Spring 6-2018

Development of a Monodisperse Oligomeric Hemoglobin-Based Oxygen Carrier for Acute Blood Replacement Therapy

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Western Washington University

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By
Leah Huey

Accepted in Partial Completion
Of the Requirements for the Degree
Bachelor of Science with
Honors in Biochemistry

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Leah Huey

June 1st, 2018

Development of a Monodisperse Oligomeric Hemoglobin-Based Oxygen Carrier for Acute Blood Replacement Therapy

A Thesis
Presented to
The Faculty of
The Chemistry Department of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Bachelor of Science with
Honors in Biochemistry

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Leah Huey
June, 2018

Abstract

Our long-term goal is to develop a monodisperse high molecular weight hemoglobin-based oxygen carrier (HBOC) for clinical care. One short-term aim is to ligate hemoglobin (Hb) molecules to a dendritic scaffold utilizing "click-chemistry". Towards this goal, we have genetically modified the C-terminus of one of the α subunits of a di-α globin to contain the *S. aureus* sortase A recognition sequence (LPETG) and we have expressed the modified globin in *E. coli*. Here, we demonstrate that these Hbs can be site-specifically functionalized through sortase-mediated ligation of peptides containing dibenzocyclooctyne (DBCO). We further demonstrated proof-of-concept by conjugating an azide-funtionalized peptide with a fluorescent tag, 6-carboxyfluorescein (6-FAM) to Hb(DBCO). Additionally, work has been done to crystallize Hb with a site-specific mutant βN108K, which promotes T-state stability of Hb (low oxygen affinity). Cell-free Hb is known to have high oxygen affinity and by determining the structural basis for improving T-state stability in our novel Hbs we hope to design our HBOC with ideal characteristics for reversible oxygen binding. This work establishes that we can functionalize hemoglobin with "click-chemistry" groups such as cyclooctyne, and conjugate that group to an azide with the ultimate goal of decorating azide-functionalized dendrimers with hemoglobin molecules to transport and exchange oxygen in the body.

Acknowledgements

To the following individuals, thank you for making this work possible:

Dr. Spencer Anthony-Cahill, my advisor, for challenging me to always dig deeper, providing guidance when I wasn't sure how, and encouragement when I needed it the most.

Dr. Clint Spiegel for his guidance with crystallization and X-ray diffraction.

Dr. John Antos for his expertise in mass spectrometry and protein engineering as well as continued enthusiasm for this project.

Past and present lab members for providing an engaging environment in which to work in. Specifically, Johann Sigurjonsson for providing the training and support necessary to do this research and Dedeepya Gudipati for help with the protein expression and purification.

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List of Abbreviations

WHO	World Health Organization
Hb	hemoglobin
HBOC	hemoglobin-based oxygen carrier
metHb	methemoglobin
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
FDA	Food and Drug Administration
scHb	single chain hemoglobin
DBCO	dibenzocyclooctyne
T-state	tense state
R-state	relaxed state
2,3-BPG	2,3-bisphosphoglyceric acid
cp	circularly-permuted
sHb	sortase-tagged hemoglobin
SM	stabilizing mutations
LB	luria bertani
TB	terrific broth
IPTG	isopropyl β-thiogalactopyranoside
IMAC	immobilized metal affinity chromatography
CV	column volumes
MWCO	molecular weight cut off
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

ESI-MS	electrospray ionization mass spectrometry
CMS	compact mass spectrometer
HPLC	high-performance liquid chromatography
NMP	N-methyl-2-pyrolidone
FITC	fluorescein isothiocyanate
NTA	nitrilotriacetic acid
6-FAM	fluorescein
PEG	polyethylene glycol

Introduction

*Blood transfusions***.** Currently, blood transfusion is the standard of care for blood loss. Hospitals rely on blood donations to provide this life-saving treatment, and many operations cannot be performed without a blood transfusion. According to the World Health Organization (WHO), in high income countries blood transfusions are most commonly performed for cardiovascular and transplant surgery, trauma, and various hematological malignancies. In low income countries blood transfusions are most needed for pregnancy-related complications and severe childhood anemia[1].

Blood transfusions require donations from healthy volunteers and this poses significant challenges. In many low and middle-income countries, the current level of blood donations does not meet the need for blood transfusions. High-income countries have a whole blood donation rate of 32.1 donations per 1,000 population per year, however, in low-income countries the donation rate is 4.6 donations per 1,000 population per year[1] (Figure 1). In addition, there are low retention rates of blood donations because of blood transfusion safety. A number of

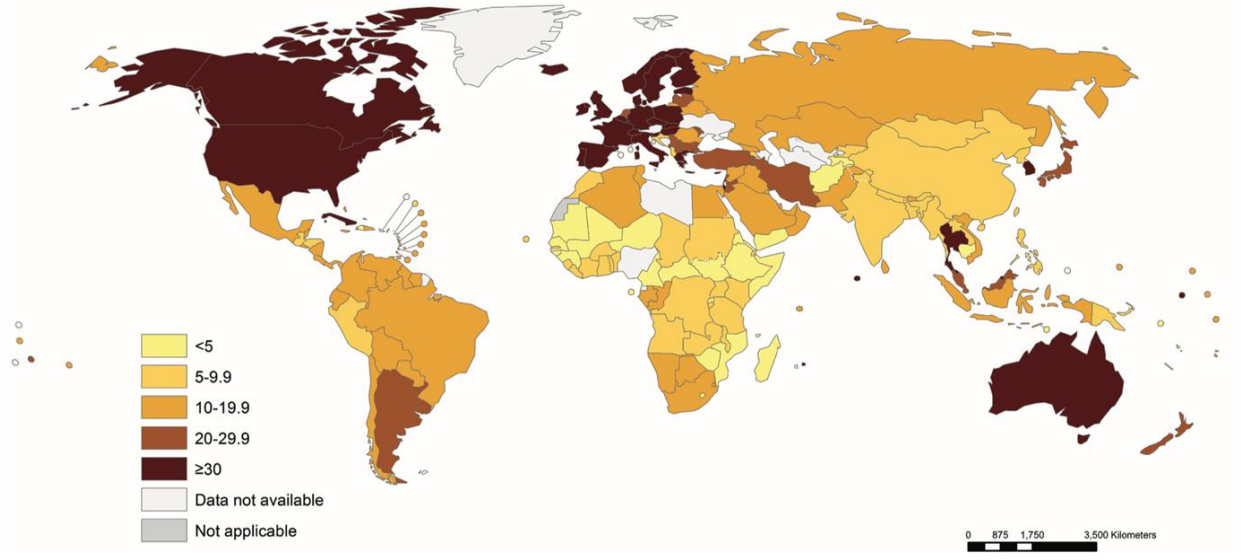


Figure 1. Whole blood donations per 1,000 population in 2013[1]. Source: World Health Organization.

countries have a high percentage of transfusion-transmissible infections in the population, but not enough adequate resources to screen for them[1]. Identification of transfusion-transmissible infections, as well as the expiration of donated blood results in a discard rate ranging from 7% - 11% of total donated blood around the world[1]. In the US, while blood donations meet the requirement, they rarely exceed it so storage of blood donations is not feasible[2].

Of the many functions of blood, one of the most essential is the transportation of oxygen from our lungs to our tissues. Without oxygen, cells are unable to produce the energy necessary for our body to function. Loss of blood causes a deprivation of oxygen to cells and thus requires an immediate response, such as a red blood cell transfusion. The protein responsible for oxygen transport, hemoglobin (Hb), is housed inside the red blood cell, and so when a patient is given red blood cells, they are also given Hb. Hb is a 64 kDa protein with 2 α and 2 β subunits. Each subunit contains a hemin group which chelates to Fe^{2+} and binds to oxygen. The red blood cell provides protection to Hb, separates Hb from reactive species, and provides the enzymes and small molecules necessary for Hb to function[3,4]. In addition, the red blood cell poses some challenges to blood transfusions. The antigens on the surface of red blood cells require blood type matching and red blood cells cannot be mass produced; they must be donated by healthy volunteers.

An alternative to red blood cell transfusions. In order to alleviate some of the demand for blood transfusions, researchers have been working to develop an alternative to red blood cell transfusions. The function of this therapeutic would be to transport oxygen and so one of the approaches has been to modify the natural carrier of oxygen, hemoglobin, and therefore develop a hemoglobin-based oxygen carrier (HBOC). By modifying Hb to function properly cell-

free, patients could be given this therapeutic to alleviate immediate concerns of oxygen transportation. While a hemoglobin-based therapeutic could not address all of the functions of blood, it would reduce the need for red blood cell transfusions for patients dealing with trauma or other conditions involving acute blood loss. Of the many challenges associated with HBOCs, one is Hb's small size relative to the red blood cell. Cell-free Hb is small enough to extravasate through the blood vessel wall and into the interstitial space between endothelial cells and smooth muscle[5]. In this space nitric oxide is a signaling molecule that relaxes smooth muscle cells[6]. When oxygenated, Hb mixes with nitric oxide, nitric oxide enters the distal pocket, and is converted to nitrate (NO_3^-) while Hb is converted to methemoglobin (metHb)[7]. When this occurs, smooth muscle is unable to receive the signal to relax which results in an increase in mean arterial blood pressure and other smooth-muscle effects such as gastrointestinal discomfort[8]. These undesirable effects can be reduced by increasing the molecular weight of the cell-free Hb [9] (Figure 2).

In addition to increased extravasation, the Hb $\alpha_2\beta_2$ heterotetramer has reduced stability in a cell-free environment which results in its dissociation into $\alpha\beta$ heterodimers. These dimers are small enough to be rapidly cleared by glomerular filtration in the kidneys or by a Hb scavenging receptor in the liver. As $\alpha\beta$ dimers, Hb is quickly cleared from the blood and therefore nonfunctional[10]. Increased renal filtration can also result in renal damage caused by the overload of the glomerular filtration capacity in the kidneys[11,3,4]. For this reason, Hb stability and molecular weight need to be addressed when developing a functional HBOC.

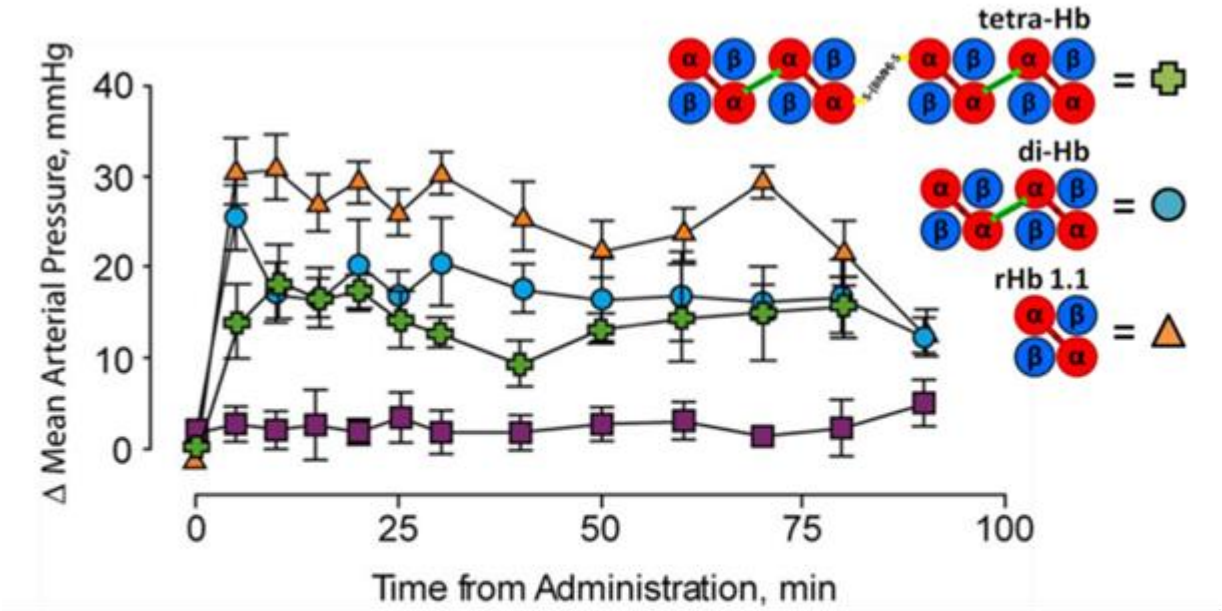


Figure 2. Change in mean arterial pressure post injection of hemoglobin constructs into rats. Hemoglobin constructs include single hemoglobin molecule, rHb1.1 (orange filled triangles), di-Hb (blue filled circles), tetra-Hb (green filled crosses), or human serum albumin as a control (50 mg/mL, purple filled squares)[9]

Another problem associated with cell-free Hb is the auto-oxidation of the iron chelated in the heme group from Fe^{2+} to Fe^{3+}. The oxidation to Fe^{3+} forms metHb which is unable to transport and exchange oxygen[12]. Heme loss is also accelerated in the methemoglobin state because of a reduction in stability[13]. For cell-free Hb the oxidation reaction occurs over a few hours and is essentially irreversible, whereas in the red blood cell there are antioxidants such as NAD(P)H that maintain Hb in the reduced Fe^{2+} state[14].

