBO-NHS : Hb (Fig. 27) showed four large molecular weight species ranging from 3 to 8 αXL-Hb tetramers per PEG backbone with the predominant species being 4 Hb : 1 PEG backbone (Figure 27B). Comparison of the two samples (Figure 26 and 27) shows that the 1:1 activation produces less speciation than the 2:1 activation but reproducibility in heterogeneity amongst samples is nearly identical in the 2:1 sample. Moreover, AUC data for the 2:1 activated product looked similar to prior inside-out PEGylated polyHb (Chapter 3). Prior inside-out PEGylated polyHb polymers utilized the only two Cys residues, Cys β93, to conjugate a XL-Hb tetramer onto the PEG backbone.

With the same number of reactive sites to PEGylate compared to the previous method, it was expected that azide-alkyne inside-out PEGylation would look similar. However, the predominant species is 4 Hb : 1 PEG, one tetramer more than prior products (Chapter 3). Figure 32 is a schematic representation of the major PEG αXL-Hb product. It has been found that larger Hb polymers are unable to scavenge NO *in vivo* because they cannot get close enough to the endothelial lining making a larger Hb nanoparticle more desirable (Matheson et al. 2002; Palmer 2006). Furthermore, oncotic pressure is relative to the number of particles in solution than the molecular weights of said components. Thus, larger Hb particles have the potential to reduce harmful changes in pressor activity upon administration.

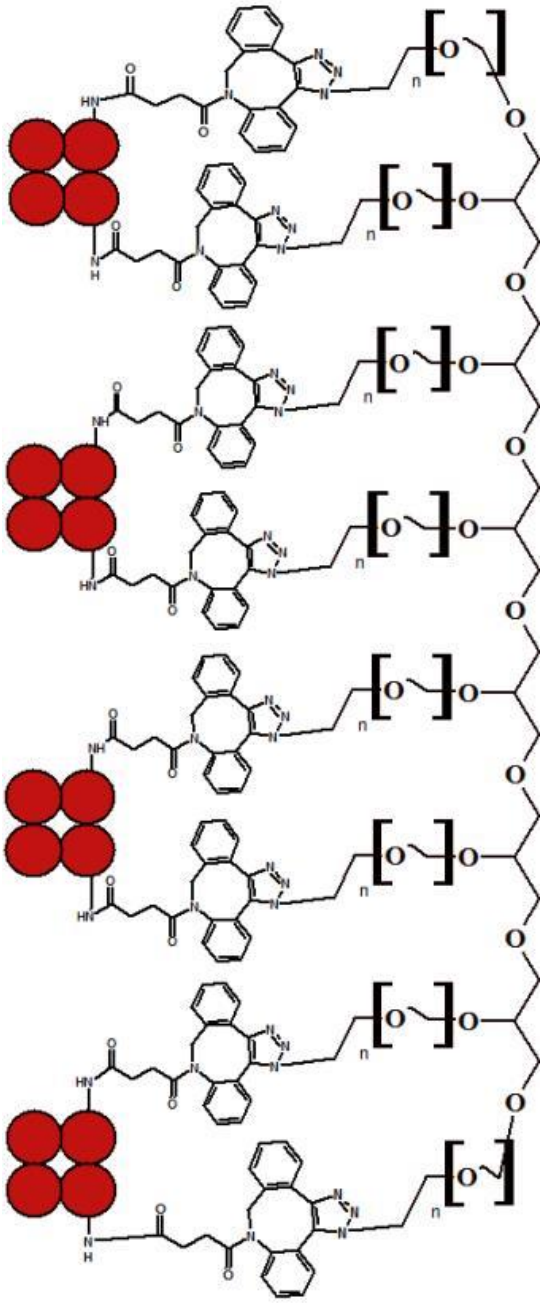


Figure 32. Proposed schematic of Inside-out PEGylated crosslinked hemoglobin. It is more probable that two arms will bind to a single tetramer than one arm binding to an individual tetramer. The major inside-out PEGylated hemoglobin polymer species produced is depicted above (4 Hb : 1 PEG).

