Hydrazine as a Nucleophilic Anchor in The Formation of Hemoglobin Bis-tetramers

By

Daniel R. Bator

A thesis submitted in conformity with the requirements For the Degree of Masters of Science, Graduate Department of Chemistry, University of Toronto

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Abstract

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Masters of Science, 2011

Daniel Bator, Graduate Department of Chemistry, University of Toronto

Two alternative compounds have been prepared and studied, as alternatives to the benzyl amine in the preparation of hemoglobin bis-tetramers. The reactivity of these compounds towards the activated ester groups on trimesoyl tris(3,5-dibromosalicylate), and 3,5-dibromosalicylate benzyl ester has been studied, as well as their functionality in a range of acidic solutions. Initial rate kinetics was used to determine rate constants for hydrazinolysis, and hydrolysis of the two esters. The objective was to find a suitable functional group that would be less basic than the benzyl amine yet still exhibit good nucleophilicity in the preparation of hemoglobin bis-tetramers. Hydrolysis being the main side reaction limiting the yield, could be slowed down in an acidic solution, but the nucleophile used could not be protonated in the process. It has been found that the benzyl hydrazine was the most effective and able to react with cross-linked hemoglobin to give the most product.
Acknowledgements

I would like to express my gratitude to Professor Ronald H. Kluger for his guidance, help, and insight, throughout the course of my work.

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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HBOC</td>
<td>Hemoglobin Based Oxygen Carrier</td>
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<tr>
<td>TTDS</td>
<td>Trimesoyl tris(3,5-dibromosalicylate)</td>
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<tr>
<td>DBS</td>
<td>3-5-di-bromo-salicylic benzyl ester</td>
</tr>
<tr>
<td>DPG</td>
<td>2,3-diphosphoglycerate site</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<tr>
<td>MOPS</td>
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</tr>
<tr>
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<td>Tetrafluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>UV-Vis</td>
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CHAPTER I:

Introduction

1.1 Background and previous work

The search for an alternative to red blood cells for oxygen transport has led to the creation of hemoglobin based oxygen carriers (HBOC). This is an approach that uses hemoglobin as the main oxygen delivery system and has many potential advantages over traditional blood transfusions. Although highly sensitive screenings are in place to assure safety, risks of disease transmission remain for those relying on donor blood. Careful blood-type matching is another safety issue that could be eliminated with a successful HBOC product. Lastly, donated blood suffers from a short shelf life, from which shortages can occur from time to time. Therefore, a need for an affordable, safe, and universal, alternative blood substitute is evident, and its development could benefit many people.¹²

The properties of cell free hemoglobin have been thoroughly studied for some time. Normal adult hemoglobin is a tetrameric protein consisting of 4 chains, α₂β₂, with a total molecular weight of 64,450 Daltons. Buried within each chain is the iron containing heme group, which binds one molecule of oxygen per heme.³ Within each hemoglobin there is a central cavity with a high concentration of positive charges called the 2,3-diphosphoglycerate (DPG) site. This site normally regulates hemoglobin’s affinity for oxygen. This has also been identified as excellent site for specific modification of the protein.⁴
Chemical modification of hemoglobin has been of particular interest and is desirable for a number of reasons. The tetrameric form is the fully functional form of hemoglobin but in circulation the protein can dissociates into two nonfunctional αβ-dimers. The dissociated αβ-dimers can quickly be excreted and may even cause kidney damage.\textsuperscript{5,6} For this reason modification of the protein is necessary to keep it functional and in circulation. However an important consideration when modifying hemoglobin is ensuring the behavior of the protein is unchanged. Physical properties, such as a high cooperativity and oxygen binding affinity, need to be retained.

A variety of cross-linking reagents have been developed to modify mammalian hemoglobin. Some are non-specific, while others have specificity with respect to the site of modification on the protein. The most successful of these developed by Kluger and co-workers is the reagent trimesoyl tris(3,5-dibromosalicylate) (TTDS). Consisting of three activated esters, with 3,5-dibromosalicylic acid leaving groups, the reaction of TTDS with deoxyhemoglobin is selective for the amino groups of the Lys 82 residue on each β subunit. This selectivity studied by Klotz and co-workers\textsuperscript{20} using di-asprins found that the Lys 82 is exposed and readily reacts with esters. The distance between the β82Lys→β82Lys is also bridged easily by short chain (~8 Å) modification. This chemical modification also converts hemoglobin from a nucleophilic protein to an electrophilic one, were the remaining unreacted ester group provides a reaction site for further modification and conjugation.\textsuperscript{7,8} However, two side products are also obtained when TTDS is reacted with hemoglobin; a
triply linked hemoglobin β1Val-β82Lys-β82Lys, and a doubly linked β82Lys-β82Lys with the third ester group hydrolyzed.