*Strategies for developing HBOCs***.** The goal of HBOC development is to modify Hb so that it can function properly outside of a red blood cell. Many HBOCs have been tested in clinical trials but none has been approved by the FDA for use in humans. While there are many challenges associated with cell-free Hb, through rational design and protein modification these challenges can be overcome. In addition, by understanding the characteristics of HBOCs that

have been developed in the past, we can learn from both successes and failures. The three main strategies for modifying Hb have been polymerization, conjugation, and cross-linking[3,15] (Figure 3).

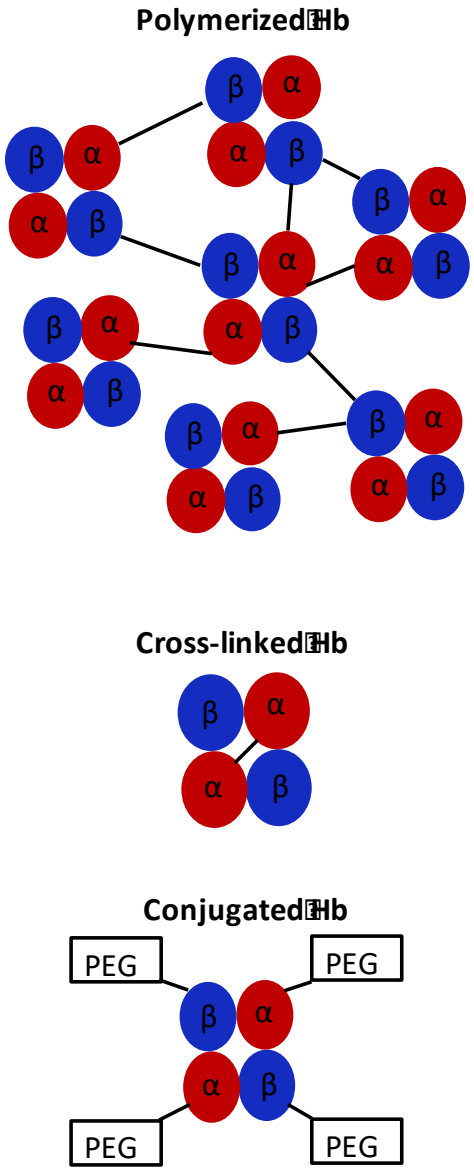


Figure 3. Strategies for development of HBOCs. Non-specific polymerized Hb (top), chemically or genetically cross-linked Hb (middle), Hb conjugated with PEG (bottom). Adapted from Stowell et al.[3]

Polymerization increases the size of HBOCs; thus, its main effect is to increase retention times in the blood. Polyheme, developed by Northfield laboratories, and Hemopure, developed by Biopure Corporation, are both glutaraldehyde-polymerized Hb products[16]. Polymerization results in an ultra-high molecular weight molecule, and so with this approach, retention times increased from the rapid clearance of cell-free Hb to a half-life of 16-20 hours for Hemopure and 24 hours for Polyheme[10]. The drawback to this approach is that the reaction, which links surface lysines on neighboring Hb together, is non-specific, and so the resulting product is a heterogeneous mixture of polymers[10,16].

Conjugation, similar to polymerization, increases the apparent molecular size of Hb in order to increase retention time and to reduce extravasation. Sangart, Inc used this approach to develop Hemospan, which was made by conjugating polyethylene glycol to Hb by site-specific addition[17]. Hemospan was found to have a half-

life of 14-23 hours in patients, but it also appeared that Hemospan was eliminated from the blood by the scavenger receptor CD163[10]. In addition Hemospan had an overall high oxygen affinity and an increase loss of hemin and so while it went through early clinical trials, its development was eventually terminated[15,17].

Lastly cross-linking Hbs subunits, both chemically or genetically, increases stability and prevents dissociation of Hb into heterodimers. HemAssist, developed separately by Baxter and the US army, chemically cross-linked the α subunits in purified human Hb with bis(3,5-dibromosalicyl) fumarate. This approach increased stability and resulted in a half-life of 2.1-4.3 hours in humans[10]. When tested more rigorously in humans, however, results indicated vasoconstriction and ultimately increased adverse effects in comparison with the standard blood transfusion[18]. Optro, developed by Somatogen, genetically cross-linked the α subunits creating a di-α subunit with a single glycine inserted between the α subunits[19]. While this produced a stable hemoglobin, other problems were still present including high oxygen affinity, nitric oxide scavenging, auto-oxidation, and hemin loss[20].

*Developing a monodisperse HBOC***.** While previous HBOCs have shown many advantages, each had its disadvantages. The most prominent problems are Hb stability in the blood and nitric oxide scavenging. To address these problems our strategy has been to develop a polymeric Hb molecule that has a defined molecular weight and size. Based on evidence shown in Figure 1, increased molecular weight of Hb will reduce mean arterial pressure caused by nitric oxide scavenging. In addition, creating a molecule with one size will eliminate the smaller molecular weight products that are associated with increased extravasation and blood pressure elevation. Initially, the strategy for this work was to develop a recombinant single-chain Hb

(scHb) in three main steps. The first was to create a circularly permuted β globin by modifying the N and C-terminal sites[21]. The second was to then link the α and β globins together to create a single chain αβ dimer (sc-αcpβ) and then lastly to link the α-globins together by inserting a single glycine linker (Figure 4)[22]. We envisioned this sc-Hb as a monomer unit to be used in the construction of a recombinant poly-Hb of defined molecular weight.

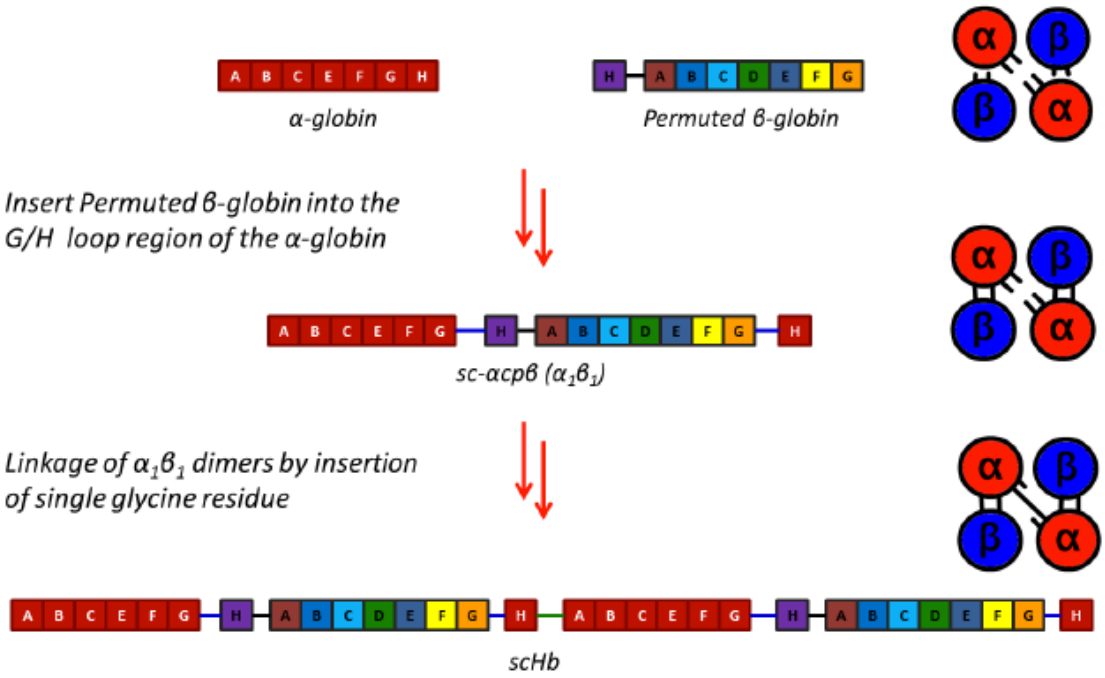


Figure 4. Scheme for scHb development. α-globin and circularly permuted β globins were linked together to create sc-αcpβ. This was followed by linking the two dimers together with a glycine linker to create scHb.[19,20]

The scHb was successfully expressed, however, in a very low protein yield[22]. This was mitigated by adding additional stabilizing mutants β G16A, β H116I, α G15A, and β K82D, which were reported by Graves *et al.* and Weickert *et al*[23,24] to increase stability and expression yields of globins. Unfortunately, even with the addition of these stabilizing mutations, the expression

yield remained too low for this approach to poly-Hb to be economically feasible. Therefore, a new approach needed to be taken to create a Hb polymer with a defined molecular weight.

Sortase-mediated monodisperse HBOC. The approach we have taken combines genetic and chemical modifications of Hb to develop an HBOC polymer that is a defined molecular weight and size and can also be produced on a large scale. Our method is based on the concept of decorating a central scaffold with functional Hb molecules. With highly efficient click chemistry Hb molecules will be attached to a commercially available azide dendrimer, which will contain 4-12 sites for Hb attachment (Figure 5). The attachment will be achieved with a cyclo-alkyne azide addition which needs no metal catalyst but is instead catalyzed by the strain produced from the cyclo-alkyne[25].

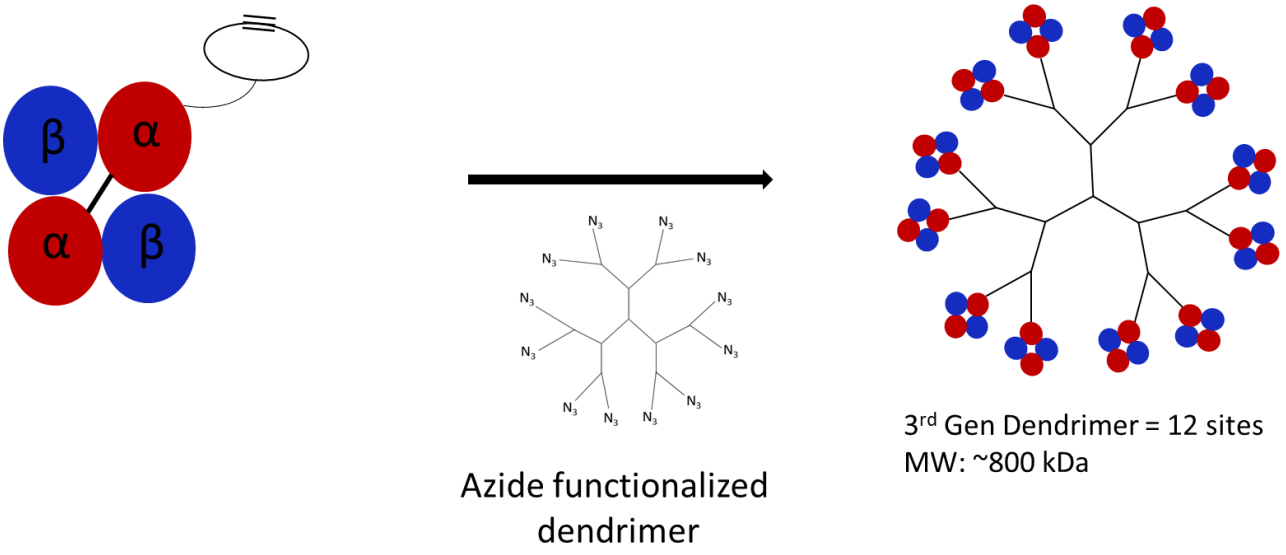


Figure 5. Scheme for development of oligomeric, monodisperse HBOC. A cyclooctyne-modified Hb molecule will be added to a azide functionalized dendrimer.