Shown here, inside-out PEG αXL-Hb can be classified as both a dendremeric and poly Hb structure (Kluger and Zhang 2003). In the past poly hemoglobins have been produced via azide-alkyne copper mediated click chemistry (Foot, Lui, Kluger 2009; Singh et al. 2015b; Yang and Kluger 2010). Conversely, this is the first poly Hb to be generated without using copper mediation, but rather a cyclic strained alkyne. With the reaction allowing to proceed without copper, there is no concern of contamination of the product with an undesirable metal. Other polymeric dimers, trimers, and octamers of Hb have been generated under different conditions. These have been created by both chemical modifications and genetic mutations to the Hb tetramer (Bobofchak et al. 2003; Fablet et al. 2003; Faggiano et al. 2011; Fronticelli et al. 2001; Vasseur-Godbillon et al. 2006). One of the largest poylmers was around 500 kDa and was shown to not adversly affect blood pressure upon comparision to crosslinked Hb (Bobofchak et al. 2003). It was infered that this was a result of decreased extravasation of the compound in circulation. Unfortunately, the limitations in these approaches are in the amount of genetic mutations and/or chemical synthesis required to create said products. Cyclic strained mediated azide-alkyne inside-out PEGylation makes similar sized Hb polymers with commercially available reagents. Moreover, the methods presented here can be easily translated to use for other proteins.

Stroma free Hb dissociates into αβ dimers in circulation which is readily filtered out by the kidneys and highly nephrotoxic. Dissociation of the tetramer is

thus circumvented through crosslinking (White and Olsen 1987). The fraction denatured curves (Figure 29) show that this modification also increases protein stability. The melting temperature for αXL-Hb has been previously reported and agrees with the experimentaly determend value presented in this paper (T_m = 53.5 °C) (Olsen 1994; White and Olsen 1987; Yang and Olsen 1991). Upon PEGylation, the stability of the tetramer decreases (T_m = 46.1 °C) but is still more stable than stroma free Hb (T_m = 41.3 °C). The observed decrease in T_m was also seen in ealier inside-out PEGylated Hb polymers (Chapter 3) indicating that crosslinking plays a larger role affecting Hb properties than PEGylation.

Autoxidation experiments were carried out to assess the speed at which the Hb samples oxidized at biological temperatures. Within a RBC is a naturally reducing environment that helps maintain Hb in the functional Fe^{2+} state. Upon extraction from the RBC, Hb more readily oxidizes to the Fe^{3+} state. Former studies have found that crosslinking under deoxy conditions increase the rate of autoxidation (Yang and Olsen 1989). The data here agrees with those findings and αXL-Hb oxidizes 1.78 times faster than that of stroma free Hb (k_{rel}). Following PEGylation, the rate of oxidation increases to 2.39 times faster than that of stroma free Hb (Table 5). Figure 30 shows the characteristic spectral changes of the PEGylated sample as it was allowed to oxidize. Previous studies have shown this trend in oxidation rates. Acharya and colleagues have previously shown that PEGylated Hb species oxidize two to three times faster than their unmodified

counterparts (Hu et al. 2008). The increase in k_{rel} to PEGylated species has been attributed to PEGs water rich nature. When PEG sequesters water molecules it brings them into closer proximity of the Hb tetramer and allows for an increase in water mediated oxidation of the iron center. However, when the magnitude of change is compared following the progression of protein modification, PEGylation does not increase k_{rel} as much as crosslinking. Stroma free Hb to αXL-Hb shows an increased rate of oxidation by 78%, where αXL-Hb to PEG αXL-Hb shows an increase of 61% (Table 5). Consequently, the rate of oxidation is affected more so by crosslinking than modification of the Lys residue and subsequent PEGylation. Fortunately, it is speculated that the increased rate of oxidation seen at 37 °C *in vitro* will be less compared to that *in vivo* due to the antioxidant agents present within plasma (Hu et al. 2008; Snyder et al. 1987; Yang and Olsen 1989).