![Figure 1. Structure of trimesoyl tris (3,5-dibromosalicylate) (TTDS)](image)

When several of the first generation HBOCs, which were cross-linked to stabilize the tetrameric structure, were subject to clinical studies the reported results of these trials found serious side effects in most cases, such as increased blood pressure. It was suggested that the scavenging of nitric oxide, an important signaling compound in blood vessel relaxation\textsuperscript{9,10}, could be responsible for the vasoactivity. It is well known that hemoglobin has a high affinity for nitric oxide, and it was believed that the relatively small protein could easily ‘leak’ through the capillary walls into the interstitial space and scavenge NO. Since much larger Red cells do not encounter this problem, it was then reasonable to expect that increasing the size of the HBOC would be necessary to avoid this problem.
One method in increasing the size of the HBOC is to modify it with derivatives of multiple chains of polyethylene glycol (PEG). This increases the size by engulfing the protein in a bulky sphere of PEG chains. Although this was found to suppress the vasoactivity, the modification also altered the protein’s physical behavior by decreasing the cooperativity and high oxygen affinity, making it unsuitable as an oxygen carrier.\textsuperscript{11} Another approach was achieved by Kluger and co-workers, in linking two hemoglobin together to form bis-tetramer. The most successful of these approaches uses TTDS to first cross-link hemoglobin. The third unreacted ester of TTDS is then modified to include an azide end, which undergoes click chemistry with a di-alkyne ligand, and another cross-linked hemoglobin.\textsuperscript{12} This reaction should produce a solution of hemoglobin bis-tetramers. The yield for the final click reaction is near 100%, but during the initial cross-linking, TTDS is susceptible to hydrolysis of one of its three esters, producing a cross-linked hemoglobin free acid side-product that limits the overall yield.
Scheme 1. Current method used to synthesize hemoglobin bis-tetramers.
1.2 Work Contributing to this thesis

Previous research provides a basis for the design of an improved HBOC compound through the production of hemoglobin bis-tetramers. Key modifications that must be incorporated are a cross-linking of the protein to maintain its tetrameric form, along with an increase in size to limit NO scavenging. All this must also be accomplished without changing the fundamental behavior of the protein and in good yields, so as not to waste any of the valuable starting hemoglobin material.

The objective of this work was to optimize the current synthetic route in the production of hemoglobin bis-tetramers even further. This would be done by focusing on the first step where TTDS cross-links hemoglobin. By substituting the current nucleophilic azide with one that could function in a more acidic environment, the reaction could be carried out at a lower pH, which would limit the amount of hydrolysis occurring, and thus limit the production of the free acid side product. This would increase the overall yield and provide an efficient route to the production of a potential HBOC. This work has therefore led to the study of various hydrazine and hydrazide functional groups and their respective reactions and use as nucleophiles. It has also led to the development of a novel hydrazine compounds that can be used as a new nucleophilic anchors in biological systems, to undergo click chemistry.
CHAPTER II:

Experimental

2.1 Methods and Materials

Commercial reagents and solvents used in these experiments were obtained from Sigma Aldrich and used without further purification. Buffers were made using doubly distilled and deionized water, and the reagents used for preparation of the buffers were of analytical grade or better. The purity of newly synthesized materials was assessed by a combination of NMR spectroscopy, and in some cases, mass spectrometry. Proton and Carbon NMR spectra were obtained at 300 MHz, and 100 MHz, respectively. Solutions of human hemoglobin were obtained from Hemosol Inc.

2.2 Synthesis of benzoic acid hydrazide

**Methyl Benzoate Ester.** H₂SO₂ (5mL) was added drop wise to a stirred suspension of methanol (100ml) and benzoic acid (1.22g, 10mmol) and was left to reflux overnight. The reaction was checked on TLC plate for completion. Once completed roughly 2/3 of the methanol was removed by a rotovapor, and the rest was then neutralized with NaH₂CO₃ (~5g). The organic layer was extracted using DCM. Then it was washed with water and brine, dried using magnesium sulfate and lastly the remainder of methanol was removed by a rotovapor. The residue left behind was dissolved in pure DCM, and passed
through a silica plug to eliminate any starting material left over. Yield was 0.92 g (82%).