For this method to work, Hb needs to contain a cyclo-alkyne tag which will be added with the help of the transpeptidase, sortase A. Found in many bacteria, but specifically in *S. aureus*, sortase attaches proteins to the peptidoglycan cell wall by recognizing a LPXTG motif,

cleaving between the threonine and glycine to form a thioester intermediate where an incoming N-terminal glycine can act as a nucleophile to form a new peptide bond[26].

In this way, sortase can attach small peptides with an N-terminal glycine to any protein containing the LPXTG motif (Figure 6). With this in mind we synthesized Hb to contain a C-

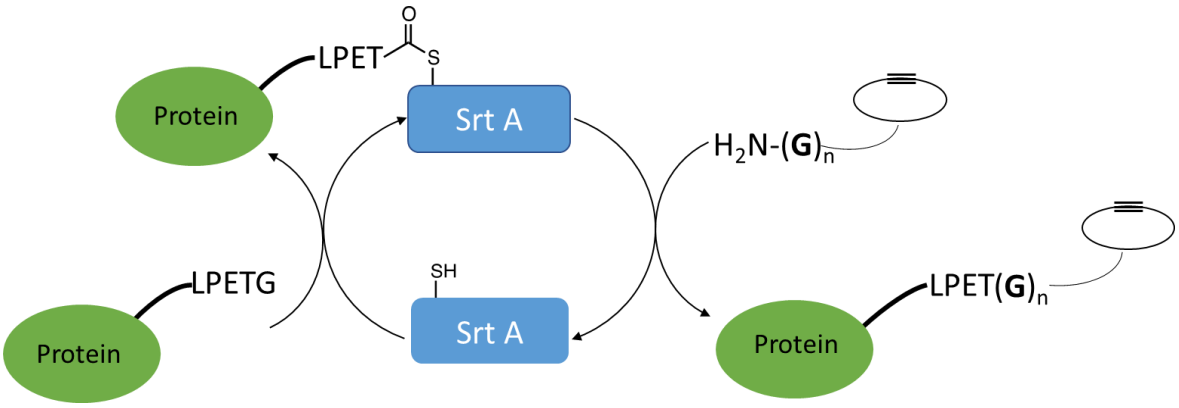


Figure 6. Sortase-mediated ligation. Sortase A recognizes the LPETG motif on a protein, forms an enzyme-bound thioester intermediate which is attacked by a glycine nucleophile derivatized with a cyclooctyne.

terminal LPETG sortase A tag on the α subunit. With sortase A as a catalyst, we added a small peptide derivatized with dibenzocycloocytne [GGK(DBCO)] to Hb (Figure 8). To create a more stable construct we further modified Hb to be di-α by covalently linking the α subunits with a glycine linker. Lastly, we included the 4 stabilizing mutations mentioned above (β G16A, β H116I, α G15A, and β K82D) and included a hexa-histidine tag for purification (Figure 7). These modifications produced a stable Hb molecule that can be easily purified as well as modified by sortase A (Figure 8).

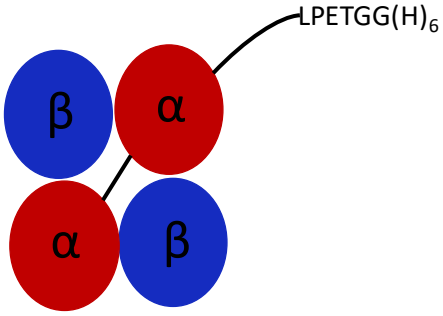


Figure 7. Hb building block. A novel recombinant Hb was designed to include a glycine linker between the α subunits as well as a sortase tag followed by a hexa-histidine tag on the C-terminus of the di- α subunit.

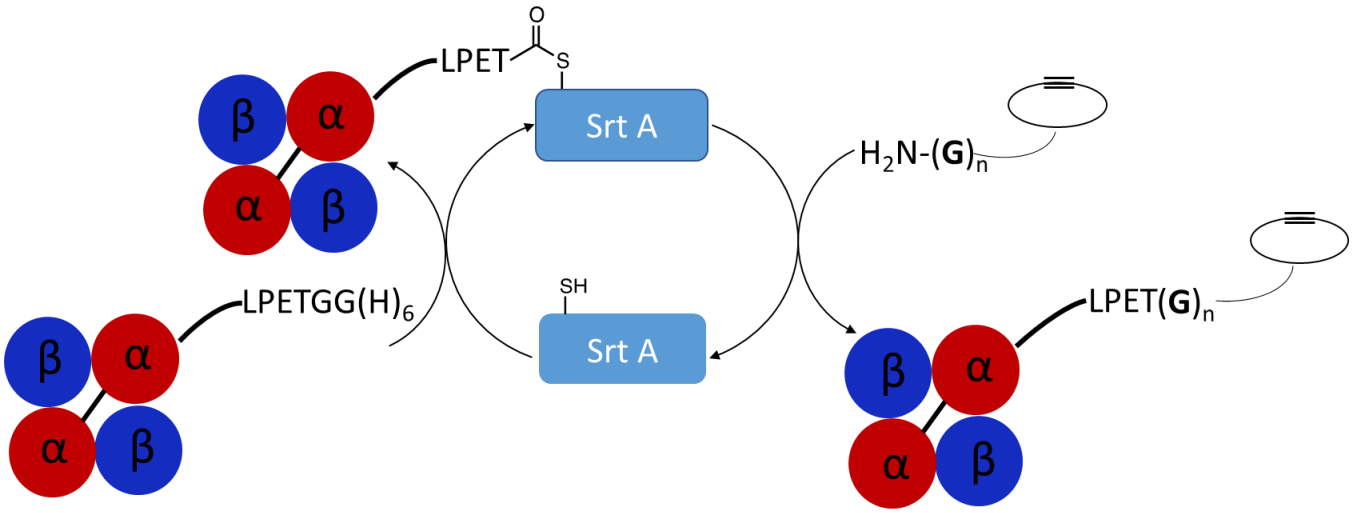


Figure 8. Sortase-mediated Hb ligation. Sortase A recognizes the LPETG motif on Hb, forms an enzyme-bound thioester intermediate which is attacked by a glycine nucleophile derivatized with a cyclooctyne.

Modulating oxygen affinity. In addition to working to develop an HBOC with a high molecular weight, we also wanted to focus on the other physical properties of Hb to ensure our product has optimal functional properties. To transport oxygen efficiently, Hb has 2 conformations. The first is the high oxygen affinity relaxed state (R-state) and the other is the low oxygen affinity tense-state (T state). These 2 conformations allow R-state Hb to bind to oxygen in the lungs and then to release oxygen by switching to the T state when it reaches various tissues in our body. Hb's conformational changes are modulated by many allosteric regulators including 2,3-bisphosphoglyceric acid (2,3-BPG), protons, and CO_2 [3]. One regulator, 2,3-BPG, is found at high concentration in red blood cells and is able to promote the change from R-state to T-state[4]. Cell-free Hb, however, is not regulated in this way because of the much lower concentration of 2,3-BPG in the blood plasma. For this reason, cell-free Hb has high oxygen affinity, which reduces the release of oxygen compared to Hb in red blood cells[27].

To alleviate this problem, mutations have been made to increase T-state stability in Hb. One in particular, βN108K, reduces Hb's oxygen affinity to a value similar to that of whole blood[28]. In order to understand how this mutation affects the stability of the R and T states in our circularly permuted (cp) Hbs, work has been done to crystallize N108K in the α-cpβ Hb construct. With X-ray diffraction data, the structure of the modified Hb can be solved revealing the structural details for this Hb variant, which may be different from those observed in the wild-type Hb. So far, we have been unable to get good resolution on X-ray diffraction data. Many crystal conditions have been tested but with further optimization X-ray diffraction data could be a possibility.

The work described in this thesis suggests that our new approach shows merit in achieving our long-term goal. We have successfully expressed and purified di-α-cpβ sHb, ligated DBCO to it with sortase A, and conjugated an azide functionalized peptide to di-α(DBCO)-cpβ sHb. Overall, the development of our polymeric HBOC has focused on reducing the effects of nitric oxide scavenging and increasing hemoglobin T-state stability. Through a combination of genetic modifications and mutations as well as chemical adaptations we plan to develop an oligomeric monodisperse product that can be used for acute blood replacement. Continued work is necessary to produce a poly-Hb in high yield and to optimize its properties. The development of a safe and effective HBOC will address the need for an alternative to red blood cell transfusions and therefore has the potential to save lives.

Materials and Methods

Protein Expression

Gene design. A di-α subunit with a sortase A tag (LPETG) followed by 6 histidines on the C-terminal end was synthesized and cloned into a pUC plasmid which contained circularly-permuted β Hb subunit with 4 stabilizing mutations (β G16A, β H116I, α G15A, and β K82D) (Di-α cp-β sHb). The gene was then subcloned into an expression vector derived from the pDLIII13-e plasmid described by Hoffman *et al*.[29].

Transformation. The expression plasmid was transformed into chemically competent BL21 *E. coli* cells by adding 1 μl of the plasmid to 50 μl of *E. coli* cells, which were then placed on ice for 30 minutes. Cells were incubated at 42°C for 1 minute, immediately placed back on ice for 3 minutes, re-suspended in 1 mL Luria Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl), and incubated at 37°C for 45 minutes in a shaker. Following this outgrowth, increasing amounts (25, 50, 100 μl) of transformed cells were added onto 3 LB tetracycline agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 14 g/L agar, 23 μM tetracycline, pH 7.0) and incubated for 26 hours at 37°C. Two distinct colonies were picked and placed in 10 mL LB-tetracycline (23 μM tetracycline) broth and incubated at 37°C for 12 hours in a shaker to make seed stocks (1:1 overnight broth:50% v/v glycerol), which were then stored at -80°C.

Protein expression. Two different protein constructs were expressed, the first was α cp-β Hb 4SM with the additional mutation βN108K (N108K Hb) and the second was di-α cp-β sHb (sHb). The first, N108K Hb, was previously transformed into BL21 *E. coli* cells and seedstocks

were obtained from Johann Sigurjonsson. The cells were first grown by inoculating 10 mL LB-tetracycline (23 μM tetracycline) with 1 μL seed-stock and incubated at 37°C for 12 hours in a shaker. Cells were then added to 1 L of Terrific Broth (TB) medium [12 g/L tryptone, 24 g/L yeast extract, 4% (v/v) glycerol, 2.31 g/L KH_2PO_4, 16.43 g/L K_2HPO_4/trihydrate] with 23 μM tetracycline and grown at 37°C with shaking at 200 rpm and induced with 1 mM isopropyl β-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 1.8. The temperature was reduced to 33.5°C and cells were grown for an additional 5 hours, with the addition of 2 mL of a stock hemin solution (12.5 g/L hemin, 200 mM NaOH) added every hour for a total of 0.05 g of hemin added per liter. Cells were harvested by centrifugation at 5,000 x g for 10 min at 4°C (Thermo Scientific Sorvall Lynx 4000) and frozen in liquid nitrogen to be stored at -80°C.

The second construct, sHb, was transformed as described above and cells were grown and induced as described for N108K Hb, except that the temperature was kept constant at 33.5°C and during induction 100mL of 20% (w/v) glucose was also added in addition to ITPG to increase expression.

Protein Purification

Cell lysis. Cell pellets were thawed and resuspended in lysis buffer [N108K Hb (50 mM Tris-HCl, pH 8.5, 17 mM NaCl); sHb (20 mM Tris-HCl, pH 8.5, 300 mM NaCl, 10 mM imidazole)]. Cells were sonicated (power output: 5 @ 50% duty cycle, Branson Intruments, Inc. Sonifier, model 450) on ice for a total of 90 seconds (3 x 30 sec pulses) with 60 seconds rest between every 30 second pulse. The resulting lysate was clarified by centrifugation at 40,000 x g for 30 min at 4°C. For the N108K Hb, 2 mM $Zn(OAc)_2$ was added to the lysate and an additional

centrifugation was performed at 40,000 x g for 30 min at 4°C. Supernatants from each construct were collected and filtered through a 0.22 μm cellulose acetate syringe filter.

