REACTION OF DIMETHYL TRISULFIDE WITH HEMOGLOBIN

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ABSTRACT

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When samples of blood were spiked with the novel cyanide antidote dimethyl trisulfide (DMTS), the color of the blood was observed to darken. Additionally, recoveries of DMTS from spiked blood were low, and the loss of DMTS was more pronounced in 5-day old blood samples than in equivalently spiked 4-month old blood samples. It was hypothesized that DMTS oxidizes hemoglobin (Hb) in the red blood cells to yield methemoglobin (metHb), methanethiol (MeSH), methyl hydrogen disulfide (MeSSH), and hydrogen sulfide (H_2S). The aim of this research was to determine the reactants, products, and chemical kinetics of the reactions of DMTS with Hb isolated from red blood cells, and with Hb in blood. The changes induced in the Hb absorption spectrum by the addition of DMTS were found to closely match those induced by the known metHb former sodium nitrite. These spectral shifts indicating the conversion of Hb to metHb were observed systematically when DMTS was added to red blood cell or Hb solutions. The formation of metHb was monitored as a function of time following the addition of a known amount of DMTS. The rate of the reaction of DMTS with Hb increased in the presence of the reducing agent dithionite (DT). H_2S, MeSH, and MeSSH were expected as reaction products, but were not directly observed in headspace samples. 2,4-dithiapentane had not been predicted as a reaction product, but was observed in the headspace above reaction mixtures of DMTS and Hb. The 2,4-dithiapentane is hypothesized to be a reaction product of MeSH with formaldehyde-based polymers in the vial cap.

KEY WORDS: Dimethyl trisulfide, Hemoglobin, Methemoglobin, Dithionite, Blood, Headspace sampling, Kinetics.

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CHAPTER I

Introduction

Cyanide intoxication and antidotes

Cyanide (CN) is produced by various organisms and plants. It has been used as a suicidal, homicidal, chemical warfare, and genocidal agent.[1] CN inhibits the activity of the enzyme cytochrome c oxidase, which is the terminal oxidase of the mitochondrial respiratory chain. CN therefore impairs the cell's oxygen utilization and ATP production.[2] The CN intoxication is initiated when CN penetrates into a protein crevice of the cytochrome c oxidase, where it temporarily bonds before binding the heme iron.[1, 3] Most mammals are able to endogenously detoxify small amounts of CN. The major route of endogenous CN detoxification involves converting CN to the less toxic thiocyanate, with the help of sulfurtransferases, such as rhodanese and mercaptopyruvate sulfurtransferase.[4] The basic reaction with rhodanese involves a transfer of the sulfur from thiosulfate (S donor) to the enzyme, forming a persulfide intermediate, which is then transferred irreversibly to CN to yield thiocyanate.[4] However, the penetration of sodium thiosulfate into the mitochondria, where the rhodanese localized, is limited.[1] The sulfane sulfur source (such as thiosulfate) generally has protonated conjugate acid, and deprotonated conjugate base forms that are in equilibrium. The conjugate bases (ionized sulfanes) react with CN to form SCN^-.[1] Nitrites and cobalt compounds have also been studied and used for CN detoxification.[1] Nitrite generates metHb, which binds CN with high affinity to form cyanomethemoglobin.[1] The addition of sodium pyruvate to the nitrite-thiosulfate antidotal combination enhances the protective effect against CN.[1] Sodium pyruvate detoxifies CN by reacting with it to form cyanohydrins.[1, 5] Cobalt compounds successfully compete with

cytochrome c oxidase for CN through the formation of stable cobalt CN complexes.[6] The standard CN antidotes used in the United States currently are hydroxocobalamin (Cyanokit®),[7] and the combination therapy of sodium thiosulfate and sodium nitrite (Nithiodote®).[8]

Dimethyl trisulfide

Dimethyl trisulfide (DMTS) was found to be more efficacious as an antidote against CN poisoning than the Cyanokit® and the Nithiodote® therapies.[9] DMTS contains three consecutive divalent (sulfenyl) sulfur atoms, and is found naturally in plants such as garlic, cabbage, and broccoli. Like other sulfanes (RS_nR), DMTS is expected to undergo disproportionation reactions (below).[10, 11]

$$2RS_nR \rightarrow RS_{n+1}R + RS_{n-1}R$$

The disproportionation of DMTS to disulfane and tetrasulfane is accelerated at elevated temperatures.[12] At room temperature, small amounts of di- and tetrasulfanes are formed via this reaction (Figure 1).

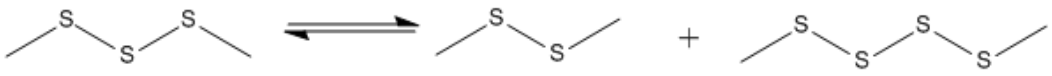


Figure 1. DMTS disproportionation.

Hemoglobin

Hemoglobin (Hb) is the iron-containing protein in the red blood cells of all vertebrates that delivers oxygen to tissues in the body. Mammalian Hb is an $\alpha_2\beta_2$ tetramer, where α and β refer to subunits with different amino acid sequences.[13] Each subunit is comprised of a heme which is a porphyrin ring containing four pyrrole groups linked by

methene bridges. The heme is wrapped in a hydrophobic pocket between the E and F helices.[13] The iron (II) is coordinated by the four N atoms from the porphyrin and one N atom from a Histidine (F8) side chain.[13]

In blood, the iron (II) ion in Hb reversibly binds oxygen by a coordinate covalent bond to form oxyhemoglobin (HbO_2), which has a brilliant red color.[14] The heme bound O_2 is stabilized by hydrogen bonds to the E7 Histidine. There are two hydrophobic side chains on the O_2-binding side of the heme, Valine E11 and Phenylalanine CD1, helping hold the heme in place. These side chains swing open as the protein breathes, allowing O_2 in and out. Deoxyhemoglobin (deoxyHb) is the Hb in which no ligand is bound, and it has a different color and absorption spectrum than HbO_2.[13] Vertebrate Hb is in equilibrium between the deoxy and the oxy structures.[15] CO, NO and H_2S all bond to the heme iron with greater affinity than O_2.[13]

Hb oxidation

In Hb, the globin inhibits the oxidation of Fe(II) to Fe(III). When this oxidation occurs, the resulting Fe(III) hemoglobin is called methemoglobin (metHb). MetHb has a dark brown color, and does not bind O_2 effectively. MetHb is reduced back to deoxyHb naturally in the presence of endogenous metHb reductase *in vivo*.[16]

It is well known that Hb is easily oxidized by various oxidants when it is found outside the protective environment of the red blood cell.[17, 18] The most common oxidant is O_2. When O_2 binds to the heme iron, the structure of the oxygenated state is $Fe^{3+}O_2^-$. Normally, when the oxygen leaves, it returns the extra electron to the iron. However, occasionally this does not occur, and the $Fe^{3+}O_2^-$ dissociates to yield metHb and the superoxide anion O_2^-.[19] This process is known as the autoxidation of Hb.

Nitrite is known to oxidize HbO_2 to metHb. The reaction of nitrite with HbO_2 is described below:[20]

$$4HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4\text{metHb} + 4NO_3^- + O_2 + 2H_2O$$

Sulfhemoglobin (SHb) can be generated[21-23] by the reaction of sulfides, such as H_2S, with Hb. Research shows that H_2S might be released continuously and slowly in the presence of dithionite (DT) from serum and tissues.[23] Reducing agents such as DT catalyze the formation of SHb in the presence of HbO_2 and trace amounts of H_2S.[23, 24] The sulfide does not bind to the O_2 binding site of the heme, but rather to the porphyrin ring that surrounds this site. SHb can undergo one electron transfer to form sulfmethemoglobin (SmHb). The SmHb reverses back to SHb in the presence of a suitable reducing agent, such as DT, or reductase in blood cells.[21, 25] The formation of SHb decreases with increasing pH.[25]

According to Marcus theory, Hb can be oxidized to metHb via either an outer or an inner sphere process.[40,42] In the outer sphere process, the oxidant and heme iron (II) retain full coordination shells, and the electron jumps from electron donor to electron accepter without a large change in structure. In the inner sphere process, the oxidant binds to the sixth coordination position of the Hb.[40,41]

Hb absorption spectra

Absorption spectroscopy can be employed for rapid and reliable quantification of Hb.

Generally, the absorption bands (Q bands) that are responsible for the red to purple color of the porphyrin, are present in the visible region between 500-700 nm.[26] The Q band

absorbances are centered near: 540 nm, and 576 nm in HbO_2; 554 nm in deoxyHb; 500 and 630 nm in metHb; 619-623 nm in SHb. In SmHb, the 620 nm peak decreases, and a smaller peak (1/10 or 1/20 of the 620 nm peak) appears at 717 nm.[21, 27] A very sharp intense Soret band in porphyrin, which is sensitive to substituent groups, appears around 400 nm in the near UV region.[26] In porphyrin compounds, the Soret band arises primarily due to an electron dipole moment that allows π-π^* transitions.[28] Experimentally, the Soret peak is at 413-414 nm in HbO_2, 430 nm in deoxyHb and 405 nm in metHb.[27] The Soret peak for SHb is red shifted relative to HbO_2.[22]

The molar absorptivity of the Hb Soret band is about 10-fold greater than the Q band molar absorptivities, so the spectrophotometric quantitation in the Soret region is more sensitive. The Soret absorbance has a second advantage in that it is seen in the spectra of all Hb species.[29]

Research goal and overview of experiments

The primary aim of this research was to develop a deeper understanding of the initial reactions of DMTS with Hb. Specifically, the experimental goal was to determine the reactants, products, and chemical kinetics of the *in vitro* reactions of DMTS with Hb isolated from blood, and with Hb in red blood cells. The investigations were carried out using standard methods, including: gas chromatography mass spectrometry (GC-MS) with headspace (HS) sampling, UV-vis absorption spectroscopy, and high performance liquid chromatography (HPLC).

This research was initiated by experiments to validate a GC-MS method for determining DMTS from blood. As part of this study, the effect of blood storage age on DMTS recoveries was investigated. The DMTS recoveries from the HS above aliquots of

5-day and 4-month old sheep blood that had been identically spiked with DMTS were found to be significantly different. In the same experiment, it was noted that the addition of DMTS to blood caused the blood to darken (Figure 2). Because the oxidation of Hb to metHb is a well-known cause of darkening in blood, it was hypothesized that the low percent recovery of DMTS from blood might be due to the oxidation of Hb to metHb by DMTS.

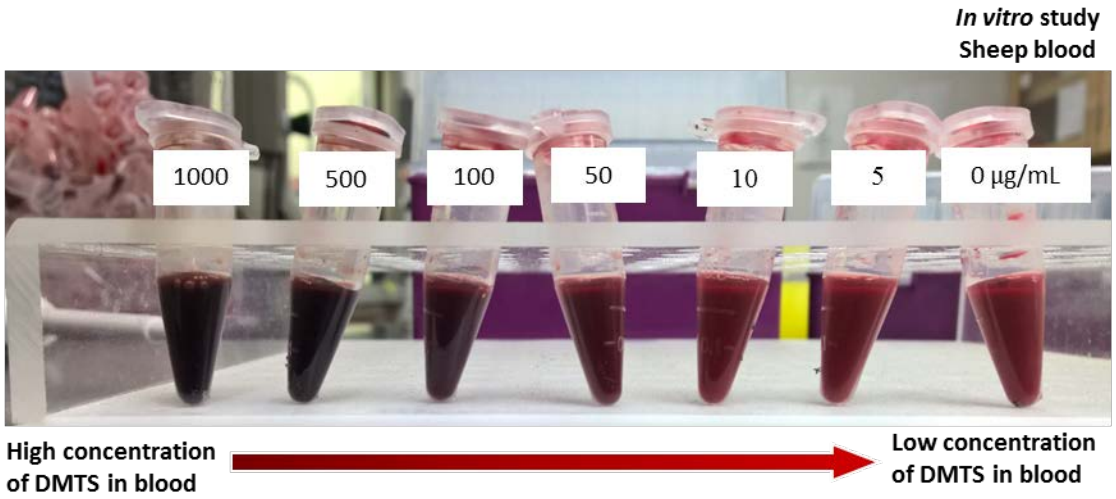


Figure 2. DMTS concentration effects on blood color. Picture was taken by Lorand Kiss.

The hypothesized reactions between DMTS and Hb are illustrated below.

$$CH_3SSSCH_3 + 2Fe^{2+}(HbO_2) + 2H^+ \rightarrow 2Fe^{3+}(metHb) + CH_3SSH + CH_3SH$$

or

$$CH_3SSSCH_3 + 4Fe^{2+}(HbO_2) + 4H^+ \rightarrow 4Fe^{3+}(metHb) + 2CH_3SH + H_2S$$

To test this hypothesis, experiments were set up to: (1) compare Hb spectral changes upon addition of DMTS to those obtained upon the addition of $NaNO_2$, which is a known metHb former; (2) follow the kinetics of the reaction between DMTS and Hb in

blood and in phosphate buffered saline (PBS) solutions using UV-vis absorption spectroscopy, and (3) characterize the components gathered from the head space above DMTS/blood reaction mixture phosphate buffered saline (PBS) solutions by GC-MS.