\(^1\)H NMR\((\text{CDCl}_3)\) : \(\delta 3.92\ (s, 3\text{H}, \text{CH}_3)\), \(\delta 7.4\ (\text{dd}, 2\text{H}, \text{ArH})\), \(\delta 7.48\ (\text{dt}, 1\text{H}, \text{ArH})\), \(\delta 8.02\ (\text{dd}, 2\text{H}, \text{ArH})\).

**Benzoic Acid Hydrazide.** Methyl benzoate ester (0.92, 7.5mmol) was dissolved in methanol (20mL) and hydrazine hydrate (2mL, 38mmol) was added slowly into the solution while stirring. The solution was heated to a reflux and set to react overnight. Once the reaction was complete the methanol was removed by a rotovapor and the residue was cooled in an ice bath, which caused the product to precipitate out. The product was recrystallized in isopropyl to get pure product. Yield was 0.31g (45%). \(^1\)H NMR\((\text{CDCl}_3)\) : \(\delta 4.55\ (s, 2\text{H}, \text{NH}_2)\), \(\delta 7.52\ (m, 3\text{H}, \text{ArH})\), \(\delta 7.88\ (s, 2\text{H}, \text{ArH})\), \(\delta 9.8\ (s, 1\text{H}, \text{NH})\).

\[\text{Figure 2 : Structure of Benzoic Acid Hydrazide}\]

2.2.1 **Synthesis of Azido benzoic acid hydrazide**

**(p-Bromomethyl) Methyl Benzoate Ester.** \(\text{H}_2\text{SO}_2\) (2mL) was added slowly, over the course of 15min to a stirred suspension of methanol (100ml) and \((p\text{-bromomethyl})\text{benzoic acid (1.0g, 4.7mmol)}\) and was left to reflux overnight. Once the reaction was completed roughly 2/3 of the methanol was removed by a rotovapor. The remaining solution was neutralized
with NaH₂CO₃ (~ 5g). Then the organic layer was extracted using DCM, washed with water and brine, dried using magnesium sulfate, and lastly the remainder of methanol was removed using the rotovapor. The residue left behind was then dissolved in pure DCM, and passed through a silica plug to eliminate any starting material left over. Yield was 0.95 g (90%). ¹H NMR(CDCl₃): δ 3.92 (s, 3H, CH₃), δ 4.48 (s, 2H, CH₂), δ 7.51 (dt, 2H, ArH), δ 8.10 (dd, 2H, ArH).

(p-Bromomethyl) Benzoic Hydrazide. (p-bromomethyl) methyl benzoate ester (0.5g, 2.2mmol) was dissolved in ethanol (20mL), to which hydrazine hydrate (1.5mL, 30mmol) was added drop wise. The solution was left to reflux for a period of 24hrs, after which another equivalent of hydrazine hydrate (1.5mL, 30mmol) was added. This was once again was left to reflux for another 24hrs. Once the reaction was finished, the ethanol was removed by a rotovapor, and ethyl acetate was added instead. Brine was used to remove the aqueous layer, and the remaining organic layer was dried using magnesium sulfate, and the solvent was removed by a rotovapor to get an oily residue. Yield was 0.28 g (61%). ¹H NMR(CDCl₃): δ 4.40 (s, 2H, CH₂), δ 4.52 (s, 2H, NH₂), δ 7.50 (dt, 2H, ArH), δ 8.08 (dd, 2H, ArH), δ 9.77 (s, 2H, NH)

Azido Benzoic Hydrazide. NaN₃ (0.58g, 9mmol) was partially dissolved in DMF (10mL). (p-bromomethyl) benzoic hydrazide (1g, 4.36mmol) was added to that solution and was left to reflux at 100°C for 24hrs. Once the reaction was completed, the aqueous layer was
extracted using water (100mL). The remaining organic layer was washed with diethyl ether, then brine, and then dried with magnesium sulfate. The solution was then placed in a freezer over night, and the product precipitated out. Yield was 0.31g (37%). $^1$H NMR(CDCl$_3$) : δ 4.41 (s, 2H, CH$_2$), δ 4.48 (s, 2H, NH$_2$), δ 7.51 (dt, 2H, ArH), δ 8.1 (dd, 2H, ArH), δ 9.77 (s, 2H, NH). $^{13}$C NMR (CDCl$_3$) δ 52 (s), δ 60.5 (s), δ 126 (s), δ 129.5 (s), δ 130.5 (s), δ 144 (s), δ 172(s).