Immobilized metal affinity chromatography (IMAC). To purify Hb constructs a column was packed with 10 mL GE Healthcare Fast Flow Chelating Sepharose resin. The column was washed with 4 column volumes (CV) of 200 mM NaCl, 2 CV 20 mM $Zn(OAc)_2$, and 6 CV 200 mM NaCl. N108K Hb lysate was then loaded onto the column and washed with 8 CV 20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, then 2 CV 250 mM Tris-HCl, pH 8.5, followed by 6 CV 20 mM Tris-HCl, pH 8.5. The protein was eluted with 20 mM Tris-HCl, pH 8.5, 15 mM EDTA.

For the sHb construct the column was prepared with the same resin and washed with 4 CV of 0.2 M NaCl, 0.5 CV 0.2 M $NiSO_4$, 4 CV 20 mM NaOAC, 0.5 M NaCl pH 4, and 5 CV 0.2 M NaCl. Lysate was loaded onto the column and washed with 4 CV lysis buffer (20 mM Tris-HCl, pH 8.5, 300 mM NaCl, 10 mM imidazole), followed by 4 CV wash buffer (20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 50 mM imidazole). The protein was eluted with 20 mM Tris-HCl, pH 8.5, 50 mM NaCl, 300 mM imidazole.

Dialysis. Buffer exchange was performed by adding the protein solution to Fisherbrand dialysis tubing with a molecular weight cutoff (MWCO) of 6,000-8,000 Da. Tubing was placed in 2 L of Buffer A (20 mM Tris-HCl, pH 8.5) and incubated for 24 hours at 4˚C. Hb samples were collected and concentrated to less than 1 mL with Amicon Ultra concentrators with 10,000 MWCO.

Anion-exchange chromatography. Protein was further purified with a Mono-Q 10/100 GL strong anion exchange column (8 mL CV). Protein samples from IMAC were centrifuged for 10 min at 10,000 x g (Eppendorf centrifuge 5415D) to pellet any insoluble impurities. The

Mono-Q 10/100 column was equilibrated with 5 CV water, 1 CV Buffer A, 1 CV Buffer B (20 mM Tris-HCl, 0.5 M NaCl, pH 8.5), then 5 CV Buffer A at 4 ml/min. Protein was then injected onto the column and eluted with a gradient that started at 100% Buffer A, 0% Buffer B and increased to 100% Buffer B and 0% Buffer A over 20 CV. Eluent, which was monitored at 280 nm, was collected, and samples with a red tint were kept. The presence of hemoglobin in the samples was confirmed with UV-Vis spectroscopy (see below).

Size exclusion chromatography. The α cp-β Hb 4SM N108K construct was further purified with an Amersham Pharmacia Biotech Superdex 75 HR 10/30 column (24 mL CV). The column was equilibrated with 2 CV water and then 2 CV ammonium acetate, pH 8.5 at 0.5 mL/min. The protein sample was centrifuged for 10 min at 10,000 x g prior to injection onto the column. Eluent with a red tint was collected.

Protein Characterization

UV-Vis spectroscopy. Purifed Hb samples were characterized by UV-visible spectroscopy with a Thermo Scientific Nanodrop 1000 Spectrophotometer. The absorbance of the Soret band at 415 nm was measured to calculate Hb concentration (the extinction coefficient of oxy-Hb = 12,500 M^{-1} cm^{-1}).

SDS-PAGE. Samples were prepared by 1:1 dilution with 2X Bio-Rad Laemmli sample buffer. Samples were added to wells in a stacking gel with a 15% resolving layer [15% (v/v) acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% (v/v) SDS, 0.1% (v/v) APS, TEMED] and a 5% stacking layer [5% (v/v) acrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% (v/v) SDS, 0.1% (v/v) APS, TEMED]. To track protein migration Thermo Scientific PageRuler Prestained Protein Ladder was added to an

empty lane. Electrophoresis was carried out for 30 min at 90 V, which forced the samples to travel through the stacking layer. The voltage was increased to 150 V until the samples had traveled to the bottom of the gel. The gel was removed and then imaged. For fluorescence imaging the gel was immediately scanned using a Bio Rad Gel Doc EZ imager. Following the image, the gel was incubated in Coommassie protein stain [50% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R250], followed by destain (1:3:4 glacial acetic acid:methanol:water solution). Images of Coomassie-stained gels were obtained with a Bio Rad Gel Doc EZ imager.

Protein mass spectrometry. All electrospray ionization mass spectrometry (ESI-MS) analysis were done on an Advion expression CMS instrument attached to a Thermo Scientific Dionex Ultimate 3000 HPLC system. Protein samples were analyzed by ESI-MS on an Aeris 3.6 μm WIDEPORE XB-C8 200 Å LC column using Method A [Solvent A = 5% acetonitrile, 0.1% formic acid; Solvent B = acetonitrile, 0.1% formic acid (mobile phase). Flow rate = 4 mL/min. Gradient = 10% solvent B, 90% solvent A (0.0-2.0 min), 10% solvent B to 90% solvent B (2.0-12.0 min), hold 90% solvent B (12.0-14.0 min), 90% solvent B to 10% solvent B (14.0-14.1 min), equilibrate back to 10% solvent B, 90% solvent A (14.1-17.0 min)]. Spectra were analyzed with MestReNova and reconstructed with Analyst.

Conjugation and ligation of sHb

Synthesis of GGK(DBCO). The peptide Fmoc-GGK, obtained from Sierra Reed and John Antos, was ligated to dibenzocyclooctyne (DBCO), deprotected and purified. To ligate Fmoc-GGK to DBCO 5.49 μmol DBCO-NHS was mixed with 11 μmol Fmoc-GGK, 32.9 μmol DIPEA, and

N-methyl-2-pyrrolidone (NMP) in a total reaction volume of 100 μl. The reaction mixture was incubated at 25°C for 25 min and product formation was confirmed with ESI-MS on a Kinetex 2.6 μm, C18 100 Å LC column (100 x 2.1 mm, C18, Phenomenex) using Method B [Solvent A = 5% acetonitrile, 0.1% formic acid; Solvent B = acetonitrile, 0.1% formic acid (mobile phase). Flow rate = 0.4 mL/min. Gradient = 100% solvent A (0.0-1.0 min), 0% solvent B to 90% solvent B (1.0-7.0 min), hold 90% solvent B (7.0-9.0 min), 90% solvent B to 100% solvent A (9.0-9.1 min), equilibrate at 100% solvent A (9.1-12.0 min)] (Expected mass for Fmoc-GGK(DBCO) = 768.3 Da, observed mass = 768.3 Da). Product was then deprotected by mixing with 20% (v/v) piperidine followed by incubation at 25°C for 45 min. The reaction was monitored by ESI-MS on the Kinetex C18 column using Method B and determined to be complete by 45 min (expected mass for GGK(DBCO) = 546.3 Da, observed mass = 547.3 Da). The reaction mixture was then purified with HPLC on the Luna 5u C18(2) 100 Å column (250 x 10 mm) (semi-prep, Phenomenex) using Method C [Solvent A = 5% acetonitrile, 0.1% formic acid; Solvent B = acetonitrile, 0.1% formic acid (mobile phase). Flow rate = 4 mL/min. Gradient = 10% solvent B, 90% solvent A (0.0-2.0 min), 10% solvent B to 90% solvent B (2.0-15.0 min), hold 90% solvent B (15.0-17.0 min), 90% solvent B to 10% solvent B (17.0-17.1 min), equilibrate back to 10% solvent B, 90% solvent A (17.1-20.0 min)]. Eluent was monitored at 280 nm as well as 300 nm and the peak eluting at about 8.5 min was collected. Formic acid and acetonitrile were removed from the collected sample by rotary evaporation and the sample was then frozen over dry ice and lyophilized to remove any remaining solvent. The purified peptide was resolubilized in 100 μl water and 20 μl DMSO and its identity was confirmed by LC-ESI-MS with Method B as described above (Figure

A1 and A2). Final concentration of the GGK-DBCO stock solution was determined using UV-Vis at λ_{max} = 309 nm and a molar extinction coefficient of 12,000 M^{-1} cm^{-1}.

Sortase-mediated ligation. Di-α cp-β sHb was ligated to the peptide GGK(FITC) (FITC = fluorescein isothiocyanate) as a proof-of-concept prior to ligation to GGK(DBCO). In ligation reactions 50 μM di-α cp-β sHb was mixed with 100 μM GGK(FITC) (obtained from Sierra Reed and John Antos) and 5 μM sortase A heptamutant in sortase buffer (500 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 100 mM $CaCl_2$). The reaction was incubated for 150 min at 25 °C with samples removed and denatured every 30 min for SDS-PAGE and mass spectrometry. The di-α cp-β sHb ligation to GGK(DBCO) was repeated in the same manner except that the reaction was monitored for 120 min.

Purification of di-α(DBCO) cp-β sHb product. Di-α(DBCO) cp-β sHb was purified from the sortase-mediated ligation reaction mixture with IMAC. The IMAC column [3 mL Thermo Scientific HisPur Ni-NTA (nitrilotriacetic acid)resin] was prepared by washing the packed resin with 10 CV water followed by 10 CV loading buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole). The reaction mixture was then loaded onto the equilibrated column and washed with 10 CV loading buffer. Di-α(DBCO) cp-β sHb was then eluted with a low imidazole buffer (20 mM Tris pH 8.0, 150 mM NaCl, 50 mM imidazole) and di-α cp-β sHb was eluted with a high imidazole buffer (20 mM Tris pH 8.0, 150 mM NaCl, 250 mM imidazole). Eluents were initially characterized with UV-Vis spectrometry and then samples containing hemoglobin were characterized with SDS-PAGE and mass spectrometry.

Cyclooctyne azide conjugation. Purified di-α(DBCO) cp-β sHb was then conjugated to fluorescent 6-FAM (fluorescein)-azide by adding 20 μM di-α(DBCO) cp-β sHb to 10 μM 6-FAM-

azide (obtained from Sierra Reed and John Antos). The reaction was incubated at 25°C for 60 min and samples were taken and denatured every 15 min for analysis with SDS-PAGE and mass spectrometry.

Crystallization

Crystal trays. A total of 30 crystal trays were made and each tray was made with varying buffer conditions, protein concentration, and temperature (Table A1). Initially, the top of each well was coated with petroleum jelly to help create a seal and according to the conditions, and 500 μl of the specified buffer was placed in each well in a 24-well tray. On a cover slide, 1-2 μl of buffer was mixed with 1 μl of protein solution, producing a single drop. When specified in Table 1, 40 μl of Hampton Research Al's Oil was added on top of the protein:buffer drop. The cover slide was then flipped over and placed on the top of the well, where the slide was then pushed down and turned to ensure a good seal. The tray was placed in a dark area at the specified temperature and crystal growth was monitored by observation using a Motic digital light microscope.

X-ray diffraction. Crystals were collected based on size, structure, and color. To collect a crystal, 15% glycerol cryo buffer (specified buffer with 15% glycerol) was added to the crystal slide followed by 30% glycerol cryo buffer (specified buffer with 30% glycerol). Crystals were then captured with a crystal loop and quickly transferred to liquid nitrogen. Crystals were stored in liquid nitrogen until diffraction data was collected. X-ray diffraction data were collected on Rigaku XtaLAB crystal X-ray diffractometer. A total of 3 images were collected with steps of 30 degrees.

Results

With the goal of developing a high molecular weight monodisperse hemoglobin, we expressed 2 Hb constructs to study further. The first construct, N108K Hb, was expressed in order to determine the structure of the low oxygen affinity mutant by X-ray crystallography. The second construct, sHb, was expressed to test the efficacy of sortase-mediated ligation, followed by azide-alkyne conjugation.

Protein expression and purification. Both protein constructs were successfully expressed in BL21 *E. coli* cells*.* After the IMAC purification step N108K Hb was recovered in a crude yield of 35 mg/L. N108K Hb was further purified with anion exchange chromatography followed by size exclusion chromatography to >95% homogeneity based on densitometry performed on SDS-PAGE gels (Figure 9).