Hb is commonly sold as a mixture of metHb and Hb, because metHb is more stable and often forms spontaneously upon storage. In 1967 Dixon and McIntosh[30] developed a method for reducing metHb to form HbO_2. In this method, a band of the smaller DT molecules is first run onto a desalting column, where they are trapped in the pores of the gel beads. After a DT band has been established, a metHb sample is eluted through the column. As is standard in gel filtration, the larger metHb traverses the column more quickly than the smaller DT molecules.[31, 32]

In this approach for reducing metHb back to Hb, a small amount of the reducing agent DT can leak through the column, to contaminate the reduced Hb fractions. Dialysis and centrifugal ultrafiltration[33] are two methods that can be used to lower the concentration of DT in the reduced Hb fractions. The primary basis for separation in both approaches is a porous filter that retains Hb while passing DT.

The success of the DT removal can be tested by Ellman's reagent (DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid)). The disulfide bond in $DTNB^{2-}$ (at pH 7.4) is cleaved by thiols (RSH) and one of the products, 2-nitro-5-thiobenzoic acid dianion (TNB^{2-}) (Figure 3), gives an intense yellow color and the absorption spectrum shows the characteristic TNB^{2-} peak centered at 409 nm in dilute salt solution (0.1 M NaOH or 0.1 M phosphate buffer with 1 mM EDTA, pH 7.27).[34, 35]

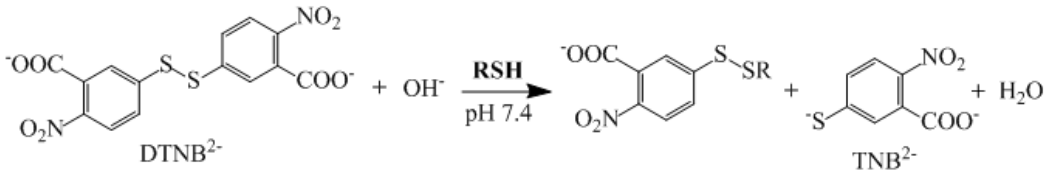


Figure 3. Reaction of $DTNB^{2-}$ with a R-SH at pH 7.4.

CHAPTER II

Materials and Methods

The chemicals used in these experiments were: dimethyl trisulfide (DMTS) (≥ 98%, Sigma Aldrich, LOT: MKBL7080V, MKBW6624V), ethanol, polysorbate 80 (PS80, Alfa Aesar, LOT: L13315), sodium chloride (NaCl), sodium nitrite ($NaNO_2$) (≥98.4%, Fisher Scientific, LOT: S347-500), dibutyl disulfide (DBDS) (97%, Aldrich, LOT: 07423HIV), deionized water (DI water) (type I), sodium hydrosulfite (85+%, Alfa Aesar, LOT: W30B038), 5,5´-dithiobis(2-nitrobenzoic acid) (98%, Alfa Aesar, LOT: 10170346), methanethiol sodium salt (NaMeS), heparin sodium salt (Sigma Aldrich, H3393-250KU), bovine hemoglobin (Hb, Abnova, LOT: 33N14542).

Blood samples included: rat blood drawn from male rats, (315-320 g, Charles River Breeding Laboratories), rabbit blood (COB 308T), sheep blood (Carolina biological supply company).

These experiments made extensive use of sample preparation equipment, gas chromatography-mass spectrometry (GC-MS), and UV-vis spectrophotometry.

Sampling and sample preparation equipment was purchased from VWR: hotplate/stirrer, fixed speed mini vortexer, Ultrasonic cleaner (VWR, Model 97043-992), crimp seal HS sampling vials (2, 5, 10 mL, 223687) with rubber septum and crimp caps. The pH meter (Orion Star A111, Item ID: 663001C920282) was purchased from Thermo Fisher.

GC-MS related equipment was purchased from Agilent: 7890A gas chromatograph, 5975C quadrupole mass spectrometer, DB-5MS, 30m*0.25mm, 0.1 µm column. Carrier gas is helium (ultra high purity). Liner: ultra inert, splitless, straight, 2 mm id, and splitless,

straight, 1.5 mm. Headspace (HS) sampling was carried out using solid phase microextraction (SPME) fibers coated respectively with PDMS, 100 µm and CAR/PDMS stableflex, 85µm; 2 mL vials with screwed caps and PTFE/RS septa (Part No. 5182-0714, 5182-0717).

Microsep™ Advance Centrifugal Devices (10K MWCO, Pall Corporation) were used for filtering DT by centrifuge and PD 10 columns (Sephadex™ G-25M, GE Healthcare) were used for reducing metHb.

A Jasco V-550 UV-vis Spectrophotometer, and an Ocean Optics UV-vis spectrometer were used in these experiments. The Ocean Optics UV-vis spectrometer consisted of UV-vis lamp DH 2000 BAL, spectrometer HR 2000+, optical fiber QP450-1-XSR OOS-001463-04, 1 cm cuvette holder, and quartz and glass cuvettes with 1 cm optical pathlengths.

A. Interactions of DMTS with blood

A1 Effect of blood storage on DMTS recoveries

Rat blood that had been stored for 5-day and 4-month periods, was spiked with DMTS. After allowing the DMTS to interact with each blood sample for 5 min, the spiked blood was diluted again with salt solution and internal standard solution to a final DMTS concentration of 59.5 µM (7.5 µg/mL). The final sample was incubated for 5 min, and SPME samples were gathered using a 10-min sampling period from the HS in each reaction vial. Gas chromatograms and mass spectra were collected from these DMTS-spiked blood samples. Each experiment was done once.

Instrumental Settings. The column temperature was initially held at 40 °C for 1 min, then ramped at 60 °C /min to 280 °C for 4 min. The run time was 8 min. The inlet

mode was initially splitless for 1 min; the pressure was 7.02 psi; the total flow was 19 mL/min; the septum purge flow was 3 mL/min; and the purge flow to split vent was 15 mL/min at 1 min. The mass spectrometer acquisition mode was selected ion monitoring (SIM). The selected ions had m/z ratios of 126.00, 126.20, 126.30, 178.10, 178.30, and 178.40. The dwell time (the time the detector spends collecting ions of one mass to charge ratio) was 10 ms for all ions.

Solutions Preparation. A 20% (w/w) aqueous NaCl solution was prepared by dissolving 5 g NaCl in 20 mL water. A 15% PS80 solution (15% (w/w) aqueous Polysorbate 80 solution) was prepared by mixing 15 g PS80 with 85 g DI water, hand-vortexing for 5 min, magnetically stirring until the solution was transparent (~30 min), and hand-shaking for 1 min. The solution was stored in the refrigerator overnight. The 15% PS80 solution was made in the same way for all experiments.

A 0.1 mg/mL DBDS solution was prepared by adding 50 µL of 10 mg/mL DBDS stock solution to 4950 µL of 15% PS80 solution in a 5 mL glass vial, and vortexing. A 1.0 mg/mL DMTS solution was made by adding 200 µL 10 mg/mL DMTS solution to 1800 µL 15% PS80 solution and vortexing. Then 500 µL of 1.00 mg/mL DMTS was mixed with 500 µL 15% PS80 solution, and vortexed to prepare a 0.50 mg/mL DMTS solution.

Doping of old blood with DMTS. Heparin is an anticoagulant that prevents the formation of clots, and the growth of existing clots, within the blood. One microliter of 50 mg/mL heparin stock solution was mixed with 180 µL of 4-month old refrigerated sheep blood, vortexed, spiked with 20 µL of 0.5 mg/mL DMTS solution, crimp sealed, vortexed for 0.5 min, and then incubated at room temperature for 5 min. A 75 µL aliquot of this DMTS spiked blood was mixed with 400 µL NaCl solution (20%, (w/w)) and 25 µL of 0.1

mg/mL DBDS (internal standard) solution in a 2 mL GC vial. The vial was crimp sealed, vortexed, incubated for 5 min at room temperature, and headspace sampled by a SPME fiber (Figure 4). The SPME sample was analyzed by GC-MS as described previously.

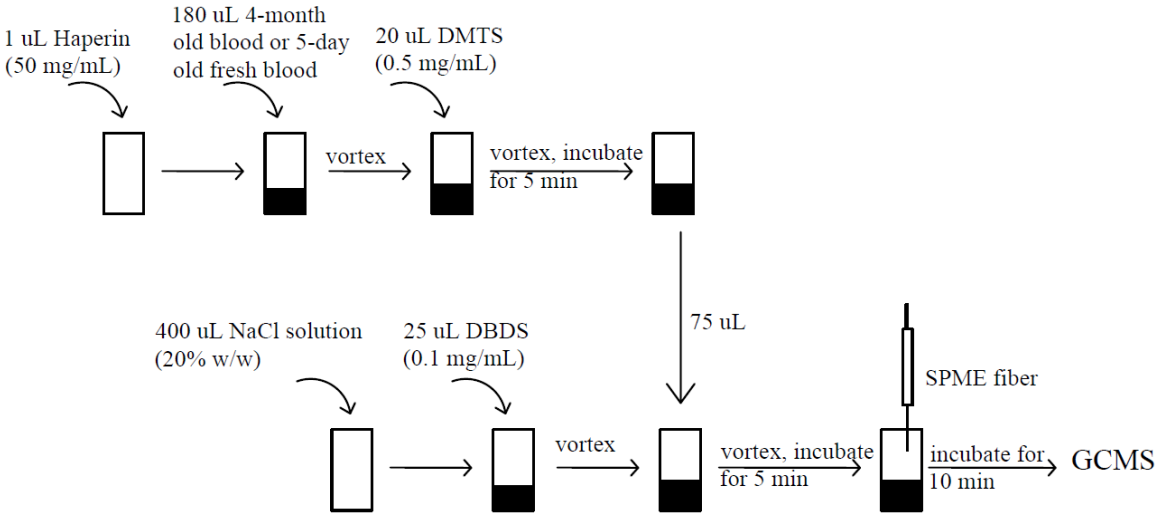


Figure 4. Preparation of DMTS-spiked blood (4-month and 5-day old) samples.

Doping of fresh sheep blood with DMTS. Fifteen microliters of heparin stock solution were transferred into 3 mL of 5-day old sheep blood, which was then vortexed. Twenty microliters of 0.5 mg/mL DMTS solution was then spiked into 180 µL of heparin treated blood and vortexed. The remaining sample preparation and analysis steps were the same as those described for the old blood sample (Figure 4).

A2 Comparing the effects of $NaNO_2$ and DMTS on blood spectra

The goal of this experiment was to compare the changes induced in the Hb absorption bands by DMTS and $NaNO_2$. UV-vis absorption spectra of untreated, $NaNO_2$-treated, and DMTS treated samples of diluted (1/1000) rat blood samples were collected using a Jasco UV-vis spectrometer. In each diluted sample the concentration of Hb was estimated to be 2.33 µM. The concentration of $NaNO_2$ in the $NaNO_2$-treated sample was

72.5 µM (5 µg/mL), and the mole ratio of $NaNO_2$ to Hb was estimated to be 31:1. The concentration of DMTS in the DMTS-treated sample was 79.4 µM (10 µg/mL), and the mole ratio of DMTS to Hb was estimated to be 34:1. Two replicates of each sample were prepared and analyzed as shown in Figure 5.

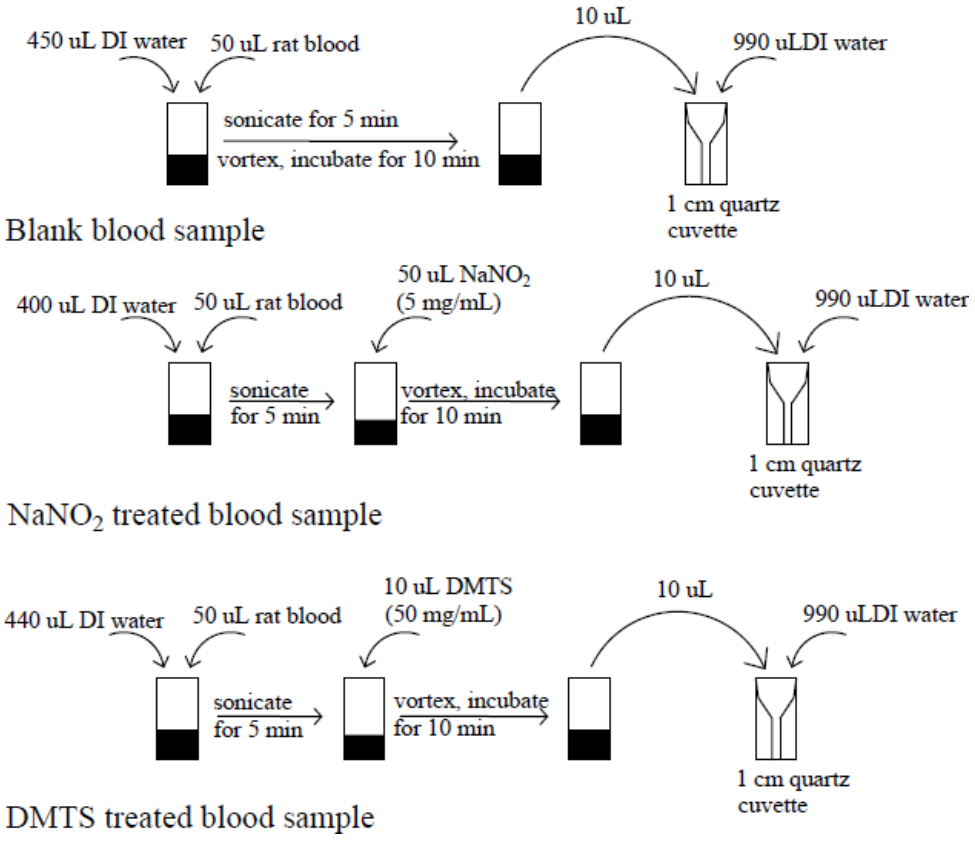


Figure 5. Preparation of blank, $NaNO_2$-, and DMTS-spiked blood samples. Two replicates of each sample were prepared. These were analyzed by UV-vis absorption spectroscopy.