Figure 3 : Structure of Azido Benzoic Acid Hydrazide

2.3 Synthesis of benzyl hydrazine

**Benzyl Hydrazine.** Hydrazine (5mL, 100mmol) was added to a solution of methanol (15mL). The solution was cooled down to 0°C and benzyl bromide (1.2mL, 10mmol) was slowly added while the solution was stirring. After 3 hours the reaction was complete so the methanol was removed using a rotovapor. The product from the remaining solution was extracted with diethyl ether. The solution was then dried using magnesium sulfate and the remaining ether was removed by a rotovapor. To purify the product the residue was distilled and the fraction boiling at 115°C was collected. Yield was 0.28g (21%). $^1$H NMR(CDCl$_3$) : δ 3.38 (d, 2H, NH$_2$), δ 3.42 (d, H, NH), δ 3.72 (s, 2H, CH$_2$), δ 7.29 (m, 5H, ArH).
2.4 Cross-linking and analysis of hemoglobin

Carbonmonoxyhemoglobin was passed through a Sephadex G-25 gel-filtration column, equilibrated with 0.1M buffer at various pH, in a refrigerator at 4°C. In the lower pH region, from pH 5.5-6.5 MES buffer was used, from pH 6.5-7.5 MOPS was used, and in the higher region pH 7.5-9.0 Tris buffer was used. Once the solution was equilibrated to the new pH it was collected in a round bottom flask and placed into a rotary apparatus under a stream of O₂ for 2 hours, to give oxyhemoglobin. During this period this flask was photo-irradiated using a visible light lamp, and placed in an ice bath. The oxyhemoglobin was then deoxygenated under a stream of N₂ in a water bath at 37°C for 2 hours.

The cross-linking agent TTDS was added directly to the solution of deoxyhemoglobin at the end of the 2 hours with minimum exposure to air. The ratio of TTDS to hemoglobin that was used was always 3:1. It was found that by 30 min, that sufficient cross-linking had taken place to which the nucleophile (either the benzyl hydrazide, or benzyl hydrazine) could be added. The reaction was then left for another 4 -6 hours, with samples being taken every hour, and either stored in the freezer for later analysis, or directly analyzed using the HPLC.
Modified hemoglobin was then analyzed using reverse-phase HPLC. A 330 Å pore size C-4 Vydac column (4.6 x 250 mm) was employed to monitor the modification produced in the reaction of hemoglobin with TTDS. Modified and unmodified globin chains were separated using developers containing 0.1% TFA along with a gradient beginning with 20% and ending at 60% acetonitrile in water. The eluent was monitored at 220nm.\textsuperscript{12,14} To verify globin fragments, retention times were compared to native unmodified hemoglobin runs taken that same day.

2.5 Kinetics

The UV/Vis kinetics were performed using a Cintra 40 UV/Vis spectrometer. Large stock buffer solutions were made up of either 0.1 M MES pH 6.5, or 0.1 Tris pH 9, at 37°C. These were used to create smaller stocks of various concentrations of nucleophile, ranging from 1mM – 15mM. Right before every experiment, fresh solutions of either ester (TTDS, or DBS ester) were also made using the stock buffer solution. As soon as these were made, they were used in the experiment. The concentration of the ester solutions was made to be higher, so when diluted in the nucleophile solution, the final concentration would always be 0.5mM.

Along with the benzyl hydrazine, and benzoic hydrazide, a benzyl amine was used to react with either TTDS, or DBS ester. The appearance of the 3,5 di-bromo salicylic acid leaving group was monitored at λ 312nm. The rate of aminolysis or hydrazinolysis was determined for both esters, where possible. All the kinetics were performed at 37°C using
various concentrations of nucleophile. Because TTDS is a tri-ester, the rate constants were determined for only the first leaving group by using the initial rate method (5% of the total reaction). In the case of TTDS the initial rate method \( k_0 \) was determined by:

\[
    k_0 = \frac{3(\Delta A / \Delta t)}{A_\infty}
\]

where \( k_0 \) is the pseudo first-order rate constant, \( (\Delta A / \Delta t) \) is the initial change in absorbance over time, and \( A_\infty \) is the absorbance at the end of the reaction. The factor of 3 is used only with TTDS to account for the three leaving groups.