Crosslinking not only affects the stability of the Hb tetramer but also oxygen affinity. Similar CD spectra between Hb samples (Figure 28) elucidated that differences in oxygen affinity are not correlated with changes in secondary structure but rather crosslinking protocol. Oxygen binding studies determined that azide-alkyne mediated inside-out PEGylation does not influence the oxygen affinity of Hb. The P_{50} values (Table 5) show that the crosslinking methodology dictates the capacity for Hb to bind and release oxygen (PEG αXL-Hb P_{50} = 31.97 torr, αXL-Hb P_{50} = 27.57 torr). This has been observed in past inside-out PEGylated Hb samples and is novel to this form of PEGylation (Chapter 3). Traditional PEGylated Hb

polymers that coat the exterior of Hb with multiple PEG chains have been shown to increase oxygen affinity independent of PEGylation methodology (Hu et al. 2007; Li et al. 2008; Li et al. 2009; Lui et al. 2012). Addition of IHP, a 2,3-BPG mimetic, shifted the oxygen binding curve to the right in all the samples (Walder et al. 1979; Walder, Walder, Arnone 1980). A shift that lowers oxygen affinity has been previously reported for αXL-Hb and shows that the compound is still accesable to Hb in the PEGylated product. Decreases in experimentally determined Hill coefficients were observed post crosslinking and following PEGylation. Cooperativity is reduced if Hb loses its ability to rotate freely when converting from T to R state upon binding oxygen. Crosslinking adds a covalent linker in the central cavity restricting the tetramers rotation, and thus reducing the experimentally determined n seen in Table 5 (Hb = 2.91, αXL-Hb = 1.39). Covalently binding PEG to the XL tetramer further resticts movement and slightly decreases the cooperativity (PEG αXL-Hb n = 1.26). However, this decrease does not affect the oxygen delivery capabilities of the product. The experimentally determined P_{50} values of the αXL-Hb and PEG αXL-Hb are similar to that of Hb in a RBC. Thus, it is expected that PEG αXL-Hb will deliver oxygen in a nondiscriminatory manner.

Conclusion

We have successfully produced a second inside-out PEGylated Hb complex through a different combination of crosslinking and PEGylation protocols. Concurrent application of crosslinking and inside-out PEGylation have the potential

to mitigate problems seen in previous HBOCs. The oxygen affinity of the product is similar to that of Hb within a RBC. In addition, this method produced a larger polymer in comparison to that of thiol-maleimide inside-out PEGylation (Chapter 3). In theory, the polymer should be able to deliver more oxygen per particle than the PEG βXL-Hb product. With an extra Hb tetramer, on average, covalently linked to the PEG backbone per particle allows for a higher amount of oxygen to be delivered to the tissues. Inside-out PEGylation has applications past HBOC development. We have provided another way to engineer large protein complexes, without the need for traditional CuAAC. The results reported here promote the versatility that multi-arm inside-out PEGylation possesses. Inside-out PEGylation is not restricted to one method of click chemistry or to a specific protein. We believe that this method of PEGylation will be effective in the synthesis of other biomacromolecules.

CHAPTER V

FUTURE WORK AND CONCLUSIONS

Future Work

To determine the efficacy of the inside-out PEGylated Hbs as HBOCs, cell cultures, and/or animal studies need to be conducted. Often, after product generation and characterization of a new HBOC, animal studies are conducted. However, cell studies may be more appropriate before testing on an entire organism. Animal studies are costly and require an immense amount of product. Before identifying if the product delivers oxygen and/or produces ROS *in vivo*, it should be determined whether or not an immune response will be elicited. Ideally, the first avenue for research to determine this is through cell cultures. In the past these have not been readily utilized to characterize HBOC products, except a few that used immunological cells and tissues. These experiments looked at immune responses induced by the production of cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Zhu et al. 2011). IL-6 is a pro-inflammatory cytokine and anti-inflammatory myokine, where TNF-α stimulates inflammation, apoptotic cell death, and fever. These cytokines are expressed in myocardial tissue and can be monitored and quantified with the use of ELISA. If an immune response is generated, the product will do more harm than good. This can easily be identified prior to animal studies through this technique.

In addition, cell studies with HeLa cells should be looked into to evaluate the products safety prior to animal studies. Cell studies of this nature have not been utilized in the past to characterize HBOC products. It is my belief that it should be another standard practice prior to animal studies. This study would be able to identify if the product will induce cell death, possibly from ROS production. Furthermore, these studies can be conducted under normoxic and hypoxic conditions, evaluating any changes in oxygen levels that may be seen *in vivo*.