Stock solutions preparation. The stock solutions used in this experiment were 5 mg/mL aq $NaNO_2$ (20.8 mg solid $NaNO_2$ in 4.16 mL DI water), and 50 mg/mL ethanolic DMTS (51.4 mg pure DMTS in 1 mL ethanol).

Rat blood solutions preparation. The rat blood samples used in this experiment included an untreated control, a $NaNO_2$ treated blood sample, and a DMTS treated blood

sample. The Hb concentration in blood[36] is around 150 g/L, the molecular weight[43] of Hb is 64500 g/mol, therefore the concentration of Hb in rat blood is roughly 2.33 mM.

The untreated control was prepared in two steps. First, 1/10 dilution of rat blood was prepared by mixing 50 μL of rat blood with 450 μL DI water, sonicating for 5 min, and then incubating for 10 min. From this, a 1/1000 dilution of rat blood was prepared by mixing 10 μL of the 1/10 solution with 990 μL DI water in a 1 cm quartz cuvette. This was the control sample from which spectra were collected. Two replicates were prepared.

The $NaNO_2$ treated blood sample was prepared in two steps. A 50 μL aliquot of rat blood was mixed with 400 μL DI water, and sonicated for 5 min. Then 50 μL $NaNO_2$ (5 mg/mL aqueous solution) was added. This solution was vortexed and incubated for 10 min to yield a 1/10 dilution of the rat blood that was spiked with $NaNO_2$. A 1/1000 dilution was prepared by mixing 10 μL of the 1/10 spiked blood with 990 μL DI water in a 1 cm quartz cuvette, followed by hand shaking. This was the $NaNO_2$ treated blood sample from which spectra were collected. Two replicates were prepared.

The DMTS-treated blood sample was prepared in two steps. A 50 μL aliquot of rat blood was mixed with 440 μL DI water (Figure 6), and sonicated for 5 min. Then 10 μL of 50 mg/mL ethanolic DMTS was added. This solution was vortexed and incubated for 10 min to yield a 1/10 dilution of the rat blood spiked with DMTS. A 1/1000 dilution was then prepared by mixing 10 μL of the 1/10 spiked blood with 990 μL DI water in a 1 cm quartz cuvette, followed by hand shaking. This was the DMTS-treated blood sample from which spectra were collected. Two replicates were prepared.

Instrumental Settings. Absorption spectra of all samples were measured using a Jasco UV-vis spectrometer (Scan range: 350-600 nm, step size: 1 nm, scan speed: 400 nm/min, band width: 2 nm).

A3 Probing the DMTS-to-Hb reaction ratio

In an initial attempt to determine the DMTS to Hb reaction ratio, UV-vis spectra were gathered from a sample of diluted blood as the concentration of DMTS in the sample was incrementally increased from 0 to 4X the estimated Hb concentration. The initial sample was a 3 mL aliquot of 0.1% (v/v) rat blood in water held in a cuvette with a stir bar, and capped with a septum. Baseline spectra of the dilute blood sample were taken at approximately 80 s intervals. Samples were stirred between spectra, but not while spectra were being collected. Following this, the 0.1% diluted blood sample was repeatedly spiked with small volumes of 0.5 mg/mL DMTS using a 10 µL syringe. Following each spike, the sample was stirred outside the spectrometer. Every 2 min the sample was returned to the spectrometer and a UV-vis spectrum was collected. When the Soret peak near 414 nm stopped shifting, the next spike was applied. The spiking sequence is recorded in Table 1, and an experimental flow diagram is given in Figure 6. The experiment was only done once.

Table 1

Sequence of DMTS spikes added to blood

Volume of 0.5 µg/mL aq. DMTS solution added each time (µL)	Amount of DMTS in cuvette (nmol)	Concentration of DMTS in cuvette (µM)
0	0	0
1	4	1.333
1	8	2.665
2	16	5.326
2	24	7.984
2	32	10.64
2	40	13.29
2	48	15.94
2	56	18.58
2	64	21.22
2	72	23.86
2	80	26.49
4	96	31.75
4	112	36.99

Instrument settings. Following each spike, spectra were collected with the Jasco UV-vis spectrometer (range: 350-600 nm, scan rate: 400 nm/min, bandwidth: 2 nm). Reference was empty cuvette for measurement. DI water spectrum was taken first as reference for analysis.

Stock blood solution preparation. A 2% (v/v) blood stock solution was made by transferring 10 μL rat blood into 490 μL DI water, vortexing, and sonicating for 5 min.

DMTS solutions. A 250.6 mg sample of DMTS was diluted to 5 mL with ethanol to prepare a 50 mg/mL DMTS solution. A 20 μL aliquot of the 50 mg/mL DMTS solution was mixed with 980 μL DI water to obtain a 1 mg/mL DMTS solution. A 500 μL aliquot of the 1 mg/mL DMTS solution was diluted with 500 μL The DI water to prepare a 0.5 mg/mL DMTS solution.

Recording Soret Shift as a function of DMTS concentration. A 2850 μL of DI water was added to a quartz cuvette and a reference spectrum was collected. To this was added 150 μL of 2% (v/v) blood stock solution. Hb concentrations in rat blood have been reported to range from 80-150 g/L (1.24-2.33 mM) being lower in embryos and higher in adults.[36] Based on an adult Hb concentration of 2.33 mM, the 3 mL sample contained 28 nmol of heme iron.

The 3 mL blood sample was capped with a Teflon septum and stirred for 2 min on a magnetic stirrer outside the spectrometer, and then returned to the spectrometer where baseline spectra were gathered. Then 1 μL of 0.5 mg/mL DMTS was injected into the cuvette with a 10 μL syringe. The sample was stirred externally for 2 min, and then returned to the spectrometer for analysis. Spectra were collected by Jasco UV-vis spectrometer (Range 350-600 nm, scan speed 400 nm/min, bandwidth 2 nm. Reference was empty cuvette for measurement. The DI water spectrum was taken first as reference for analysis.) every 1 min 22 sec until the wavelength of the Soret peak remained constant.

This process of DMTS spiking, external stirring for 2 min, and collection of UV-vis absorption spectra was repeated until 112 nmol of DMTS had been added to the 3 mL

diluted blood sample, and the Soret peak had stopped shifting between additions (Figure 6).

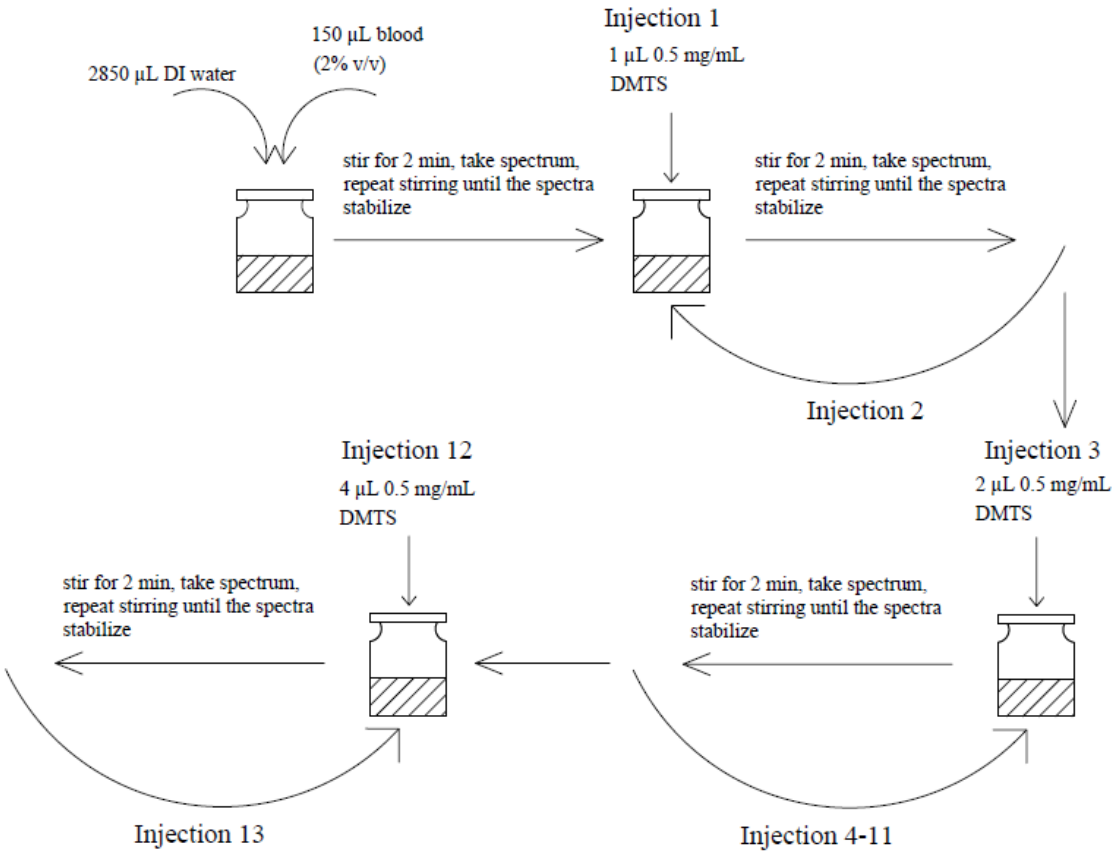


Figure 6. Preparation of successive additions of DMTS to blood *in vitro*.

A4 Comparing analyte (DMTS) and internal standard (DBDS) reactivity in blood.

The aim of this experiment was to study the kinetics of the reaction between DMTS and Hb in blood. Specifically, diluted sheep blood was spiked with DMTS. The concentration of DMTS in the final solution was calculated to be 12 mM (15 µg/mL). The blood was diluted to 13.5%, the concentration of Hb in blood was estimated to be 0.24 mM. The mole ratio of DMTS to Hb was 50:1. DMTS was allowed to interact with each blood sample for different times (0, 5, and 30 min). SPME samples were collected using a

10 min sampling period from the HS in each reaction vial. These SPME samples were then manually injected to initiate the collection of a chromatogram, and associated mass spectrum. There was no replicate for this experiment.

Solutions preparation. A 10 mg/mL DMTS stock solution was prepared by diluting 100 mg DMTS to 10 mL with 15% PS80 solution, and vortexing. A 10 mg/mL DBDS stock solution was prepared by diluting 100 mg DBDS to 10 mL with 15% PS80 solution, and vortexing. A stock blood solution was made mixing 20 µL of heparin solution with 4 mL of 26-day old sheep blood.

A 600 µL aliquot of 10 mg/mL DMTS was mixed with 5400 µL 15% PS80 solution to make 6 mL of a 1 mg/mL DMTS solution. This was mixed with an equal volume of 15% PS80 solution to prepare a 0.5 mg/mL DMTS solution. A 0.1 mg/mL DBDS internal standard solution was prepared by mixing 50µL of 10 mg/mL DBDS with 4950 µL 15% PS80 solution.

Preparation of the 0, 5, and 30 min incubation period samples. A 20 µL DMTS (1 mg/mL) was added to 180 µL of heparinized sheep blood and vortexed for seconds. A 75 µL aliquot of this spiked blood was immediately transferred into vial containing 25µL of DBDS (0.1 mg/mL) and 400 µL of DI water. The vial was crimped, vortexed, incubated for 5 min at room temperature, and then the HS sampled with a SPME fiber for 10 min. The SPME sample was analyzed by GC-MS. Two other samples were prepared and analyzed in an identical manner (Figure 7), except that the DMTS spiked blood was left to incubate for 5 and 10 min respectively, prior to mixing with water and internal standard. The concentration of Hb in sheep blood is calculated to be 1.78 mM.[44]

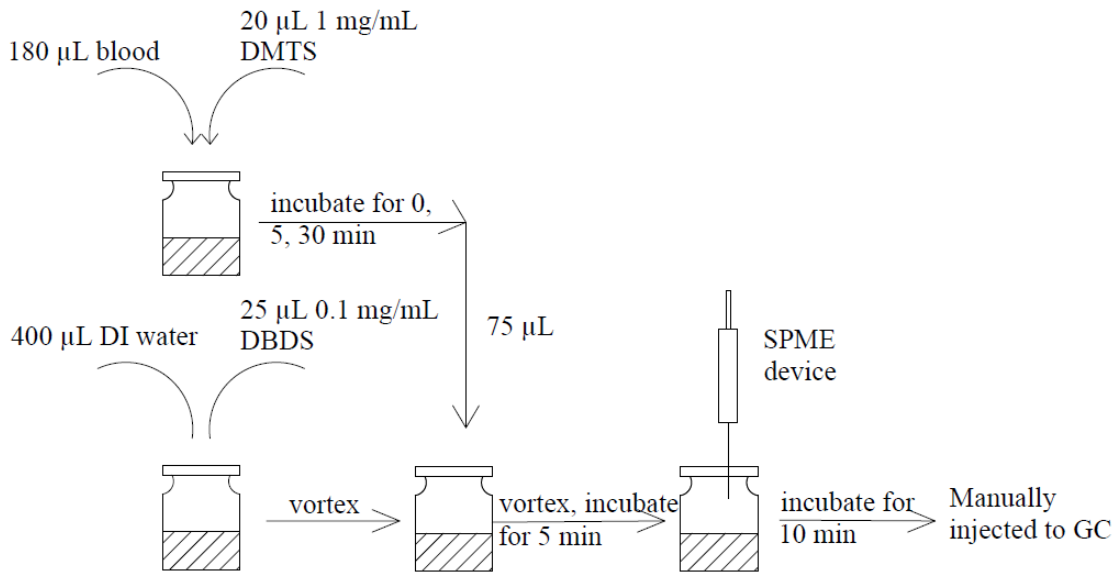


Figure 7. Preparation of incubation time effect on DMTS in blood *in vitro*.