The pseudo first-order rate constants, \( k_0 \), was determined by using the slopes of absorbance verse time, and was then plotted verse the total nucleophile concentration to determine the second-order rate constant for aminolysis, or hydrazinolysis, \( k_N \). The slopes were also corrected for the amount of nucleophile in basic form (applied only for benzylamine), and each \( k_0 \) value used was the average of three runs. The pseudo first-order rate constant for hydrolysis \( k_{\text{hydr}} \) was determined from the \( y \)-intercept of the slope of \( k_0 \) vs total nucleophile concentration. The kinetics procedure was carried out and followed as described in De Stefano, V.\textsuperscript{15}
2.5.1 Synthesis of DBS Ester

Di-bromo-salicylic benzyl ester was chosen because TTDS has three leaving groups, which react at different rates, giving complexity to the reactions. DBS has only a single leaving group, which simplifies the reactions, and is much slower to hydrolyze. The synthesis was a combination of procedures from Klotz,\(^\text{16}\) and Kluger\(^\text{7}\).

**T-butyl-3,5-dibromosalicylate benzyl ester.** Starting with the carboxy protected t-butyl-3,5-dibromosalicyla (1.42g, 3.8mmol) in anhydrous THF (45mL), potassium tert-butoxide (0.45g, 4mmol) was added to the solution. The mixture was stirred at room temperature for 15min and then a solution of benzyl chloride (0.5g, 4mmol) and THF (10mL) was added dropwise over 15 min. The reaction was left overnight at room temperature. Ether (70mL) was then added to the mixture and the solution was washed with water, then dried using magnesium sulfate, and solvents were removed by a rotovapor. Yield was 1.2g, 86%.

\(^1\)H NMR(CDCl\(_3\)) : \(\delta 1.36\) (s, 9H, (CH\(_3\))\(_3\)), \(\delta 3.41\) (s, 1H, CH), \(\delta 3.96\) (s, 1H, CH), \(\delta 7.22\) (m, 5H, ArH).

**3,5-dibromosalicylic benzyl ester.** t-butyl-3,5-dibromosalicylate benzyl ester (1.2g, 3.2mmol) was dissolved in anhydrous TFA (25mL) and kept at room temperature for 2 hours. Diethyl ether (25mL) was initially added to induce crystallization, followed by a 50:50
mixture of ether and hexane (15mL), then left in a freezer overnight. Product was collected by filtration. Yield was 0.8g, 80%. $^1$H NMR(CDCl$_3$) : δ 3.41 (s, 1H, CH), δ 3.96 (s, 1H, CH), δ 4.46 (s, 1H, OH), δ 7.22 (m, 5H, ArH). $^{13}$C NMR (CDCl$_3$) : δ 121 (s), δ 121.9 (s), δ 128.4 (s), δ 132.8 (s), δ 133.2 (s), δ 136.1 (s), 137.2 (s), 139.6 (s), 140.8 (s), 148.6 (s), 163.3 (s), 164.6 (s).

Figure 5. Structure of 3,5-dibromosalicylic benzyl (DBS) ester
CHAPTER III:

Results

3.1 Modification of Hemoglobin with benzoic acid hydrazide

Scheme 2. Reaction of cross-linked hemoglobin with benzoic acid hydrazide. This can produce not only the desired product, but also the free acid by-product upon hydrolysis.

Once benzoic acid hydrazide was successfully prepared it was used to substitute the current nucleophile, a benzyl amine, in the hemoglobin bis-tetramer reaction. This allowed the reaction to be carried out in less basic medium, ranging from pH 8 - 6.5. By lowering the concentration of \([OH^-]\), base-catalyzed hydrolysis would also be limited. In this reaction TTDS was first reacted with hemoglobin for 2 hours before benzoic acid was introduced. In
the HPLC analysis the hydrolyzed peak is normalized and the area of both peaks represents all the products formed.