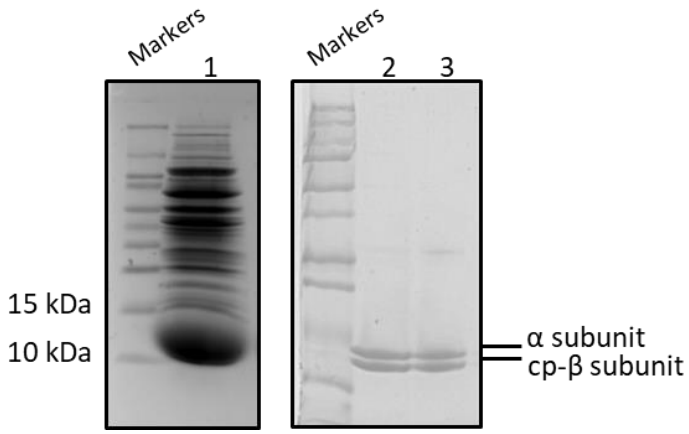


Figure 9. Purification analysis of N108K Hb by SDS-PAGE. N108K Hb was purified first with IMAC (Step 1), followed by anion exchange chromatography (Step 2) and size exclusion chromatography (Step 3).

The sHb expression was significantly lower than that for N108K Hb, with a crude yield of 1.035 mg/L after IMAC. With the hexa-histidine tag, however, we were able to get good purification after 2 steps: IMAC followed by anion exchange chromatography. The sHb was purified to 98% homogeneity based on densitometry (Figure 10).

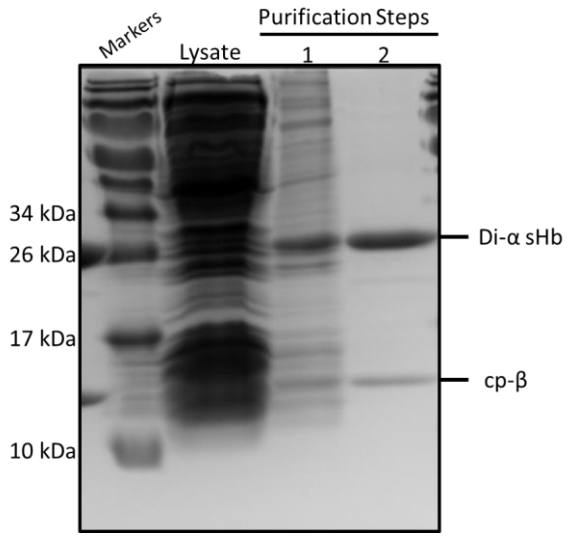


Figure 10. Purification analysis of sHb with SDS-PAGE. sHb was purified from lysate using IMAC (Step 1), followed by anion exchange chromatography (Step 2)

Sortase-mediated ligation of GGK(FITC) to sHb. In order to verify that the sortase-mediated ligation would occur with Hb, we wanted to test the reaction with a fluorescent probe so that the reaction progress could be easily visualized. Thus, we initially tested this reaction by combining sHb with GGK(FITC) and sortase A (Figure 11). The reaction occurred for

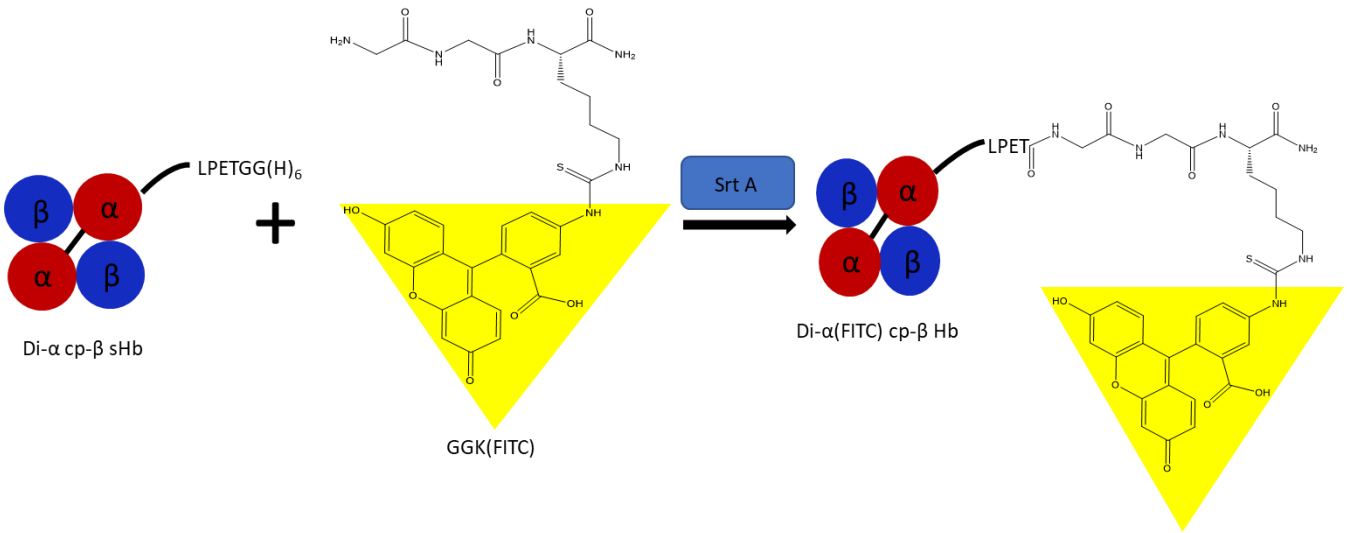


Figure 11. Sortase-mediated ligation of GGK(FITC) to sHb.

150 minutes and was analyzed both with ESI-MS and SDS-PAGE. By measuring the fluorescence in the gel as well as staining the gel with Coomassie stain we were able to monitor any changes among all of the proteins present as well as those that were modified with a fluorescent label. During the course of the reaction the migration of the cp-β bands stayed constant, whereas the fluorescence intensity of the small GGK(FITC) linker decreased and the larger di-α(FITC) band increased (Figure 12). In addition, from ESI-MS we were able to verify the presence of di-α(FITC) as well as calculate the overall reaction yield of 75% (Figure 13).

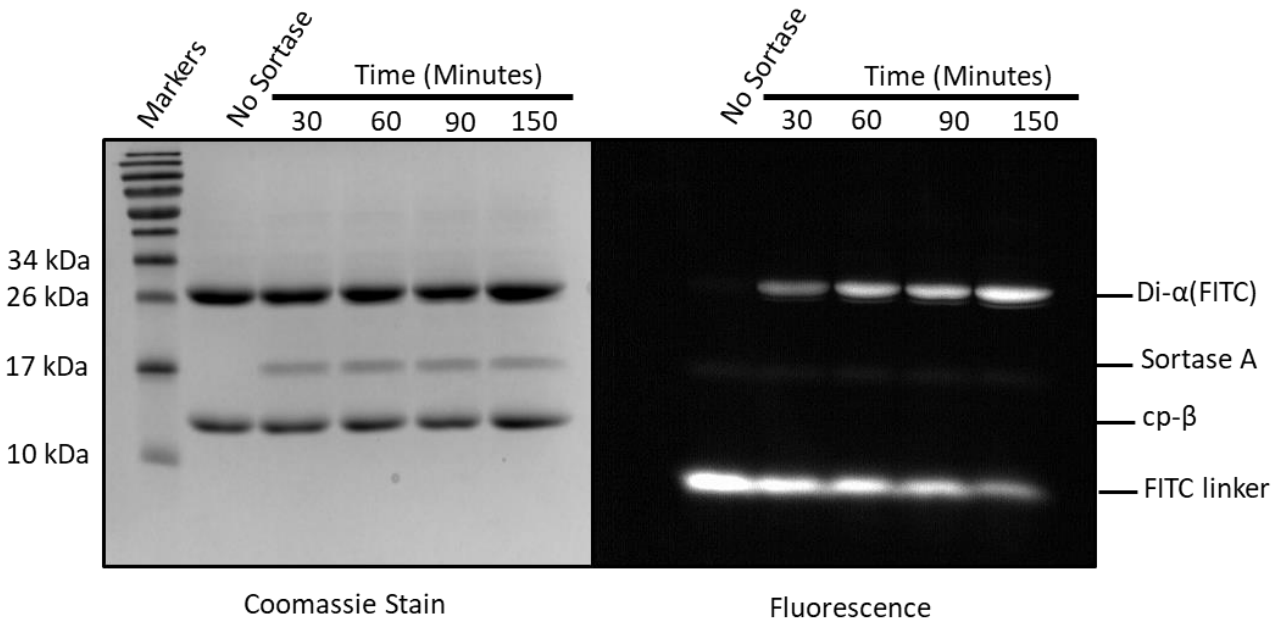


Figure 12. SDS-PAGE of GGK(FITC) ligation to sHb. Reaction ran for 150 min. Bands present include GGK(FITC) with a mass of 685.71 Da, *cp-β* with a mass of 16,532 Da, sortase A with a mass of 17,851 Da, di-α(FITC) with a mass of 32,052 Da, and di-α with a mass of 32,361 Da.

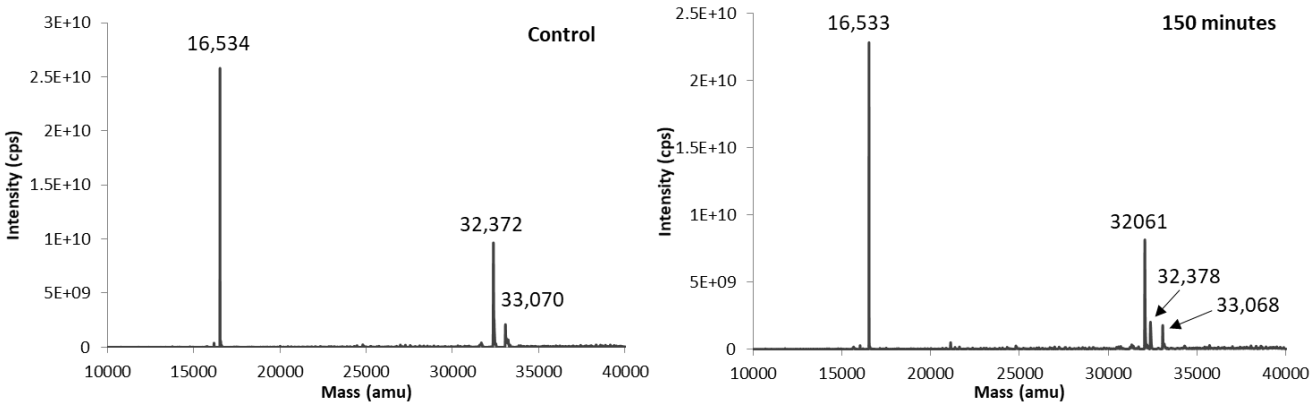


Figure 13. ESI-MS of GGK(FITC) ligation to sHb. A sample was analyzed before addition of GGK(FITC) (control) and again after 150 min. Peaks present in the spectra include *cp-β* with an expected mass of 16,532 Da, di-α(FITC) with an expected mass of 32,052 Da, di-α with an expected mass of 32,361 Da and the double charge of *cp-β* with an expected mass of 33,064 Da.

Sortase-mediated ligation of GGK(DBCO) to sHb. Once we demonstrated that the sortase-mediated ligation to sHb worked, we then wanted to add the strained cyclooctyne (DBCO) to sHb (Figure 14). Since there is no fluorescent tag in this reaction the SDS-PAGE results are less informative due to the small molecular weight difference between di-α and di-α(DBCO) (Figure 15). However, the successful ligation of DBCO to sHb was suggested by ESI-MS, which showed a prominent peak at 31,952 Da, which is just slightly below the expected mass of

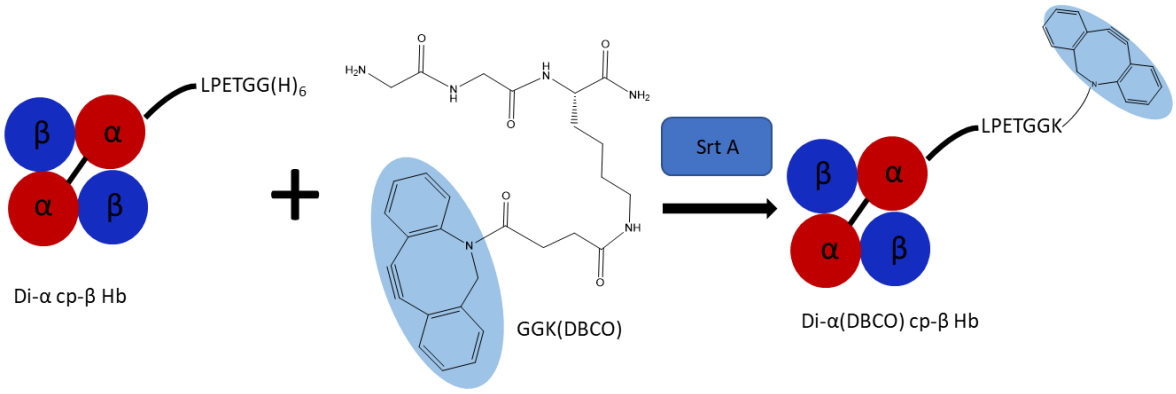


Figure 14. Sortase-mediated ligation of GGK(DBCO) to sHb.