Instrument Settings. The GC oven settings: Temperature ramping was 40 °C for 1 min, then 60 °C /min to 280 °C for 3 min, run time was 8 min, the inlet mode was split less, pressure was 7.0 psi, total flow was 19 mL/min, septum purge flow was 3 mL/min, purge flow to split vent was 15 mL/min at 1 min. MS acquisition parameters: Acquisition mode was scan mode, solvent delay was 0.00 min, scan parameters: low mass was 30.0 m/z, high mass was 500.0 m/z, threshold was 150 m/z.

A5 Kinetics of blood Hb oxidation in the presence of DMTS

The aim of this experiment was to further study the kinetics of the reaction between DMTS and Hb in blood. In this experiment, the conversion of Hb to metHb was monitored with UV-vis absorption spectroscopy as a function of time following a single large addition of DMTS to rabbit blood. The diluted rabbit blood was placed in a quartz cuvette, capped with a septum, and then spiked with DMTS (Figure 8). The concentrations of DMTS, and Hb were estimated to be 39.3, and 2.7 µM, so that the DMTS:Hb mole ratio was 14.6:1.

UV-vis spectra were continuously collected by Ocean Optics spectrometer for 4 h and by Jasco UV-vis spectrometer for 6 days. A control sample was prepared identically, except that it was not spiked with DMTS (Figure 8).

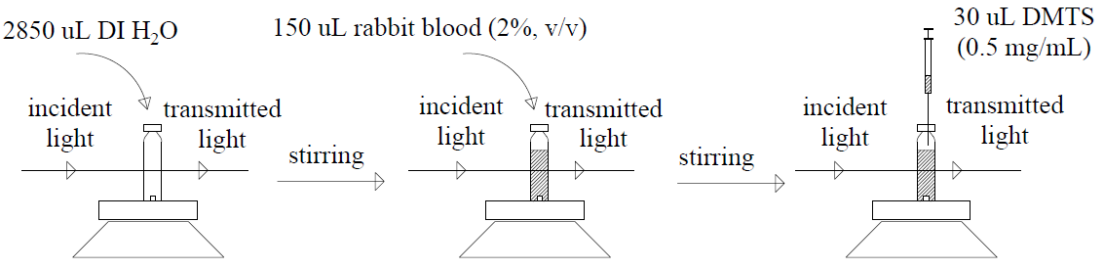


Figure 8. Preparation of single addition of DMTS to blood *in vitro*.

Blood stock solution preparation. A 2% (v/v) blood stock solution was made by mixing 10 µL rabbit blood with 490 µL DI water. The concentration of Hb in rabbit blood has been reported to be 174 g/L (2.70 mM).[38]

DMTS solutions preparation. A 50 mg/mL DMTS solution was prepared by diluting 250 mg DMTS to 5 mL with ethanol. A 1 mg/mL DMTS solution was made by transferring 20 µL DMTS (50 mg/mL) into 980 µL DI water in glass vial. A 500 µL aliquot of 1 mg/mL DMTS solution was mixed with an equal volume of DI water to prepare a 0.5 mg/mL DMTS solution.

Sample preparation. The 1 cm quartz cuvette was filled with 2850 µL DI water and a reference spectrum was collected. To this was added 150 µL of 2% rabbit blood solution. The vial was mixed and sealed. Then a 30 µL aliquot of 0.5 mg/mL DMTS solution was injected the cap septum using a 100 µL syringe that had been prerinsed with ethanol and 0.5 mg/mL DMTS solution prior to use. The final Hb and DMTS concentrations in the cuvette were estimated to be 2.70 and 39.60 µM, respectively.

Instrument settings. Spectra were collected by Ocean Optics UV-vis spectrometer for 4 h following DMTS injection (wavelength range 188.37 to 1107.73 nm, integration time 3 ms, averages 10). At this point the spectra were examined. Because the spectral changes were subtle, after 4 hours the experiment was moved to the Jasco UV-vis spectrometer (Jasco) thinking that its signal to noise ratio might be better than that on the Ocean Optics spectrometer. Following the switch, spectra were collected with the Jasco (wavelength range 300-600 nm, scan speed 400 nm/min, band width 2 nm) for the subsequent 6 days of experimentation.

In the blank blood control experiment 30 μL DI water was added into the blood solution, instead of 30 μL DMTS solution. Photographs and UV-vis absorption spectra were collected of the unspiked sample each day for 6 days. The first control spectrum was taken by the Ocean optics UV-vis spectrometer. The remaining control spectra were taken, one per day, over the next 6 days with the Jasco UV-vis spectra.

A6 Blood with DMTS and $NaNO_2$ *in vivo*

The goal of this experiment was to test the hypothesis that DMTS oxidizes Hb to metHb *in vivo*. In this experiment, either DMTS or $NaNO_2$ solution was injected into mice muscle and incubated. Blood samples were drawn at different times post-injection and analyzed using UV-vis spectroscopy.

Blood sample preparation in vivo (Dr. Petrikovics lab). A 50 mg/mL DMTS stock solution and the 9.4 mg/mL $NaNO_2$ stock solution was prepared in 15% PS80 solution. These were injected intramuscularly into CD-1 Male mice (Charles River Laboratories, Inc., Wilmington, MA). After a designated incubation time, blood was collected from the chest of each mouse. The mouse blood volume is estimated to be 79 mL/kg. For the first

mouse sample, total blood volume in the mouse was determined by $79\frac{mL}{kg} * 0.0227\ kg = 1.79\ mL$, other samples are calculated in the same way. The Hb concentration in rat blood is around 150 g/L (2.33 mM). The Hb amount in the first mouse sample body was determined as $2.33\frac{mmol}{L} * 0.00179L = 4.17$ μmol. Using this approach, the total amount of Hb in the blood of each mouse used in the experiment was estimated. The dose of reagent injected was determined as shown below.

$$V\ (\mu L) = \frac{Dose\ \left(\frac{mg}{kg}\right) * Weight\ (g)}{Stock\ \left(\frac{mg}{mL}\right)} \qquad \text{Equation 1}$$

The concentration of DMTS in rat body in the first sample can be calculated by

$$C_{DMTS\ in\ body} = C_{DMTS\ stock} * \frac{V_{injected}}{V_{total\ blood\ volume}} \qquad \text{Equation 2}$$

The experimental design is summarized in Figure 9. The estimated initial concentrations of Hb, DMTS, and $NaNO_2$ are summarized in Figure 9.

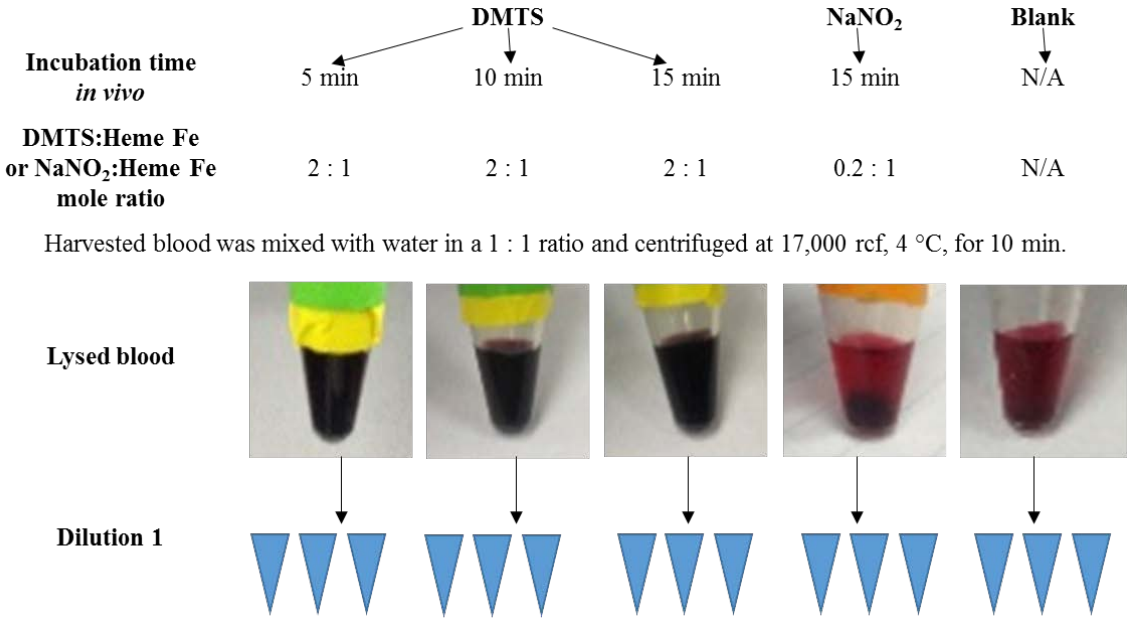


Figure 9. Experiment scheme with DMTS and $NaNO_2$ injected mice (part 1). Preparation of blood samples UV-vis spectroscopy measurement. DMTS was incubated in the mice for 5, 10, and 15 min separately and $NaNO_2$ was incubated in the mouse for 15 min.

Table 2

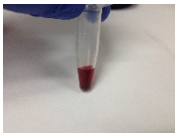
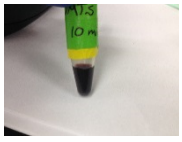
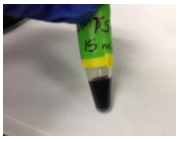
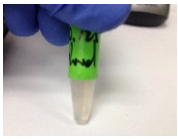
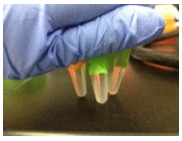
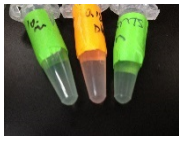
Sample information of DMTS and $NaNO_2$ treated mice

Experiment number	Dose (mg/kg)	$T_{incubation}$ (min)	Mouse Weight (g)	$V_{injection}$ (µL)	C_{stock} (mg/mL)	Blood Volume Drawn V_{blood} (mL)	Estimated Amount of Hb in blood samples (µmol)	Reagent amount injected (µmol)	$C_{reagent}$ in blood (M)	Reagent:Hb
1	DMTS 200	5	22.7	91	50	1.79	4.2	36.11	0.020	8.6:1
2	DMTS 200	10	21.5	86	50	1.70	4.0	34.13	0.020	8.6:1
3	DMTS 200	15	22.8	91	50	1.80	4.2	36.11	0.020	8.6:1
4	$NaNO_2$ 9.4	15	19.2	19.2	9.4	1.52	3.5	2.62	0.002	0.7:1
5	Control	NA	23.6	NA	NA	1.86	4.3	NA	NA	NA

Measurement after injection (Ashley Durham). One hundred microliters of whole blood were taken from each mouse, heparinized, lysed with equivalent volume of DI water, vortexed for 1 min, and then centrifuged at 17,000 rcf, 4 °C for 10 min. Five and 20 µL of lysed blood supernate were added respectively to 995 and 980 µL DI water, and vortexed for 1 min to prepare 0.25 and 1.0 % (v/v) diluted blood solutions. Equal volumes (480 µL) of the 0.25% (v/v) diluted blood solution and DI water were mixed to prepare a 0.125% (v/v) diluted blood solution. Three parallels were made for each sample. All solutions were transparent (Table 3).

Table 3

Images of blood samples from DMTS and $NaNO_2$ treated mice

	Fresh blood	Blood+DMTS 5 min	Blood+DMTS 10 min	Blood+DMTS 15 min	Blood+$NaNO_2$ 15 min
Lysed blood (50%, v/v)					
1% (v/v) series					
0.125% (v/v) series					

The 0.125% (v/v) and 1% (v/v) samples were measured in Ocean Optics UV-vis spectrometer. The integration time was 3 ms and average number was 10. The 1 cm narrow

quartz cuvette was used. The measurement took around 4 h. All diluted and stock samples were kept in the fridge after measurements.

Measurement after kept in fridge for 18 h. To check if there is any spectral change in the previous samples, the same samples (dilution 1) were measured again after stored in fridge for 18 h. Reference were also the same with the previous day. Cuvette and parameters were the same with the last measurement. Three replicates were done. The measurement took 1.5 h.

Measurement after kept in fridge for 18 h and kept in room temperature for 4 h. To check if there is any spectral change after the temperature of samples raising back to room temperature, the stock blood samples (lysed blood solution) that was stored in fridge for 18 h and kept at room temperature for 4 h were diluted to the same concentration as dilution 1 for the absorption measurements (dilution 2). The preparation method was the same as dilution 1 (Table 4**)**.