As the product peak increases, the percentage of the total area that the hydrolyzed peak represents is decreased. The product peak is increased as the reaction is carried out in progressively lower pH, yielding less hydrolyzed free acid product.

![HPLC Analysis](image)

<table>
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<th>pH Level</th>
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<th>7.5</th>
<th>7.0</th>
<th>6.5</th>
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<tr>
<td>Percent Hydrolyzed</td>
<td>84 %</td>
<td>75 %</td>
<td>57 %</td>
<td>51 %</td>
</tr>
</tbody>
</table>

Figure 6. Reverse phase HPLC of reaction of cross-linked hemoglobin with benzoic acid hydrazide at various levels of acidity. Table includes the calculated area of product that is hydrolyzed, as a percentage of all products formed. The increase in product peak indicates a larger percentage of the reaction is the desired product.
Further attempts at lowering the pH below 6.5, to decrease hydrolysis, did not give successful cross-linking between TTDS and hemoglobin. When TTDS reacts with hemoglobin it cross-links the two β subunits together, this shows up on the HPLC readout as an absence of the single β subunit peak. When the reaction between TTDS and hemoglobin was run at pH levels below 6.5, the β-peak is found, indicating that the initial modification of hemoglobin by TTDS is not taking place. This is shown in Figure 7, as unmodified native hemoglobin is compared to a reaction of TTDS with hemoglobin at pH 6.0. The overlap of the two readouts indicates that there is no modification occurring.

Figure 7. Comparison of reverse phase HPLC of native hemoglobin, to a reaction with hemoglobin and TTDS at pH 6
In order to further study the effect of the pH of the solution and the cross-linking reactions, a series of reactions were performed with just hemoglobin and TTDS. As illustrated in Figure 7, the ratio of the β and α peak is about equal in unmodified native hemoglobin but as modification takes place, the β peak starts to diminish. Figure 8 illustrates that below pH 7, the TTDS cross coupling is affected. At pH 6 there is essentially no reaction between the protein and TTDS.

![Scheme 3. Reaction of hemoglobin with TTDS at various pH.](image)

Figure 8. Reaction of TTDS and hemoglobin at various pH. The Diminishing β-peak indicates that cross-linking of hemoglobin is taking place.
Observing that the cross linking of hemoglobin was hindered at low pH, adding benzoic acid hydrazide to react with the modified protein was limited to pH 6.5. Further optimizing the conditions to those described in the experimental section, both TTDS and the benzoic hydrazide were reacted in ‘one pot,’ to give an optimum yield with 23% hydrolysis by-product, as seen below.

Scheme 4: One Pot Reaction of Hemoglobin with TTDS and Benzoic Acid Hydrazide

Figure 9. Reverse phase HPLC analysis of the final product of the reaction between cross-linked hemoglobin and benzoic acid hydrazide at 4 Hours. The amount of hydrolyzed by-product is 23%.
3.2 Modification of Hemoglobin with benzyl hydrazine

The use of benzyl hydrazine was also studied as an alternative nucleophile to the benzyl amine. The reactions conditions to optimize the yield of the product and minimize hydrolysis were carried out in the same fashion as the benzoic hydrazide. The most successful results demonstrated that hydrolysis could be further hindered, with the hydrolysis by-product occurring at 20%.

Scheme 5. One Pot Reaction of Hemoglobin with TTDS and Benzyl Hydrazine.

Figure 10. Reverse phase HPLC analysis of the final product of the reaction between cross-linked hemoglobin and benzyl hydrazine at 4 Hours. The amount of hydrolyzed by-product is 20%.
3.3 Rates of Aminolysis and Hydrolysis

The rates of hydrazinolysis and hydrolysis for benzoic hydrazide, and benzyl hydrazine with TTDS and DBS ester were studied and compared to that of benzyl amine. The alternative single di-bromo-salicylic benzyl (DBS) ester was chosen because TTDS has three leaving groups, which react at different rates, giving complexity to the reactions. The ester with a single leaving group simplifies the reaction, it also hydrolyzes at a much slower rate. The accumulation of the di-bromo-salicylic leaving group in both esters could easily be monitored at 312nm, as seen below.