Finally, animal studies should be carried out to evaluate HBOC efficacy. This is usually done by draining blood from the animal, inducing hemorrhagic shock, then infusing the animal with the HBOC product (Cabrales et al. 2005; Cabrales 2010; Cabrales et al. 2010; Conover et al. 1997; Matsumoto et al. 2005; Raat 2005). Upon administration, short and long term effects can be monitored. Short term, damage to the cardiac and vascular tissue can be monitored while examining oxygen deliver. If the animal is kept alive for longitudinal studies monitor post operatory conditions such as vascular resistance, kidney damage, gastrointestinal damage, and recovery times. While these are the first major steps in evaluating human efficacy, it should be followed by the aforementioned experiments and build upon them.

Conclusions

Presented here are two different inside-out PEGylated Hb polymers that show potential as blood substitutes. We have shown that this form of PEGylation is not restricted by the method of conjugation chemistry as seen by its success with thiol-maleimide and azide-alkyne click chemistry. Inside-out PEGylation does not alter the secondary structure of the protein while making it more stable by

comparison to the unmodified protein. Although the products are heterogeneous, their heterogeneity is reproducible amongst sample preparations. Both products are large enough in size where they should not be able to interact deleteriously in circulation with endothelial tissue. However, each product has very different oxygen delivery characteristics. The PEG βXL-Hb sample would release oxygen to highly hypoxic tissue where a PEG αXL-Hb would deliver oxygen in a non-discriminatory fashion, similar to that of Hb in RBCs.

A viable alternative to current blood transfusions has been actively pursued for several decades with minimal success. A limited supply of blood, dependent on human donors, has demanded the creation of a hemoglobin-based oxygen carrier (HBOC) from non-human sources. Such a product would deliver oxygen to tissues, providing a therapeutic response to hemorrhagic trauma. However, an effective HBOC has a utility that exceeds applications in hemorrhagic shock responses. It may also be used to treat carbon monoxide poisoning, acute anemia, organ ischemia, and cardioplegia—all conditions related to oxygen deprivation. With a myriad of potential applications, my research is centered on alleviating the dependence on human blood donations.

Blood donations must be tested for blood borne pathogens such as HIV. In The United States of America and other developed countries, testing is not exceedingly taxing but does take time and decrease the potential pool for donations. However, in less developed countries where money and testing supplies are limited, the necessity of a sustainable alternative to blood donations that is disease free

cannot be understated. Countries where HIV is more predominant already have a diminished pool to draw on for donations and our product would hopefully be able to supplement the donations, and most importantly, be produced at a minimal cost. The research maintains the idea that for the product to be viable from a production standpoint, it must be relatively simple and inexpensive to produce. The chemistry used herein to create potential HBOCs is affordable and generated from commercially available reagents that can be scaled up for mass production.

In addition to the aforementioned reasons to produce a blood substitute, the product will ideally ameliorate the need for mass blood donations in times of natural disasters or crisis. When such a disaster occurs that leads to a large population in need of blood transfusions, our product would be available in a supply greater than the available—and finite—supply of current blood transfusions. My work has the potential to provide the global community with the therapeutic potential to respond liberally to hemorrhagic trauma and other conditions of oxygen deprivation. The short and long term goals of the project address the viability of this HBOC, making it a truly successful product, focusing on both efficacy and practicality of production. Such a substitute has the potential to fill a great need in human health and bridge an economic divide to the costly alternative treatments that are currently available.

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VITA

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Webster entered the M.S. program in Chemistry at Loyola University Chicago in Fall 2011 and switched to the Ph.D. program in the Fall of 2013. For the first two years he taught five classes a semester and received a departmental teaching assistantship for one year after. This gave him the opportunity to instruct students in general chemistry, and biochemistry laboratory courses. In May 2014, he was awarded Teaching Assistant of the Year by the chemistry department. In August 2014 he received the Pre-Doctoral Teaching Fellowship from Loyola University Chicago. He also received the Summer Research Mentoring Program Fellowship in May 2015. The following year he received the Schmitt Fellowship from Loyola University Chicago in August 2015. Webster also mentored many undergraduates in research during his time at Loyola. He also served as a judge for three years at the Chicago Area Undergraduate Research Symposium.

Webster earned his Doctoral degree in Chemistry in May 2016 and is currently in medical school at MSU working on his Degree of Doctor of Osteopathic Medicine.