Table 4

Preparation of dilutions 2 from lysed blood samples

Lysed blood solutions	**Recipes for preparing each lysed blood solution**
50%, (v/v)	100 µL blood + 100 µL DI water
0.25% (v/v) series	5 µL 50% lysed blood + 995 µL DI water
0.125% (v/v) series	480 µL 0.25% lysed blood + 480 µL DI water
1% (v/v) series	20 µL 50% lysed blood + 980 µL DI water

Note: The lysed blood samples were the same sample from mice after 18 h storage in fridge followed 4 h at room temperature.

Measurement of additional DMTS/$NaNO_2$ to 0.125% (v/v) series of blood in vitro. Certain amount of DMTS or $NaNO_2$ solution were added into dilution 2 to determine whether the addition of a large excess of reagent results in a further spectral shift of the Soret peak. A 0.45 mg/mL DMTS solution was prepared by adding 9 µL DMTS stock solution (50 mg/mL, 15% PS80 solution) into 991 µL DI water (Table 5).

Table 5

Additional DMTS/$NaNO_2$ treatment to blood dilutions 2

Samples	Volumes of reagents added to blood samples			Reagent added (nmol)	Final mole ratio of reagent to total Hb	Estimated mol ratio of reagent:Hb *in vivo* (ref.)
	$V_{DMTS\ 1}$ (µL)	$V_{DMTS\ 2}$ (µL)	V_{NaNO2} (µL)			
Blank blood 1	NA	20	NA	7937	2832:1	NA
Blank blood 2	NA	NA	20	2725	945:1	NA
DMTS treated blood 5 min	9	18	NA	32/7143	11:1/2563:1	8.6:1
DMTS treated blood 10 min	9	18	NA	32/7143	11:1/2563:1	8.6:1
DMTS treated blood 15 min	9	18	NA	32/7143	11:1/2563:1	8.6:1
$NaNO_2$ treated blood 15 min	NA	NA	5/5/10	681/681/1362	243:1/486:1/973:1	0.73:1

Note: DMTS solution 1 was 0.45 mg/mL, DMTS solution 2 was 50 mg/mL NaNO2 was 9.4 mg/mL. The Hb amount in the cuvette was estimated to be 2.8 nmol.

Based on the preparation of the 0.125% lysed blood sample, the amount of Hb in the cuvette was estimated to be 2.8 nmol (Equation 3).

$$\frac{150\ g}{L} * \frac{mol}{64500\ g} * \frac{100\ \mu L}{200\ \mu L} * \frac{5\ \mu L}{1000\ \mu L} * \frac{480\ \mu L}{960\ \mu L} * 960\ \mu L = 2.8\ nmol \qquad \text{Equation 3}$$

The amount of DMTS injected into the rat was calculated to be 8.6 times larger than the amount of Hb present in the rat's blood. *After the t*wo late additions of DMTS to the lysed blood samples, the amount of DMTS was estimated to be 11 times more than the Hb amount in the cuvette.

For the blank blood (control sample), $NaNO_2$ and DMTS amount added was calculated separately below.

$$9.4\frac{mg}{mL} * \frac{mol}{69g} * 20\ \mu L = 2725\ nmol \qquad \text{Equation 4}$$

$$50\frac{mg}{mL} * \frac{mol}{126g} * 20\ \mu L = 7937\ nmol \qquad \text{Equation 5}$$

The amount of DMTS and $NaNO_2$ amount added to the rest samples were calculated in the same way. The sample treatments were shown in Figure 10.

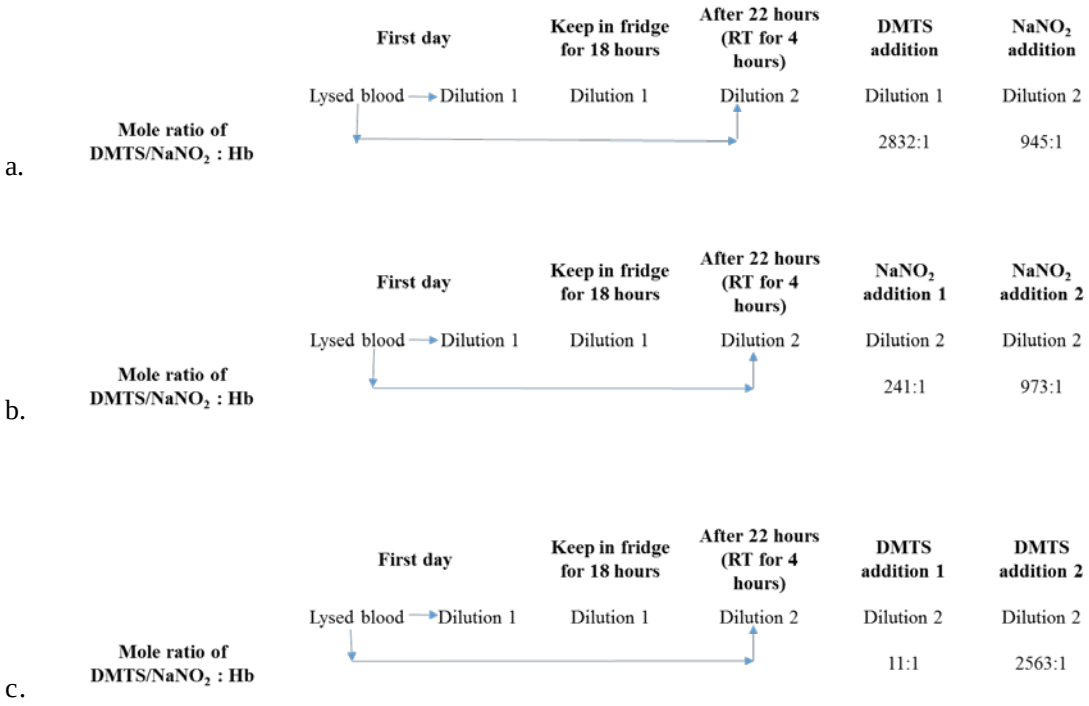


Figure 10. Scheme with DMTS and $NaNO_2$ injected to mice (part 2). Further treatment to the dilution after 22 h with additional DMTS and $NaNO_2$. (a) Treatment to blank blood by DMTS and $NaNO_2$. (b) Treatment to $NaNO_2$ treated blood sample by additional $NaNO_2$. (c) Treatment to all DMTS treated blood samples, including 5 min, 10 min, and 15 min incubation time sample.

B. Segue experiments (Moving from blood to Hb in PBS)

Introduction

A phosphate buffered saline solution of Hb was used in place of blood to study the DMTS reaction with Hb in a simpler environment. The bovine Hb used in these experiments was delivered as metHb, since metHb is more stable upon storage than HbO_2. To mimic *in vivo* conditions, the metHb was converted to deoxyHb by DT.[30] The metHb was reduced by running metHb solution through a column that had been preloaded with a band reducing band of DT. This approach reduced the time of interaction between DT and Hb to minimize deleterious side reactions. Different ratios of DT were added to metHb solution separately and the spectra was monitored by UV-vis spectrometer. The results

suggest that if similar incubation times for these mixtures are used, that DT:Hb ratios of 20:1 and greater should not be used. Alternately, it may be feasible to use such higher concentrations if the two reagents can be rapidly separated following initial mixing. The metHb reduction experiment is described below.

Phosphate Buffered Saline (PBS) solution preparation procedure. A 0.2 M aqueous mono-sodium phosphate, and a 0.2 M aqueous di-sodium phosphate stock solution were prepared. These two solutions were mixed with DI water in proportions specified by the pH table to prepare a 0.1 M phosphate buffered saline (PBS) solution with the desired pH. Then EDTA was added to the 0.1 M PBS solution to obtain an EDTA concentration of 5 mM. The EDTA was used to complex metal ions in solution that might otherwise induce protein denaturing and aggregation. A 20 mM PBS solution containing 1 mM EDTA was obtained by mixing 40 mL of 0.1 M PBS with 160mL of DI H_2O. The pH was verified by the pH meter.

B1 Determining Hb in the reduced fractions

The method described in H.B.F. Dixon and R. McIntosh, Nature, 213, 399 (Jan. 28, 1967) was followed to reduce metHb with dithionite (DT) in a PD-10 desalting column. The reference protocol[30] recommended:(1) equilibrating a 25X2.5 cm column of Sephadex G-25 with 20 mM phosphate buffer, pH 7.0, containing 1 mM EDTA; (2) applying a 2mL aliquot of DT solution (100 mg/mL DT in PBS solution) to the column; (3) applying by 1 mL of the PBS solution to the column to ensure all DT is on column; (4) applying 10 mL of sample containing oxidized Hb; (5) elute the Hb with PBS solution; (6) saturate the reduced Hb eluent with oxygen gas; (7) dialyze the oxygenated eluent against oxygen-

saturated phosphate buffer to eliminate excess DT and achieve complete conversion to HbO_2.

As shown in Figure 11, the protocol called for a column with a radius R_1=1.25 cm and a length L_1= 25 cm; however, the PD-10 desalting column that was available for this research was smaller, having a radius R_2 = 0.714 cm and a length L_2 = 5 cm.

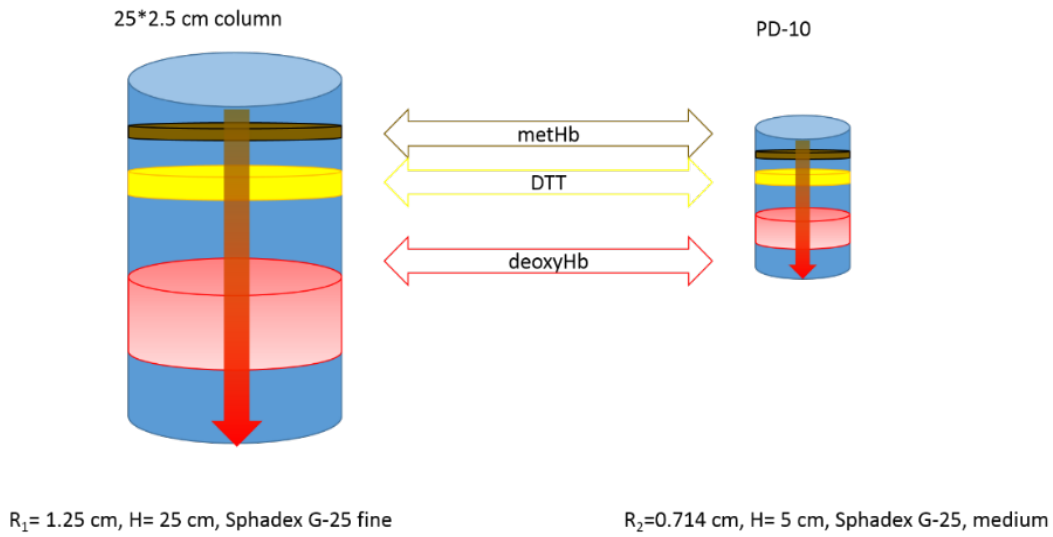


Figure 11. Scaling of amount of reactants to column size.

To accommodate the smaller PD-10 column, the amount of DT and the volume of the DT solution loaded onto the buffer were scaled respectively to 0.347 mmol, and 0.65 mL as shown in Equations 6 and 7.

$$\frac{m_1}{m_2} = \frac{200\ mg}{m_2} = \frac{{R_1}^2}{{R_2}^2}, m_2 = 65\ \text{mg} = 0.347\ \text{mmol} \qquad \text{Equation 6}$$

$$\frac{V_1}{V_2} = \frac{2mL}{V_2} = \frac{{R_1}^2}{{R_2}^2}, \text{V}_2 = 0.65\ \text{mL} \qquad \text{Equation 7}$$

The volume of the hemoglobin solution applied to the column was scaled down from 10 to 3.25 mL as shown in Equation 8.

$$\frac{V_1}{V_2} = \frac{10\ mL\ Hb}{V_2} = \frac{{R_1}^2}{{R_2}^2}, \mathrm{V_2} = 3.25\ mL \qquad \text{Equation 8}$$

In the modified protocol used in these experiments (summarized in Figure 12 and Figure 13): (1) The PD-10 column was initially equilibrated with 25 mL of PBS; (2) A 0.65 mL aliquot of 100 mg/mL DT solution was applied to the column; (3) A 1.85 mL aliquot of PBS was applied to the column to ensure all DT is on column; (4) A 3.25 mL aliquot of 10 mg/mL metHb solution was applied to the column; (5) The Hb was eluted with ~5mL of PBS solution; (6) The reduced Hb was collected in four Eppendorf tubes. Collection began with just before the red color reached the bottom of the column, and ended when no red color was observed in the eluate; (7) the eluate collected in Eppendorf tubes was microfiltered to remove precipitates; (8) the concentration was determined by the molar absorptivity of deoxyHb at 554 nm which is 13250 $M^{-1}cm^{-1}$. If a collected fraction did not show good reduction, it was discarded. (9) the remaining microfiltered filtrates were mixed together in a centrifugal concentrator and were then centrifuged at 4000 g, 4 ºC for 70 min; (10) the filtrate was tested for excess DT using a DTNB assay; (11) the Hb was resuspended in PBS and Steps 9 and 10 were repeated until no DT was detected; (12) the absorption spectra of the final reduced Hb solutions obtained were collected and the Hb concentrations was determined based on the known molar absorptivity of ty of deoxyHb at 554 nm which is 13250 $M^{-1}cm^{-1}$.