Figure 11. Two hour time overlay of the UV spectra of 0.5mM TTDS with 5mM Amine. The absorbance increase at 312nm indicates the accumulation of the leaving group 3,5-di-bromo salicylic acid due to aminolysis, as well as to a lesser extent its hydrolysis, of TTDS.
Figures 12 and 13 show the $K_0$ and the $K_N$ of the reaction of benzyl amine with TTDS. This data was used to compare the nucleophilicity of the new hydrazine, and hydrazide compounds with that of the amine.

![Graph showing the initial rate reaction of benzyl amine with TTDS and various concentrations.](image)

Figure 12: The initial rate reaction of benzyl amine with TTDS and various concentrations. The reaction was followed at 312nm, in pH 9, at 37°C.
Figure 13. Plot of benzylamine concentration vs pseudo first-order rate constant $k_0$ with TTDS. The slope is the second order rate constant for aminolysis ($k_N$), and the y-intercept is the pseudo first order rate constant for hydrolysis ($k_{hyd}$). The plot was corrected for the amount of benzylamine in basic form, and the reaction was carried out in pH 9, at 37ºC.

The next two figures show the $K_N$ for benzoic hydrazide, and benzyl hydrazine. These were performed under the same conditions as the benzyl amine. The rate constants are all summarized in table 1.
Figure 14. Plot of benzoic acid hydrazide concentration vs pseudo first-order rate constant $k_0$ with TTDS. The reaction was carried out in pH 9, at 37°C.
The rate of hydrazinolysis of benzoic acid hydrazide with TTDS was very slow, almost as slow as the competing hydrolysis reaction. Therefore the reaction was re-done at lower pH, were the competing hydrolysis reaction could be quenched, to give a more accurate rate constant for hydrazinolysis. The result was very similar, and had a much smaller error associated with it, as summarized in table 1.
Figure 16. Plot of benzoic acid hydrazide concentration vs pseudo first-order rate constant $k_0$ with TTDS. The reaction was carried out in pH 6, at 37ºC.
Table 1. The reaction rates of aminolysis/hydrazinolysis for benzyl amine, benzoic hydrazide, and benzyl hydrazine with TTDS, or DBS ester. The corresponding rate for hydrolysis is also given. All Experiments were carried out in pH 9, at 37°C, except for the benzoic acid hydrazide redo, which was done at pH 6, for a more accurate reading.

<table>
<thead>
<tr>
<th></th>
<th>TTDS</th>
<th>DSB ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aminolysis, $k_N$ (M$^{-1}$s$^{-1}$)</td>
<td>Hydrolysis, $k_{hyd}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>3.2 ± 0.2</td>
<td>2.8x10$^3$ ± 3x10$^4$</td>
</tr>
<tr>
<td>Benzoic Acid Hydrazide</td>
<td>5.1x10$^3$ ± 8x10$^4$</td>
<td>2.8x10$^3$ ± 5x10$^4$</td>
</tr>
<tr>
<td>Benzyl Hydrazine</td>
<td>7.3x10$^2$ ± 2x10$^3$</td>
<td>2.8x10$^3$ ± 3x10$^4$</td>
</tr>
<tr>
<td>Benzoic Acid Hydrazide (pH 6)</td>
<td>4.1x10$^3$ ± 3x10$^4$</td>
<td>2x10$^5$ ± 2x10$^6$</td>
</tr>
</tbody>
</table>
Our strategy in converting hemoglobin into a potential therapeutic agent requires the synthesis of hemoglobin bis-tetramers in high yield. The current methods suffer from the formation of a free acid by-product, which forms upon hydrolysis of the third activated ester of TTDS when cross-linked to hemoglobin. The by-product forms at about 25%, and renders the modified hemoglobin unreactive to further modification which is necessary in forming the bis-tetramer.\textsuperscript{12}

One approach in limiting hydrolysis and the formation of the by-product is to perform the reaction at lower pH. This would decrease the concentration of \([OH^-]\) and hinder the base-catalyzed hydrolysis that is prevalent in creating the free acid by-product.

![Scheme 6: OH\textsuperscript{-} catalyzed hydrolysis of cross-linked hemoglobin.](image)

**Scheme 6:** OH\textsuperscript{-} catalyzed hydrolysis of cross-linked hemoglobin.
Currently the nucleophile used to introduce the azide linker necessary for the click reaction in joining the two hemoglobin molecules together is a benzyl amine. This functional group has a pKa of 9.34\textsuperscript{18} and is protonated in acidic solution. This work has therefore probed the use of alternative functional groups that are less basic but are still good nucleophiles due to the α-effect from the neighboring atoms lone pair electrons.