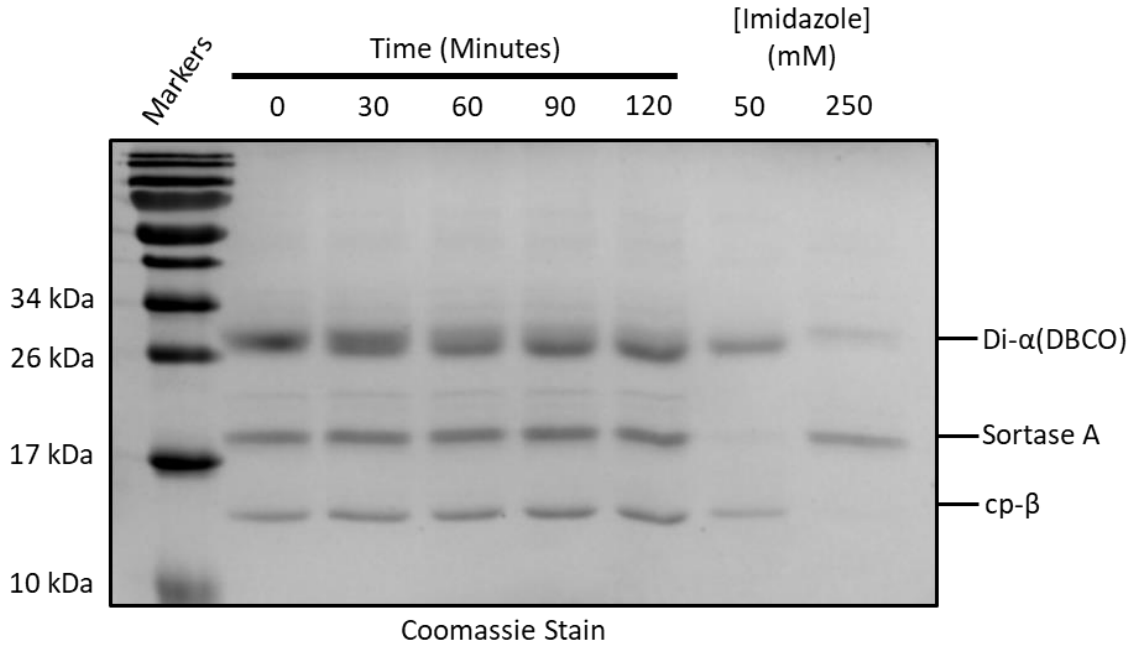


Figure 15. SDS-PAGE of GGK(DBCO) ligation to sHb followed by purification. The reaction ran for 120 min and was then purified. Samples were collected from the 50 mM imidazole elution as well as the 250 mM imidazole elution. Bands present include *cp-β* with a mass of 16,532 Da, sortase A with a mass of 17,851 Da, di-α(DBCO) with a mass of 31,969 Da, and di-α with a mass of 32,361 Da.

di-α(DBCO) of 31,969 Da. The di-α peak at 32,361 Da also decreased in intensity and a reaction yield of 87% product was calculated (Figure 16).

Purification of di-α(DBCO) cp-β sHb. Following the sortase-meditated ligation of DBCO to sHb we then wanted to establish that we could purify the desired product from the reaction mixture. To do this we took advantage of the loss of the hexa-histidine tag from sHb during the ligation. Due to several histidine residues on the surface of human Hb, Hb naturally has a moderate binding affinity for Ni^{2+} IMAC resin. Thus, while di-α(DBCO) sHb still had some affinity for the Ni^{2+} IMAC column it had a reduced affinity compared to the unligated di-α sHb and so

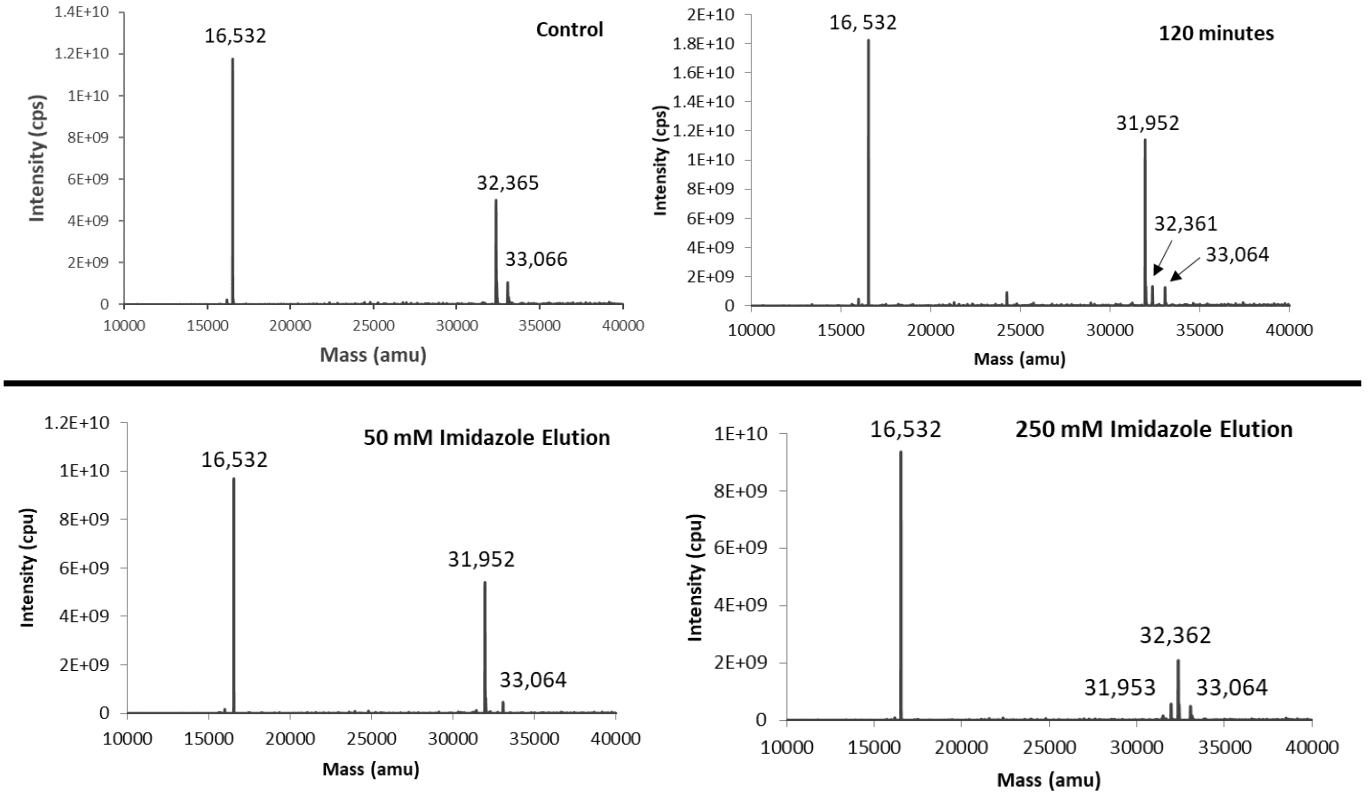


Figure 16. ESI-MS spectra of GGK(DBCO) ligation to sHb followed by purification. A sample was analyzed before addition of GGK(DBCO) (control – top left) and again after 120 min (top right). The reaction mixture was purified with IMAC resulting in a 50 mM imidazole elution of di-α(DBCO) (bottom left) and 250 mM imidazole elution of di-α (bottom right). Peaks present in the spectra include *cp-*β with an expected mass of 16,532 Da, di-α(DBCO) with an expected mass of 31,969 Da, di-α with an expected mass of 32,361 Da and the double charge of *cp-*β with an expected mass of 33,064 Da.

eluted at a lower imidazole concentration of 50 mM imidazole. The unligated di-α sHb eluted from the Ni^{2+} column at 250 mM imidazole. The SDS-PAGE showed a slightly lower band at 32 kDa in the 50 mM imidazole lane which suggests the presence of di-α(DBCO), while in the higher imidazole lane, the di-α band has slightly retarded mobilty (Figure 15). The expected lower mass for the purified DBCO-sHb was also confirmed by ESI-MS with a peak at 31,952 Da for 50 mM imidazole elution sample (Expected mass of di-α(DBCO) = 31,969 Da) compared to

the peak at 32,362 Da for the 250 mM imidazole elution sample (Expected mass of di-α = 32,359 Da) (Figure 16).

Cyclooctyne azide conjugation. The last proof-of-concept for the development of our monodisperse HBOC was the cycloocytne azide conjugation. The reaction was carried out in the absence of a copper ion catalyst with the addition of 6-FAM-azide to the purified di-α(DBCO) Hb and monitored for 60 min (Figure 17). SDS-PAGE showed that while the migration of the *cp-β* band was constant, the band for di-α was slightly retarded. Additionally, the fluorescence intensity at the di-α molecular weight increased as the reaction progressed consistent with the addition of 6-FAM to the di-α globin (Figure 18). ESI-MS also showed a peak at 32,415 Da which is slightly lower than the expected mass of 32,430 Da for di-α(DBCO-6-FAM-azide). The calculated yield was 68% (Figure 19).

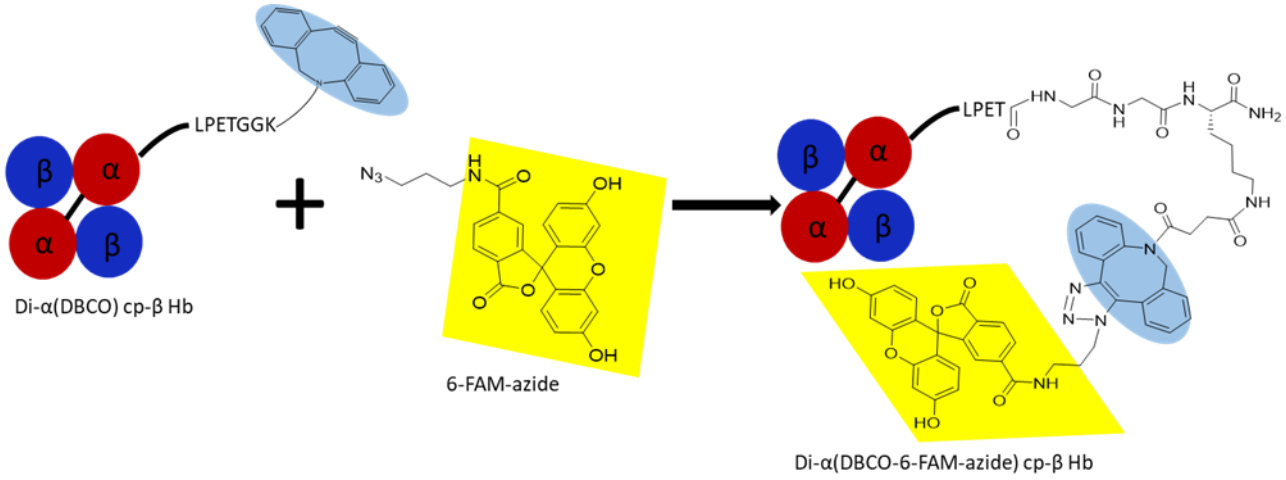


Figure 17. Cyclooctyne azide conjugation.