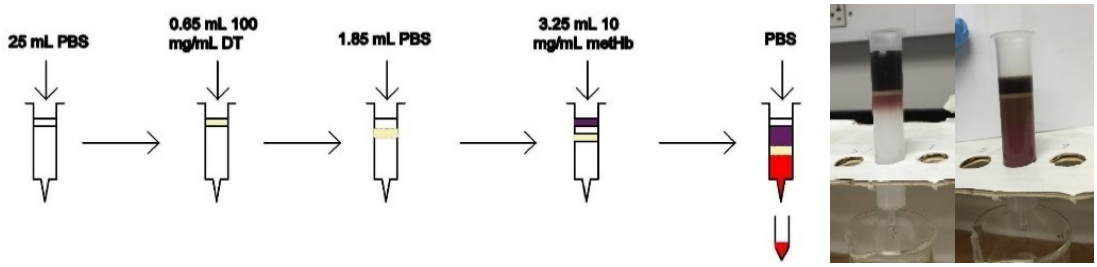


Figure 12. Scheme of reduction of metHb to Hb with DT in desalting column. Representative photographs of the PD10 column immediately following addition of metHb solution, and midway through the reduction are shown below the corresponding drawings in the schematic. Note the color difference between the dark metHb (top of the column), and lighter red Hb following reaction with DT.

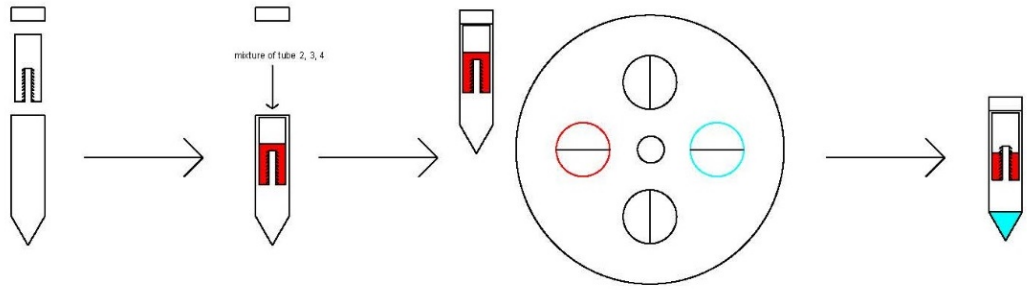


Figure 13. Scheme of DT removal from reduced Hb solution by ultrafiltration.

Ellman's reagent (DTNB) is reduced to TNB^{2-} by thiols. TNB^{2-} has an intense yellow color with a corresponding absorption maxima at 409 nm in PBS solution. In these experiments Ellman's reagent was used to detect the presence of DT contamination in the reduced HbO_2 solutions. In the initial test of this method, the HbO_2 eluate was put through one centrifugation cycle and redispersed with PBS. A 300 µL aliquot of 5 mM DTNB was added to 2 mL of 9.32 µM redispersed HbO_2 solution. The mole ratio of DTNB to Hb was 80.5 to 1. The unused portion of the redispersed HbO_2 solution was centrifuged again at 4000 g, 4 ºC for another 60 min. The absorption spectra of reduced Hb after centrifuge were collected and the concentrations were determined. The level of DT contamination

was assayed by mixing 284 µL of 0.5 mM DTNB solution, 284 µL of 69.78 µM HbO_2 and 3.4 mL PBS, and collecting a UV-Vis absorption spectrum of the resulting solution. The mole ratio of DTNB to Hb was 7:1.

B2 Ellman's reagent tests for thiols, and for DT in filtrate.

Control experiments of DTNB, including 1) DMTS with DTNB, 2) DT with DTNB, and 3) DT with DMTS were carried out in advance to eliminate thiol contributions from molecules other than DMTS metabolites. The 0.5 mM solutions of DTNB, DMTS and DT were mixed by equivalent volume in alternated orders as summarized in Table 6. All solutions were prepared in 20 mM PBS (containing 1 mM EDTA, pH 7.2)

Table 6

Reaction order of DTNB, DT and DMTS

	Trial 1	**Trial 2**	**Trial 3**
1 step	DMTS+DT 1:1	DMTS+DTNB 1:1	DT+DTNB 1:1
2 step	DMTS+DT+DTNB 1:1:1	DMTS+DTNB+DT 1:1:1	DT+DTNB+DMTS 1:1:1

The DT and DMTS with DTNB control experiment. A 5 mM DT (0.871 mg/mL) was prepared by dissolving 87.1 mg DT in 10 mL PBS solution. Then a 0.5 mM DT was prepared by dilute 500 µL 5 mM DT to 5 mL with PBS solution.

A 0.05 mM DTNB solution was prepared by diluting 500 µL 0.5 mM DTNB to 5 mL PBS.

A 502.4 mM DMTS (63.1 mg/mL) was prepared by dissolving 634.4 mg DMTS in 10 mL ethanol and a 0.5 mM DMTS was prepared by diluting 10 µL 502.4 mM DMTS to 10 mL with PBS. And a 0.05 mM DMTS was prepared by diluting 200 µL 0.5 mM DMTS to 2 mL PBS.

In the first trial, the 200 µL of 0.5 mM DMTS was first mixed with 200 L of 0.5 mM DT solution and diluted with 1.6 mL PBS in a 1 cm quartz cuvette. After taking the absorption spectrum, a 200 µL of DTNB (0.5 mM) was added to the above solution. The final concentration of each reagent was 0.045 mM. The reaction ratio was 1:1:1.

In the second trial: A 200 µL of 0.5 mM DMTS was first mixed with 200 µL of 0.5 mM DTNB solution and diluted with 1.6 mL PBS in a 1 cm quartz cuvette. After taking the absorption spectrum, a 200 µL of DT (0.5 mM) was added to the above solution. The final concentration of each reagent was 0.045 mM. The reaction ratio was 1:1:1.

In third trial, the 200 µL of 0.5 mM DTNB was first mixed with 200 µL of 0.5 mM DT solution and diluted with 1.6 mL PBS in a 1 cm quartz cuvette. After taking the absorption spectrum, a 200 µL of DT (0.5 mM) was added to the above solution. The final concentration of each reagent was 0.045 mM. The reaction ratio was 1:1:1.

The metHb reduction methods were kept being modified and optimized in the rest experiment when preparing HbO_2 solution to improve the experiment efficiency (Figure 14).

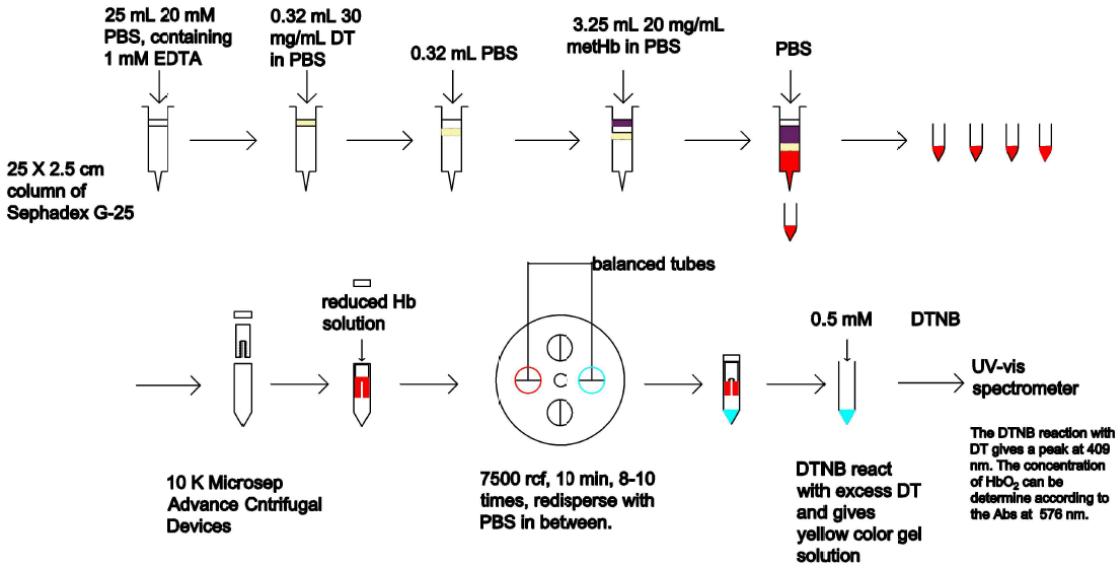


Figure 14. Optimized metHb solution reduction process.

B3 DMTS loading capacity of PBS.

To estimate how much DMTS could be loaded into an aqueous PBS solution, the saturated concentration of DMTS in PBS was found with UV-vis spectroscopy.

Solution preparation. DMTS solutions were prepared in PBS (20 mM, pH=7, 1 mM EDTA). Two parallel solutions of saturated DMTS were prepared by mixing 100 µL of DMTS with 900 µL PBS, autovortexing for 100 min, and centrifuging (5 min, 4 ºC, rcf 14000) to separate the excess DMTS from the PBS solution. The PBS supernate from the two replicate solutions was mixed to obtain a single saturated solution. Serial dilution of the saturated solution was used to prepare solutions whose concentrations were 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 that of the saturated solution, as shown in Figure 15. A UV-vis absorption spectrum was collected through a 1 cm path length of each solution.

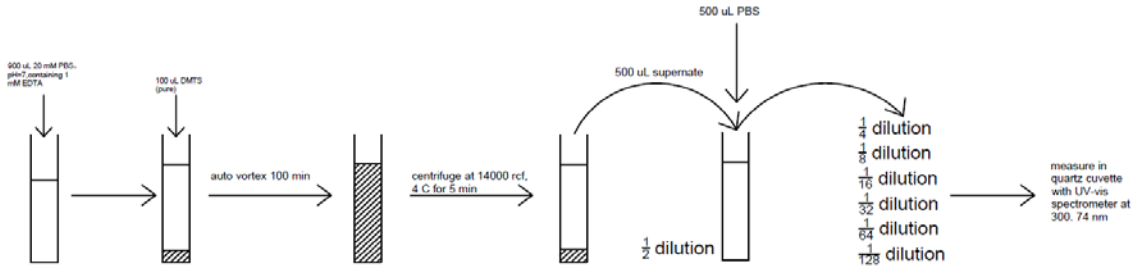


Figure 15. Preparation of dilution series from DMTS saturated solution in PBS.

A series DMTS standards with known DMTS concentrations were also prepared (Figure 16), to aid in the determination of the exact concentration of the saturated solution. The 0.22 mg/mL DMTS solution was made by adding 11.2 mg of DMTS into 50 mL PBS, vortexing for 170 min. The 0.1 mg/mL DMTS solution was made by adding 10.1 mg DMTS in 100 mL PBS, stirring for 160 min. The 0.05 mg/mL DMTS solution was made by adding 4 mL 0.1 mg/mL solution into 4 mL PBS and vortexed. And the 0.005 mg/mL solution was made by taking 0.5 mL 0.05 mg/mL solution into 4.5 mL PBS and vortexing.

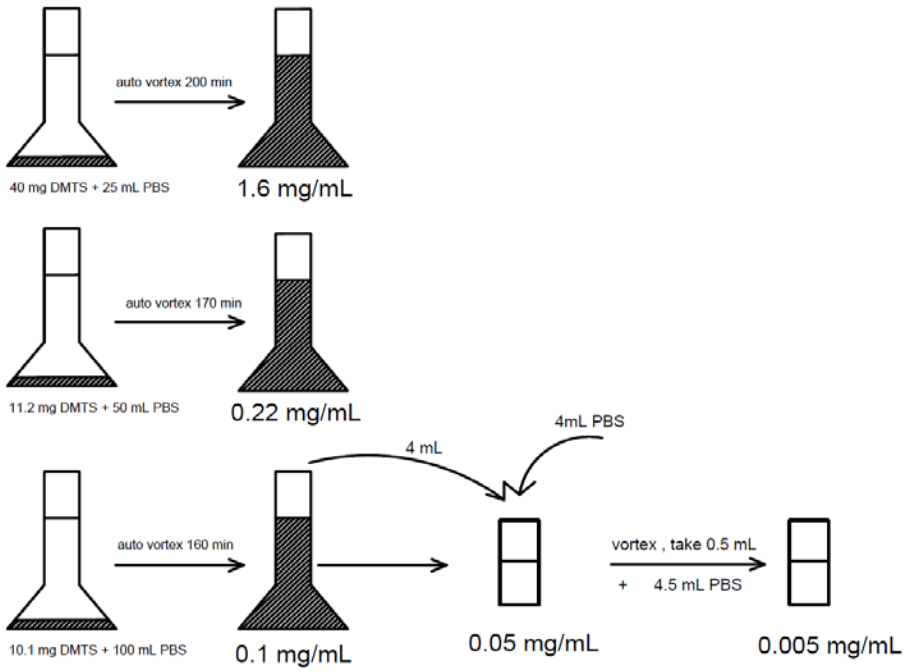


Figure 16. Preparation of DMTS standards solution in PBS.