Using the benzyl amine, and performing the reaction at lower pH, the yield decreases, as more free acid by-product forms.\textsuperscript{12} The opposite trend was observed using the benzoic acid hydrazide in Figure 6. Here we see that hydrolysis, and the formation of the by-product is decreased as the pH is lowered. This clearly indicates that the [OH\textsuperscript{-}] is mainly responsible for the hydrolysis, and as long as the nucleophile used is not protonated, the reaction yield should increase.

However a limit on far the pH can be lowered has also been found, as seen with Figure 7 and 8. These diagrams show that below a pH of 7, the cross-linking reaction between hemoglobin and TTDS begins to be affected, and by pH 6 very little cross-linking is taking place. This is due to the fact that TTDS reacts with the two β82Lys groups on each β-subunit. Although this amino acid is found in a highly cationic cavity, the amine still is protonated and exhibits the same problem we see with our benzyl amine, it loses its nucleophilicity and is not able to react with TTDS to become cross-linked.\textsuperscript{4}
Scheme 7: Protonated β82 Lys groups on hemoglobin.

It is therefore determined that the optimum pH of the reaction be 6.5, where the trade-off between TTDS reactivity, is balanced well with the base-catalyzed hydrolysis. Another approach explored was changing the solution pH, and allowing the TTDS to react at pH 8, then introducing the benzoic hydrazide at pH 6. This did not yield better results because the TTDS can be considered to be the ‘slow’ step in the reaction. TTDS is not very soluble in aqueous solution and only once it reacts with hemoglobin does it become soluble and quickly reacts with the benzoic hydrazide. By switching the solution, you either ended up with unmodified hemoglobin, or up to 50% hydrolyzed by-product.

The overall improvement using the benzoic acid hydrazide was minimal. The amount of hydrolyzed by-product was lowered from around 25% to 23%. This demonstrates that although the benzoic hydrazide can undergo the reaction at much lower pH, it isn’t as reactive as the benzyl amine. This is to be expected as the benzoic hydrazide is much less basic, which also means it is much less nucleophilic. The α-affect, which can increase the nucleophilicity of a compound, is diminished by the neighboring carboxyl group. Jencks found that the reactivity of nucleophilic reagent such as hydrazide groups, can be up to $10^3$ times less reactive then simple amine’s.
With this in mind, the benzyl hydrazine was employed. Like the amine, it did not feature any delocalizing carboxyl group, and with a basicity right between that of the benzyl amine, and benzoic hydrazide, it should be more reactive and remain reactive in the lower pH region. The improvements were once again marginal. The production of the hydrolyzed by-product was limited to 20%, as seen in Figure 10.

The kinetics study was performed to evaluate the exact reactivity of the two compounds, and compare them to that off the benzyl amine used. This would give a more accurate picture of how reactive each compound is. The reaction rates were determined for all three compounds as they reacted with either TTDS or DBS ester, along with the hydrolysis rate of each ester. From the table 1 we notice that the aminolysis rate for benzyl amine is on the order of 600 – 700 times faster than the rate for hydrazinolysis by the benzoic acid hydrazine. This demonstrates that the benzyl amine is in fact a much better nucleophile when unprotonated and better able to react with a given ester. The benzyl hydrazine is also less reactive than the benzyl amine, by about a factor of 20 – 40 times less.

Figure 17. Structure of benzoic hydrazide, benyl hydrazine, and benzyl amine, with their corresponding basicity.
effective. This is a significant improvement over the benzoic hydrazine. This also
demonstrates that for any reaction where the benzylamine is to be replaced, the
concentration of benzyl hydrazine that is needed to perform the same reaction, is going to
be much less than the amount of benzoic hydrazide needed.
CHAPTER V:

Conclusion

Decreasing the pH of the reaction is critical in limiting hydrolysis of TTDS and increasing the overall yield of hemoglobin bis-tetramers. Amine functional groups, although good nucleophiles, are protonated in acidic solutions and limit the choice of reaction conditions. Alternative functional groups, such as hydrazine, are less basic, but still act as good nucleophiles in reaction with TTDS. The benzyl hydrazine in particular can undergo the required reaction with TTDS modified hemoglobin at lower pH, which increases product yield.
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