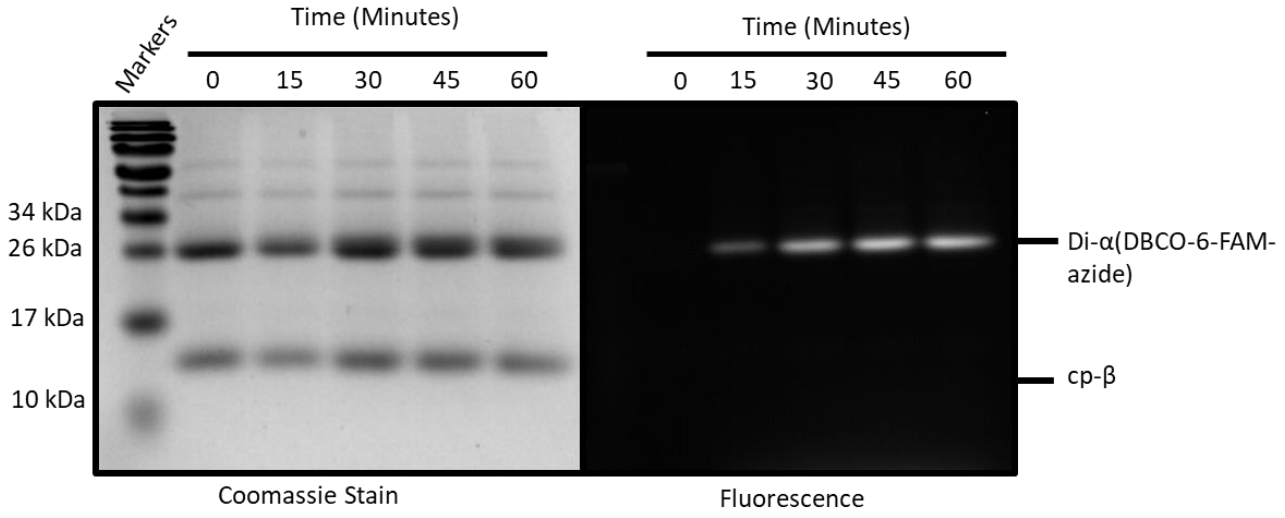


Figure 18. SDS-PAGE of 6-FAM-azide conjugation to sHb(DBCO). The reaction ran for 60 min. Bands present include *cp-β* with a mass of 16,532 Da, di-α(DBCO) with a mass of 31,969 Da, and di-α(DBCO-6-FAM-azide) with a mass of 32,430 Da.

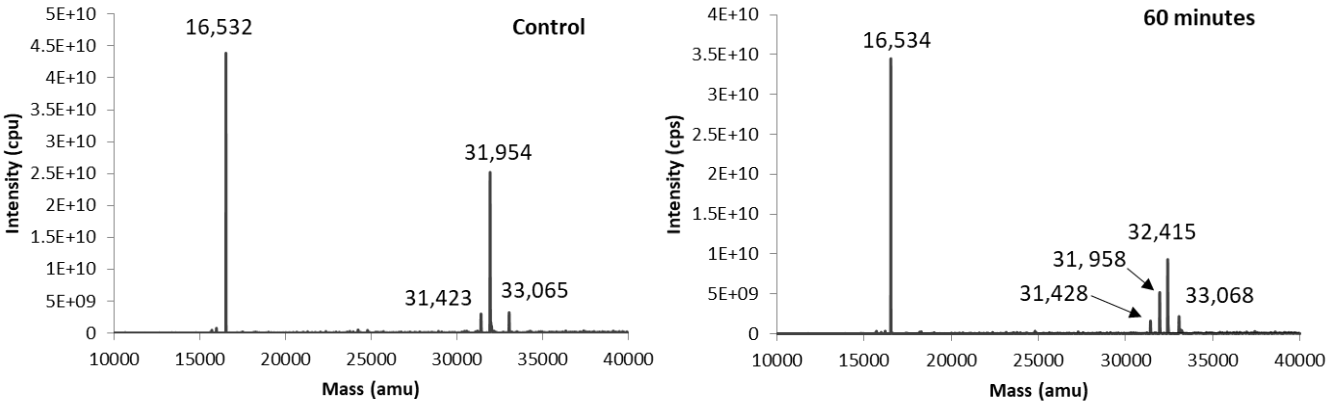
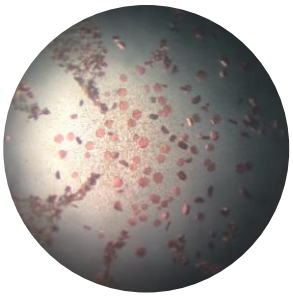
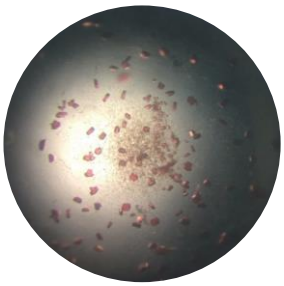


Figure 19. ESI-MS of 6-FAM-azide conjugation to sHb(DBCO). A sample was analyzed before addition of 6-FAM-azide (control) and then the reaction ran for 60 min. Peaks present in the spectra include *cp-β* with an expected mass of 16,532 Da, di-α(DBCO) with an expected mass of 31,969 Da, di-α(DBCO-6-FAM-azide) with an expected mass of 32,430 Da and the double charge of *cp-β* with an expected mass of 33,064 Da.



5 mg/ml
1:1 Protein:Buffer
PEG 3350 28%
0.1 M Bis Tris pH 5.8
4ºC

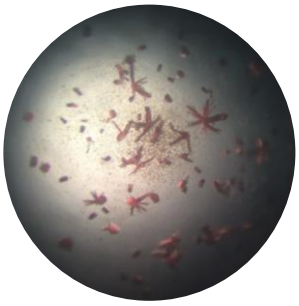


5 mg/ml
1:1 Protein:Buffer
PEG 3350 24%
0.1 M Bis Tris pH 5.8
4ºC

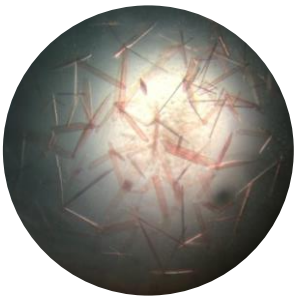
Figure 20. Crystal conditions with varying PEG concentration.

Crystallization and X-ray diffraction. A total of 31 crystal trays were made with the hanging drop method and a variety of buffer (mother liquor) compositions. Almost all trays were made with a maximum protein concentration of 5 mg/mL because at higher concentrations Hb precipitated out of solution. The ratio of protein to buffer added to the hanging drop was 1:1 or 1:2 for the same buffer conditions. The pH of the buffer, 0.1 M Bis Tris, varied from 5.8-7.5. The largest crystals with the best structure were obtained in conditions where the buffer pH was between 5.8-6.2 (Figures 20-22). Polyethylene glycol (PEG) 3350 was added with varying amounts ranging from 18-33% (w/v). Larger crystals with better morphology were obtained when the PEG concentrations was 22-28% (Figure 20-21).

Various salts were added such as sodium chloride, ammonium acetate, and ammonium sulfate which resulted in crystals with different geometric structures compared to those obtained



5 mg/ml
1:1 Protein:Buffer
PEG 3350 22%
0.1 M Bis Tris pH 6.0
10 mM NaCl
4ºC



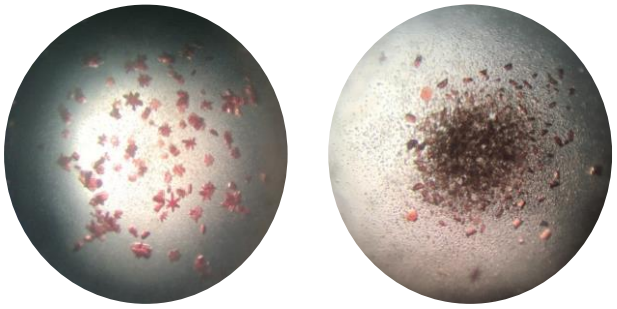
5 mg/ml
1:1 Protein:Buffer
PEG 3350 22%
0.1 M Bis Tris pH 6.0
20 mM $(NH_4)_2SO_4$
4ºC

Figure 21. Crystal conditions with the addition of sodium or ammonium sulfate.

without added salt. In the absence of added salt the crystals had a square geometry with a large surface area.

With the addition of salt, the crystals formed were long, thin and rectangular. Among the different salts conditions tested, the addition of ammonium sulfate resulted in the largest crystals with the best morphology (Figure 21). To reduce precipitation, crystal growth was slowed by adding Al's oil to some of the crystal trays, as well as reducing the temperature at which the trays were made and stored from 25°C to 4 °C (Figure 22).

Crystals with red color, regular morphology, and noticeably larger size were looped and frozen for X-ray diffraction. With the help of Professor Clint Speigel, crystals were mounted in the diffractometer and the diffraction images were collected. Unfortunately, none of these crystals diffracted with sufficient resolution for protein structure determination.



5 mg/ml
1:1 Protein:Buffer
PEG 3350 28%
0.1 M Bis Tris pH 6.2
4ºC

5 mg/ml
1:1 Protein:Buffer
PEG 3350 28%
0.1 M Bis Tris pH 6.1
25ºC

Figure 22. Crystal conditions at different temperatures.

Discussion

While the development of a monodisperse, oligomeric HBOC is by no means complete, the initial experiments reported here demonstrate the potential of our approach. We have established proof-of-concept for linking DBCO-modified Hb molecules to a scaffold that displays

azide groups. This proof-of-concept was achieved in a stepwise fashion by testing a series of reactions that could be easily monitored.

One of the biggest concerns that needs to be resolved is the low expression yield of ~1.0 mg/mL for the sHb construct. A large reason for this low expression is the inclusion of cp-β globin into the sHb construct, which is known to have a reduced expression in comparison to wt β globin. This β–globin variant was used in these initial experiments in the interest of time: we had expression vectors with the cp-β gene readily available. Now that we have established proof-of-concept for sortase A's ability to modify Hb, we will create an alternate version of sHb that co-expresses a wild-type (wt) β globin with the sortase-tag di-α globin. With the addition of wt β the expression of sHb should increase dramatically (in our experience by > 10-fold). In the unlikely chance that expression does not increase, the sortase tag on the di-α globin may be inhibiting expression. Other ways to optimize the expression yields include changing the amount of time the cells are grown, changing the temperature during the growth, or including additional nutrients. Increasing expression is necessary to support future preclinical testing of polymeric Hbs.

Once expressed and purified the sHb contruct was easily modified with reactions that produced good yields. While the yield for each reaction could be improved, the cyclooctyne azide conjugation has the most room for optimization. To increase product formation the reaction could continue for a longer period of time, and the linker, 6-FAM-azide, could be used in greater excess, such as 3x, 5x, 10x. Although tractable in the near-term, requiring a large excess of the azide reagent is not desirable; thus, optimizing yields through changes in reaction conditions will be a top priority.

To optimize the sortase-mediated reactions, more focus should be placed on minimizing side reactions. Side reactions that could be occurring include the reverse reaction which results in the re-formation of the starting sHb as well as formation of a hydrolysis product (Figure 23). The reverse reaction occurs when the cleaved peptide with an N-terminal glycine acts as a nucleophile and reforms the starting material. To prevent this, a 2x excess of the linker peptide is added in order to push the reaction forward. Another strategy to minimize the reverse reaction is to add nickel to the reaction mixture which can then chelate with the cleaved peptide to prevent it from reacting[30]. There are fewer options for preventing the hydrolysis product.