C. Interactions of DMTS with Hb

Once good fractions of reduced HbO_2 had been obtained, preparations were made to look for the products of the hypothesized reaction with DMTS, and to probe the kinetics of the reaction between DMTS and HbO_2. The hypothesized reactions were given earlier and the first of these is reproduced below.

$$CH_3SSSCH_3 + 2Fe^{2+}(HbO_2) + 2H^+ \rightarrow 2Fe^{3+}(\text{metHb}) + CH_3SSH + CH_3SH$$

First experiment (C1), a series of experiments were conducted in which the conversion of HbO_2 to metHb was followed by UV-vis absorption spectrophotometry. The goal of the first experiment (Experiment A) described in C1 was to determine whether a low concentration of residual DT affected the rate of the reaction of Hb with DMTS. Because it was laborious to remove this DT, we wanted to check if it was necessary. The goal of the second experiment (Experiment B) was to see if an excess of DT could reverse the effects of DMTS, by converting metHb back to Hb. In the third experiment (Experiment C) the reaction between DMTS and Hb was initiated with different starting concentrations of Hb and DMTS, and the formation of metHb was followed by UV-vis spectroscopy. The goal was to collect a preliminary dataset that would enable us to begin characterizing the rate law for the reaction of DMTS with Hb.

Second (C2) HPLC-UV and UV-vis absorption spectrophotometry were employed to follow, respectively, the losses of HbO_2, and DMTS as they reacted with each other. The goal was to estimate the stoichiometric coefficients in the reaction of DMTS with HbO_2.

Third (C3), an experiment was run to see if could reliably detect the presence of methanethiol (MeSH) from a control sample with our GC-MS instrument and methods. If

so, the goal was to identify its GC retention time, and mass spectrum. MeSH is one of the hypothesized products of the reaction between DMTS and HbO_2.

The fourth (C4) and fifth (C5) experiments in this section used a gas syringe, and a SPME fiber respectively to collect samples from the headspace above a reaction mixture of DMTS and HbO_2 in PBS solution. The goal was to see if we could detect the presence of methane thiol, or other new compounds in the headspace above reaction mixtures.

C1 Kinetics: Monitoring HbO_2 loss by absorption spectroscopy

All solutions used in these experiments used 20 mM PBS, 1mM EDTA as the solvent solution. All solutions were buffered at pH 6 to lower the affinity of oxygen binding to the heme in Hb.

Experiment A (Experiment K1). The conversion of HbO_2 to metHb was followed as a function of time with UV-vis spectroscopy twice: once in the presence of DT, and once in the absence of DT.

DMTS stock solution. A saturated solution of DMTS was prepared by mixing 100 µL of DMTS with 900 µL PBS and vortexing for 50 min. This solution was centrifuged at 4 ºC, rcf 14000 for 5 min. The supernate was removed. Its absorbance at 300.74 nm was measured in a 1 cm quartz cuvette. The concentration of DMTS in the supernate was calculated to be $C_{DMTS} = 3.4$ mM using the previously determined calibration equation, $A = 2.59 * C_{DMTS} - 0.0149$.

Reduced Hb preparation. HbO_2 solution was prepared by reducing metHb with DT on a desalting column. A PD-10 column was equilibrated with about 25 mL PBS. A 0.32 mL aliquot of DT solution was transferred into the column and drained into the gel with the help of 0.32 mL of PBS. A 3.25 mL aliquot of metHb was added and eluted with about

5 mL PBS. Eppendorf tubes were used to collected the red colored HbO_2 column eluate. If the solution was cloudy due to the precipitation of Hb and the other blood cell components, then it was centrifuged at 4 ºC, rcf 14000 for 5 min. A small portion of the clear supernate was diluted so that an absorption spectrum could be obtained and compared to a known HbO_2 spectrum. Representative spectra are shown in Figure 17. In all cases the match was excellent, and the clear HbO_2 supernate was used for subsequent experiments.

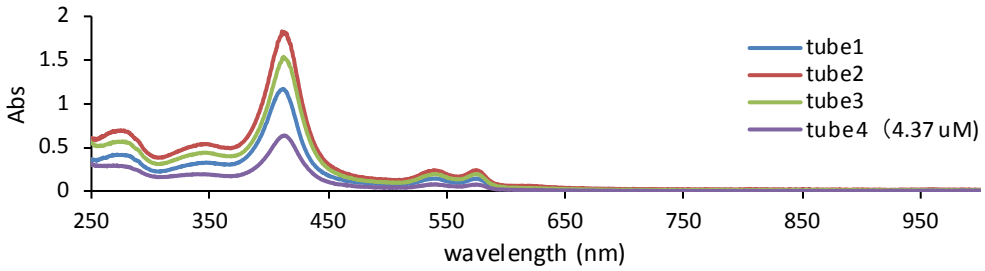


Figure 17. Absorption spectra showing HbO_2, indicating successful reduction. The samples were taken from the four reduced Hb eluate (mainly HbO_2) fractions gathered in each Eppendorf tubes from the PD10 desalting column for use in experiment K4.

The Hb was divided into two aliquots, one that was filtered to remove DT, and one that was not.Filtered aliquot. A portion of the reduced Hb solution was transferred into centrifugal ultrafiltration tubes (10K) and centrifuged at rcf 7500, 4 ºC for 10 min. The dehydrated Hb solution, was rehydrated by adding PBS solution. This filtering/rehydrating cycle was repeated until the filtrate showed no observable yellow color after addition of 0.5 mM DTNB in PBS. At that point UV-vis spectra were collected of the clear filtrate. These spectra showed the DTNB peak at near 320 nm, but not the TNB^{2-} peak that would have indicated the presence of residual DT. At this point the DT was below our detection limit and the filtration cycles were stopped. The HbO_2 in that was trapped in the filter was rehydrated. An absorbance spectrum of the rehydrated HbO_2 solution was collected.

Representative spectra of the DT free filtrate, and the rehydrated HbO_2 solution are shown in Figure 18. The HbO_2 absorbance at 576 nm was substituted into Beer's law to calculate the HbO_2 concentration in the rehydrated solution.[45]

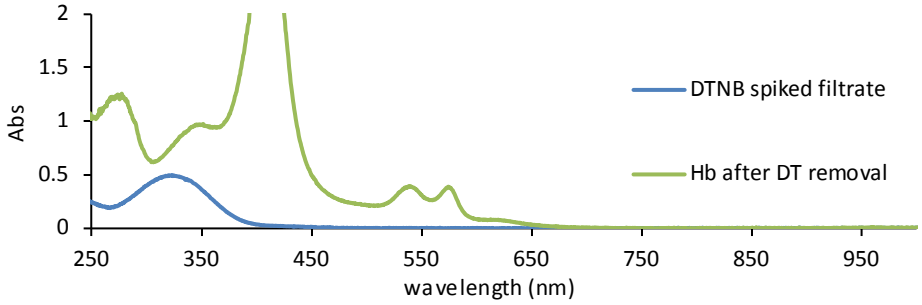


Figure 18. Absorption spectra showing removal of excess DT from Hb. Absorption spectra of HbO_2 solution after removal of DT by centrifugal ultrafiltration, and of the DTNB spiked, Hb-free filtrate collected from the 9 centrifugal wash. The lone peak in the centrifugal wash spectrum shows only unreacted DTNB, suggesting that the DT has been successfully removed from the Hb solution at this point.

Filtered HbO_2 solution was mixed with DMTS and PBS in one cuvette, and only with PBS in a second. The initial concentration of HbO_2 in both the reaction mixture and the control solution was 3.2 μM. The initial concentration of DMTS in the reaction mixture was 640 μM. The second cuvette served as a control for the oxidation of HbO_2 by components of the air. Spectra of both solutions were systematically collected as a function of time. The experiment was repeated as described above, except that unfiltered HbO_2 solution which still contained residual DT, was used in place of the filtered HbO_2. Spectra of the two reaction mixtures (HbO_2/DMTS/PBS and HbO_2/DMTS/PBS/DT) and the two controls (HbO_2/PBS and HbO_2/PBS/DT) were collected until the initial HbO_2 spectrum had been converted to a metHb spectrum.

Experiment B. The conversion of HbO_2 to metHb is known to be reversible. In the second experiment in this series, an excess of DT was added to one of metHb solutions generated in the prior experiment to reduce the metHb formed by DMTS, back to HbO_2. Spectra were collected before and after the addition.

Experiment C (Experiment K2-K4). The goal of the third experiment in this series was to collect a preliminary dataset that would enable us to begin characterizing the rate law for the reaction of DMTS with Hb. Thus, three separate reactions (K2, K3, and K4) were initiated with different starting concentrations of Hb and DMTS as summarized in Table 7. The formation of metHb in each reaction mixture was followed by UV-vis spectroscopy.

Table 7

The estimated starting conc. of HbO_2 and DMTS in experiments K2-K4

Exp. #	**$V_{Hb\ rehydrated}$ (µL)**	**$V_{DMTS\ Stock}$ (µL)**	**DMTS:Hb Mole ratio**	**Target C_{DMTS} (µM)**	**Target C_{Hb} (µM)**	**Reaction time (min)**
K2	10.7	690	166:1	800	5	173
K3	25	600	79:1	800	10	113
K4	13.7	484	116:1	600	5	70

The concentrations of the final HbO_2 stock solutions used in experiments K2-K4 are shown, along with the concentrations of other materials (DT, metHb, and DTNB) involved in preparing each HbO_2 solution are summarized in Table 8. The DMTS stock

solutions used in experiments K2, K3 and K4 (as shown in Table 7) had concentrations, respectively of 3.0, 3.3, and 3.0 mM.

Table 8

Prep. of metHb, DT and DTNB stock solutions for experiments K2-K4

		K2	**K3**	**K4**
DT (1 mL)	Conc.(mM)	287.2	287.2	172.3
DTNB (0.5 mM, 9 mL)	DTNB (mg)	50.0	50.0	30.0
metHb (0.3 mM, 5 mL)	MetHb (mg)	100.7	100	97.3
$C_{HbO2\ rehydrated}$ (μM)		**1.17**	**10.0**	**0.91**

C2 Kinetics: Monitoring DMTS loss by HPLC

In these experiments, HPLC-UV and UV-vis absorption spectrophotometry were employed to follow, respectively, the losses of HbO_2, and DMTS as they reacted with each other. The goal was to estimate the stoichiometric coefficients in the reaction of DMTS with HbO_2.

Preparation of stock solutions. The DMTS was prepared as the method illustrated in the summary of kinetics experiments. The concentration was determined to be 3.0 mM at 300.74 nm with calibration curve.A 30 mg/mL solution of DT in PBS (0.17 M) was prepared by mixing 30 mg DT stock solution with 1 mL PBS. A 20 mg/mL metHb (0.31 mM) was prepared by dissolving 100 mg metHb in 5 mL PBS. The metHb solution was reduced, and the DT removed by centrifugal ultrafiltration. The concentration of the reduced, filtered, Hb stock solution was 0.69 mM.

The target concentrations of DMTS and HbO_2 in the reaction samples were 50 and 5 µM, respectively.

UV-vis sample. A 14.6 µL of 0.69 mM HbO_2 solution (previously centrifugally filtered to remove residual DT), was added into 1.46 mL PBS and then mixed with 30 µL of 3.0 mM DMTS solution in a glass cuvette with stirring. UV-vis spectra were collected and saved every 5 min for 5.5 h.

HPLC samples. Twenty-one samples were prepared for HPLC analysis, in 2 mL glass vials with Teflon septum lids, as summarized in Table 9.

Table 9

HPLC samples and controls

Reaction progress	**0 h**	**1.5 h**	**5.5 h**
Hb/DMTS samples	3 parallels	3 parallels	3 parallels
DMTS controls	3 parallels	N/A	3 parallels
Hb controls	3 parallels	N/A	3 parallels

Nine HPLC samples contained the same 10:1 DMTS to HbO_2 reaction mixture that had been added to the cuvette for UV-vis analysis. Six of the HPLC samples were controls containing only HbO_2 in PBS solution. The final six HPLC samples were controls containing only DTMS in PBS solution.

Extraction. Because Hb will plug up an HPLC column, all 21 samples were extracted to isolate the DMTS from the Hb prior to HPLC analysis. A 500 µL aliquot was transferred from each vial to an Eppendorf tube containing 1000 µL of acetonitrile (ACN), auto-vortexed for 10 min, and centrifuged at 14000 rcf 4 C for 5 min. The supernate was

collected into a new glass vial with PTFE septum screwed cap. HPLC samples were drawn from these supernate solutions. The 21 solutions were prepared at the same time. The final ingredient added was the DMTS to initiate reaction. All vials were crimp sealed with a Teflon coated septum.

Immediately following initiation of reaction, three reaction vials, three DMTS control vials and three Hb control vials were extracted and the DMTS containing supernates stored for subsequent measured by HPLC. After 1.5 h, another three reaction vials were extracted and the DMTS containing extract was stored for HPLC analysis. After the UV-vis sample had indicated completion of the reaction (about 5.5 h) the final three reaction vials, three DMTS control vials and three Hb control vials were extracted and stored for subsequent HPLC analysis. The order in which the samples were run on the HPLC is summarized in Table 10.

Table 10

Measurement order of HPLC reaction and control samples

order	sample	order	sample
1	DMTS 0 h 1	12	Reaction 1.5 h 3
2	DMTS 0 h 2	13	Reaction 5.5 h 1
3	DMTS 0 h 3	14	Reaction 5.5 h 2
4	Hb 0 h 1	15	Reaction 5.5 h 3
5	Hb 0 h 2	16	DMTS 5.5 h 1
6	Hb 0 h 3	17	DMTS 5.5 h 2
7	Reaction 0 h 1	18	DMTS 5.5 h 3
8	Reaction 0 h 2	19	Hb 5.5 h 1
9	Reaction 0 h 3	20	Hb 5.5 h 2
10	Reaction 1.5 h 1	21	Hb 5.5 h 3
11	Reaction 1.5 h 2		

Note. D refers to DMTS solutions, H refers to HbO_2 solutions, and S refers to DMTS and HbO_2 reaction samples.