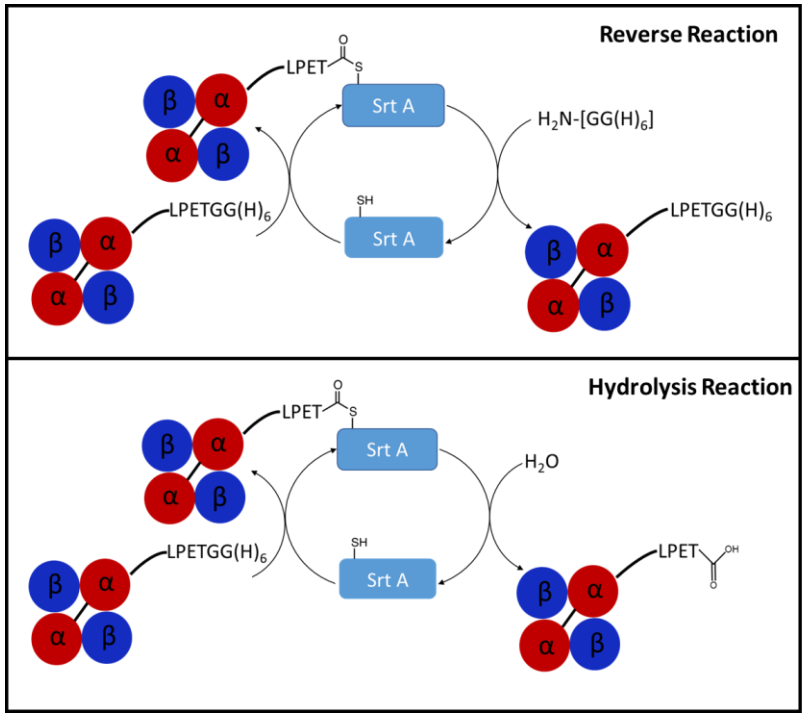


Figure 23. Side reactions during sortase-mediated ligations.

However, based on the initial experiments described above, it does not seem to be a significant problem for ligations with sHb. Only a small fraction of the total di-α added to the reaction appears to form the hydrolysis product.

The next steps for the development of this HBOC are to conjugate sHb to a larger azide scaffold. First, sHb conjugation to a small azide tetramer will be tested, followed by increasingly larger azide scaffolds. We anticipate that the structure of the scaffold will need to be optimized to allow multiple high molecular weight proteins to react with the azide groups. Commercially available scaffolds will be tested first, but these reagents have relatively short arms that may sterically prevent multiple ligations with Hb. Furthermore, it will be necessary to test the oxygen binding affinities of the therapeutic as it is developed to verify that it can reversibly bind oxygen. If as expected, the product has high oxygen binding affinity, the mutant βN108K can be incorporated into the protein, therefore reducing the oxygen affinity to a physiologically-functional level.

In support of efforts to modulate poly-Hb oxygen binding, we undertook structural studies of N108K mutants. We have not yet obtained suitable diffraction data. In order to get a high-resolution X-ray data set, the production of N108K Hb crystals needs to be further optimized. While crystal nucleation is not an issue, the formation of crystals that are large enough with good morphology for crystallography is needed. With the conditions sampled, crystals are able to form but many have irregular morphology or a small size. For the time being, this effort will be suspended until we narrow down the Hb variant that is optimal for the sortase ligation work.

Through rational design that combines genetic and chemical modifications, our strategy to generate an HBOC shows potential to provide a functional oxygen carrier for acute blood replacement. Employing sortase A to add a highly-specific, biocompatible DBCO functional group to Hb allows us to explore the potential of “click chemistry” to generate a monodisperse poly-Hb. Moreover, by investigating mutations to decrease oxygen affinity, our product will have increased capability of transporting oxygen.

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Appendix

Table A1. Crystal Tray Conditions

Tray	[Protein]	Protein:Buffer	Temperature °C	Column Condition	Variance	Row Condition	Variance	Other
1	5	1:1	25	0.1 M Bis Tris pH	5.9, 6.0, 6.1, 6.2	PEG 3350 (%)	25, 26, 27, 28, 29, 30	none
2	5	1:1	25	0.1 M Bis Tris pH	5.9, 6.0, 6.1, 6.2	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
3	5	1:1, 1:2	25	0.1 M Bis Tris pH	5.75, 6.05,6.6,7.0	PEG 3350 (%)	26, 28, 30, 32, 34 , 36	none
4	5	1:1, 1:2	25	0.1 M Bis Tris pH	6.05,6.3,6.4, 6.6	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
5	5	1:2, 1:3	25	0.1 M Bis Tris pH	6.05,6.3,6.4, 6.6	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
6	3	1:1, 1:2	25	0.1 M Bis Tris pH	6.05, 6.3, 6.4, 6.6	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
7	5	1:1, 1:2	25	0.1 M Bis Tris pH	6.0, 6.3, 6.5, 6.8	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
8	5	1:1, 1:2	25	0.1 M Bis Tris pH	6.0, 6.3, 6.5, 6.8	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
9	5	1:1, 1:2	25	NaCl (mM)	0, 10, 15, 20	PEG 3350 (%)	28, 29, 30, 31, 32, 33	0.1 M Bis Tris pH 6.0
10	5	1:1	25	NaCl (mM)	0, 10, 15, 20	PEG 3350 (%)	28, 29, 30, 31, 32, 33	0.1 M Bis Tris pH 6.8
11	5	1:1	25	0.1 M Bis Tris pH	6.0, 6.3, 4.4, 6.6	PEG 3350 (%)	28, 29, 30, 31, 32, 33	Al's oil
12	5	1:1	25	0.1 M Bis Tris pH	6.0, 6.3, 4.4, 6.6	PEG 3350 (%)	28, 29, 30, 31, 32, 33	none
13	5	1:1	4	NaCl (mM)	0, 5, 10, 20	PEG 3350 (%)	28, 29, 30, 31, 32, 33	0.1 M Bis Tris pH 6.0
14	5	1:1	4	NaCl (mM)	0, 5, 10, 20	PEG 3350 (%)	18, 20, 22, 24, 26, 28	0.1 M Bis Tris pH 6.0
15	5	1:1	4	0.1 M Bis Tris pH	5.8, 6.0, 6.2, 6.4	PEG 3350 (%)	18, 20, 22, 24, 26, 28	none
16	10	1:1	4	0.1 M Bis Tris pH	5.8, 6.0, 6.2, 6.4	PEG 3350 (%)	18, 20, 22, 24, 26, 28	none
17	5	1:1, 1:2	4	NaCl (mM)	10, 20, 30, 40	PEG 3350 (%)	22, 24, 26, 28, 30, 32	0.1 M Bis Tris pH 6.0
18	5	1:1, 1:2	4	(NH4)2SO2	0, 5, 10, 20	PEG 3350 (%)	22, 24, 26, 28, 30, 32	0.1 M Bis Tris pH 6.0
19	5	1:1	4	(NH4)2SO2	0, 5, 10, 20	PEG 3350 (%)	22, 24, 26, 28, 30, 32	0.1 M Bis Tris pH 6.0
20	5	1:1, 1:2	4	NaCl (mM)	0, 20, 40, 60	PEG 3350 (%)	22, 24, 26, 28, 30, 32	0.1 M Bis Tris pH 6.0
21	5	1:1, 1:2	4	(NH4)2SO2	0,10, 20, 30	PEG 3350 (%)	22, 24, 26, 28, 30, 32	0.1 M Bis Tris pH 6.0
22	5	1:1, 1:2	4	(NH4)2SO2	0,10, 20, 30	0.1 M Bis Tris pH	5.8, 6.1, 6.4, 6.7, 7.0, 7.5	24% PEG
23	5	1:1, 1:2	4	PEG 3350 (%)	24, 26, 28, 30	NH4(SO2)2	10, 20, 30, 40, 50, 60	0.1 M Bis Tris pH 6.0
24	5	1:1, 1:2	4	(NH4)2SO2	0,10, 20, 30	PEG 3350 (%)	18, 22, 26, 32, 36, 40	0.1 M Bis Tris pH 6.0
25	5	1:1, 1:2	4	0.1 M Bis Tris pH	6.0, 6.2, 6.4, 6.5	NH4(SO2)2	40, 50, 60, 70, 80, 90	24% PEG
26	5	1:1, 1:2	4	PEG 3350 (%)	22,23,24,26	NH4(SO2)2	40, 50, 60, 70, 80, 90	0.1 M Bis Tris pH 6.0
27	5	1:1, 1:2	4	0.1 M Bis Tris pH	6.0, 6.3,6.4, 6.5	PEG 3350 (%)	20, 22, 24, 26, 28, 30	Al's oil
28	5	1:1, 1:2	4	PEG 3350 (%)	20, 22, 24, 26	NH4(SO2)2	0, 20, 40, 60, 80, 100	0.1 M Bis Tris pH 6.5
29	5	1:1, 1:2	4	PEG 3350 (%)	18,20,24,26	NH4(SO2)2	0, 20, 40, 60, 80, 100	0.1 M Bis Tris pH 6.5
30	5	1:1, 1:2	4	PEG 3350 (%)	23, 24, 25, 26	0.1 M Bis Tris pH	6.2, 6.3, 6.4, 6.5, 6.6	none
31	5	1:1, 1:2	4	0.1 M Bis Tris pH	5.9, 6.1,6.27,6.43	PEG 3350 (%)	22, 22, 23, 23, 24, 24	none

Synthesis and purification of GGK(DBCO). The peptide GGK(DBCO) was successfully synthesized and purified. After the initial purification with Method A (see Materials and Methods) there was still residual Fmoc in solution so a second purification was carried out. Better separation of the DBCO and Fmoc peak was achieved with Method C, and we were able to purify GGK(DBCO) to >95%.

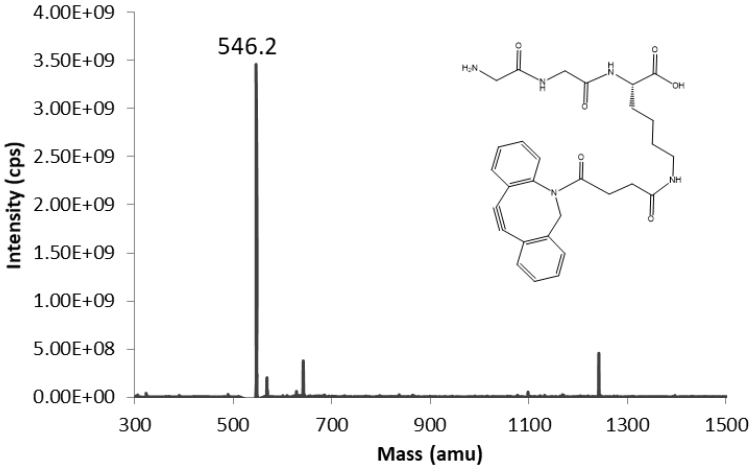

Figure A1. ESI-MS analysis of GGK(DBCO). Main peak at 546.2 Da confirmed the presence of GGK(DBCO) which has an expected mass of 546.26 Da.

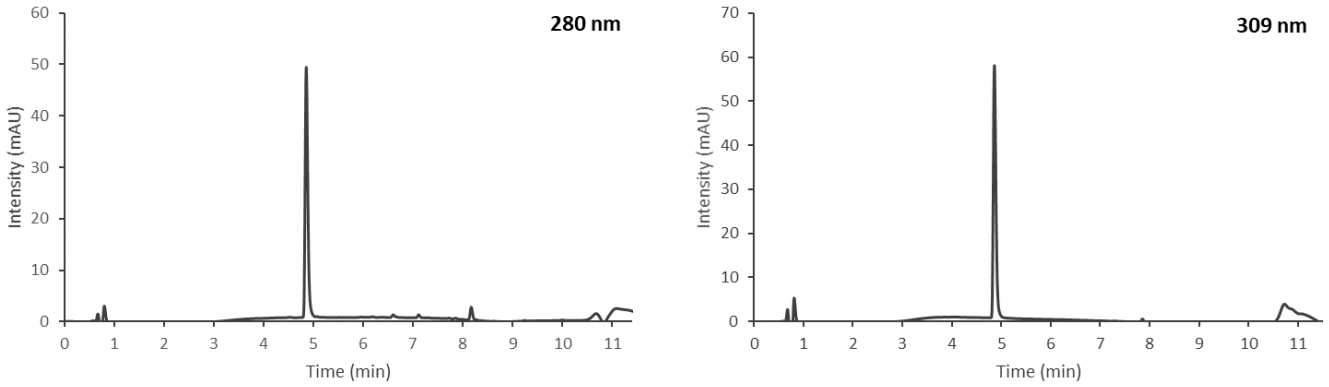

Figure A2. HPLC spectra of GGK(DBCO). The main peaks seen both at 280 nm and 309 nm confirm purity of GGK(DBCO) purity.