C3 Search for products: GC-MS analysis of MeSH standard (control)

Our starting hypothesis was that DMTS might be reduced by Hb in blood to produce metHb, and reduction products such as CH_3SH, CH_3SSH and H_2S (Equation 1 and 2). The aim of this experiment was to determine the retention time of MeSH in the GC and its mass spectrum in MS. Because MeSH is toxic, the first challenge was to design a safe approach to for generating and analyzing MeSH samples.

The National Institute for Occupational Safety and Health Recommended 15 min Exposure Limits (NIOSH REL.7/7/2016) for MeSH is 0.5 ppm by volume[48] (1 mg/m^3, $2 * 10^{-5} \frac{mol}{m^3}$ 0.02 µmol/L). The OSHA permitted exposure limit (PEL) is 10ppm which is twenty times higher. If we assume that the HS pressure above the solution in the sample

vial is 1 atm ($1.01*10^5$ Pa), then we can calculate the partial pressure of MeSH in the HS that is needed to reach the NIOSH REL (Equation 9).

$$P_{\underset{EL}{CH_3SH}} = \frac{0.5mL_{CH_3SH}}{10^6 mL_{air}} * 1.01 * 10^5 Pa = 0.051\ Pa_{CH_3SH} \qquad \text{Equation 9}$$

The relationship between the concentration of a volatile solute (like MeSH) in aqueous solution, and the vapor pressure of the same volatile solute in the HS above the solution is given by Henry's law, $C = H * P_g$, where C is the concentration of the volatile solute in the aqueous phase, P_g is the partial pressure of that species in the gas phase under equilibrium conditions, and H is the Henry solubility constant.[46] Using the literature values for Henry's constant for MeSH ($2.0 \cdot 10^{-3}$ to $5.1 \cdot 10^{-3}$ mol/m^3 Pa at 298K), the concentration of MeSH in aqueous solution that will yield a partial pressure equal to the exposure limit can be calculated at 298K using Henry's law.

$$C_{Min} = 2.0 * 10^{-3} \frac{mol}{m^3 Pa} * 0.051 Pa * \frac{1\ m^3}{1000L} = 1.01 * 10^{-7} \frac{mol}{L} \qquad \text{Equation 10}$$

$$C_{Max} = 5.1 * 10^{-3} \frac{mol}{m^3 Pa} * 0.051 Pa * \frac{1\ m^3}{1000L} = 2.60 * 10^{-7} \frac{mol}{L} \qquad \text{Equation 11}$$

Using the more conservative estimate, any aqueous solution whose MeSH concentration is still at or above 0.10 µmol/L following equilibration with the HS will produce a partial pressure above the exposure limit (0.051 Pa, or 0.5 ppm). This is close to, but below the solubility limit of 0.48 µmol/L (23.30 µg/L) for MeSH[46] at 20 ºC in water.

In aqueous solution, MeSH[47] (CH_3SH, pKa = 10.33 at 25 ºC) is always in equilibrium with methyl thiolate (CH_3S^-).

$$CH_3S^- + H^+ \leftrightarrow CH_3SH \qquad \text{Equation 12}$$

Because the methyl thiolate anion has negligible volatility, only the CH_3SH component will partition out of solution into the HS. Thus, the solution pH can be used control the relative concentrations of the volatile CH_3SH and the nonvolatile CH_3S^-, and thus the partial pressure of MeSH gas. Planning on using this strategy, we purchased a 15 wt% aqueous CH_3SNa solution, and proposed that five microliters of this stock solution be transferred quickly to a 1.5 mL glass vial with PTFE septum screwed cap in the fume hood and equilibrate for 15 min, then insert SPME fiber and incubate for 15 min, and inject in to GC.

Prior to carrying out this experiment, we calculated: (1) if the HS partial pressure of MeSH inside sampling vial would exceed the exposure limit concentration, and (2) the danger that this 5 µL sample in the sampling vial would represent if all of it were spilled into the lab and acidified. To ensure a full understanding of the safety considerations, we also calculated (3) the expected room partial pressure of MeSH that would result if the full stock bottle spilled but was not acidified; and (4) the partial pressure of MeSH that would result in the worst case scenario if the full stock bottle was spilled and completely acidified.

The molarity of sodium methyl thiolate in the 15% MeSNa stock solution, and in the diluted sample for HS sampling was calculated as shown respectively in Equation 13.

$$C_{stock} = \frac{15g_{MeSNa}}{100g_{solution}} * \frac{1.08\mathrm{g}_{solution}}{cm^3} * \frac{1mol}{70.09g_{MeSNa}} * \frac{cm^3}{mL} * \frac{m}{10^{-3}} \qquad \text{Equation 13}$$

$$= 2.3M$$

The 1.08 g/cm^3 density of stock solution was calculated based on a calibration curve ($\text{Density} = 0.3644[CH_3SNa]^2 + 0.4949[CH_3SNa] + 1$) determined by fitting literature densities for sodium methyl thiolate solutions of different concentrations.[49]

SAFETY – Four spill scenarios.

Case 1a: All of the HS sampling solution is spilled outside the hood, but not acidified

The 5 µL sample of 2.3 M MeSNa stock solution contains 11.5 µmol of MeSNa. Knowing this, the minimum volume required to bring the HS partial pressure below the exposure limit of $1.4 * 10^{-5} \frac{mol}{m^3}$ can be calculated Equation 14.

$$V_{min} = \frac{11.5 * 10^{-6}\text{mol}}{2.0 * 10^{-5} \frac{mol}{m^3}} = 0.58\ m^3 \qquad \text{Equation 14}$$

Because the lab has a volume of around 180 m^3, a complete spill and acidification of the 5 µL sample would not raise the level in the room above the exposure limit.

Since the 15% MeSNa solution is strongly basic, the sodium comes from NaOH. The concentration of NaOH in the solution will be greater than or equal to 2.3 M, leading to a solution pH greater than or equal to 13.6. At this pH, most of the analyte will be present as the nonvolatile conjugate base MeS$^-$. Knowing this we can use the alpha fraction equation for monoprotic acids to calculate the concentration of the salt that is present as the volatile MeSH at pH 13.6.

$$[\mathrm{CH_3SH}] = \mathrm{C}_{total}\left(\frac{[H^+]}{[H^+]+\mathrm{K}_a}\right) = 2.3M * \left(\frac{10^{-13.6}}{10^{-13.6}+10^{-10.33}}\right) \quad \text{Equation 15}$$

$$= 0.0012\frac{mol}{L}$$

$$P_{CH_3SH\ upper\ limit\ in\ vial} = \frac{0.0012\ \frac{mol}{L}}{2.0 * 10^{-3}\ \frac{mol}{m^3 Pa}} * \frac{1\ 000\ L}{m^3} \quad \text{Equation 16}$$

$$= 600\ Pa\ (= 0.006\ atm)$$

This calculation predicts that the vapor pressure of MeSH (600 Pa) above 5 µL of sodium methyl thiolate stock solution should be high enough to obtain a good sample for analysis (much greater than the exposure limit), and low enough to avoid causing pressure problems for the seal.

Spill scenarios for the full 25g stock solution is spilled:

Case 2a: All of the stock solution is spilled, but not acidified outside the hood

The number of moles sodium methyl thiolate (15% in water, 25g) in the entire stock solution is 0.05, and this can be used in conjunction with the NIOSH REL, and the OSHA PEL to calculate room volumes that would be needed to bring concentrations down to recommended and permitted levels (Equation 17-19).

$$\frac{15\ \mathrm{g}_{CH3SNa}}{100 g_{stock}} * \frac{25 g_{stock}}{\frac{70.09\ \mathrm{g}_{CH3SNa}}{\mathrm{mol}}} = 0.05\ \mathrm{mol}_{CH_3SNa} \quad \text{Equation 17}$$

$$V_{NIOSH\ REL} = \frac{0.05\mathrm{mol}}{2.0 * 10^{-5}\ \frac{mol}{m^3}} = 2500\ m^3 \quad \text{Equation 18}$$

$$V_{OSHA\ PEL} = \frac{0.05\text{mol}}{20 * \left(2.0 * 10^{-5}\frac{mol}{m^3}\right)} = 125\ m^3 \qquad \text{Equation 19}$$

Spaces with volumes of 2500 m^3, and 125 m^3 are required to dissipate the CH_3SH vapor from a complete spill and acidification of the stock solution respectively to the NIOSH recommended level, and the OSHA permitted level. The lab volume of ~ 180 m^3 is greater than the required OSHA PEL volume for a worst case spill with acidification, and smaller than the volume required to reach the NIOSH REL. Assuming that the ventilation systems failed, the full volume of the of the west side of the second floor would be needed to bring partial pressure below the NIOSH REL. Because diffusion is relatively slow, and because we are so acutely sensitive to the smell of these compounds, there would still, in this worst-case scenario be time to walk away from the source if somebody did not suffer an initial "knockout dose". The best safety precautions are to use this chemical in the hood, and to keep it away from acids. If it does spill, evacuate the room. The ventilation systems will reduce of MeSH below the exposure limit rapidly.

The sample preparation was carried out in the fume hood in Dr. Thompson's lab, with gloves (nitrile), and goggles using the following steps: (1) a wooden support was built to prevent the stock solution bottle from tipping. An 1.5 mL HS sampling vial was secured in a second block again to mitigate against tipping; (2) Working in the hood, 5 µL of 15% MeSH solution was transferred to the secured 1.5 mL glass vial; (3) a lid with a septum was screwed down onto the vial so that the Teflon liner of the septum faced the solution; (4) The vial was allowed to sit for 15 min to ensure that the HS and liquid were equilibrated; (5) A SPME fiber was inserted through the septum into the HS, and left in the HS for a 15

min sampling period. During this period the GCMS parameters were set (vida infra); (5) The fiber was injected to the GC-MS inlet and sit for the whole run.

GC settings. Flow rate: 20 mL/min, first splitless injection and then purge flow to split to vent at 15 mL/min at 1 min. Injector temperature is 150 ºC; septum purge flow is 19 mL/min, total flow is 19 mL/min, oven program is at 50ºC hold 1 min, 50ºC to 80ºC at 10 ºC/min, hold 5 min, 80ºC to 220 ºC at 50 ºC/min, hold for 1 min. Injector temperature: 150 ºC. Inert straight liner. MS settings. SCAN and SIM mode will be used. Scan parameters: m/z: 20-27.9, 28.1-31.9, 33-200. Sim parameters: m/z: 12-15, 33, 44-50. Dwell time: 50 µs. Two glass vacuum funnels with activated charcoal (removed from a new Britta water filter) was placed on top of GC front inlet exits to absorb the possible waste gas.

Stock solution storage. The MeSNa stock solution was stored in a desiccator, under Ar gas, in the hood. The desiccator was covered with black-out cloth to reduce light induced reactions.

C4 Search for products: GC-MS analysis of syringe headspace samples

A gas tight syringe was purchased to collect representative HS samples, assuming that volatile compounds such as MeSH, and H_2S, might not partition very effectively into the SPME fiber.

Initial control samples. A 0.6 mM solution of DMTS in PBS was prepared as described in section C1. The first eluate from the desalting column was collected at the beginning of metHb reduction, which containing mainly PBS and little DT solution, and the early fraction of reduced Hb from column was collected as well. Each sample was equilibrated for 30 min. A 400 µL HS sample was collected from each vial by gas tight syringe and locked into the syringe using a syringe lock valve. The gas was compressed to

250 µL immediately before injection. The lock valve was then opened and the gas was pushed out of the syringe into the GC injector which was set at the low temperature of 110 °C in an attempt to minimize reactions. The column temperature was held at 50 °C for 1 min, ramped at 10 °C/min to 150 °C, held at 150 °C for 5 min, ramped at 50 °C/min to 220 °C, held for 1min. The mass spectrometer was operated in scan mode.

GC-MS reaction sample, and controls. A reaction sample containing 0.8 mM DMTS and 5 µM reduced Hb solution, and a Hb control sample were prepared as described in C1, but in 2 mL glass vials sealed with screw caps and Teflon coated septa. Two replicates of the reaction mixture, and two replicates of the Hb control sample were prepared. HS samples (400 µL) were gathered from the first reaction replicate at 30 and 60 min after initiation of the reaction, and from the second reaction replicate at 135 min after initiation of the reaction. A 400 µL HS sample was gathered from the first Hb control replicate at 90 min after the start of the experiment, and from the second Hb control replicate at 165 min after the start of the experiment. The samples were analyzed by GC-MS. Injections were made in splitless mode. The inlet temperature was 110 ºC. The column temperature was held at 50 °C for 3 min, ramped at 10 °C/min to 150 °C, ramped at 20 °C/min to 220 °C, and held at 220 ºC for 3 min.

A control was also run to see how well the vials used in these experiments were holding their seals. A reaction mixture containing 0.6 mM DMTS and 5 µM Hb in PBS was prepared, and equally distributed among 12 headspace sample vials. The GC oven temperature was fixed at 150 ºC for 90 min. HS samples were sequentially taken from each vial, and injected one at a time while the GC was running. Each gave rise to a new DMTS peak in the spectrum. Because DMTS was present in a thousand-fold excess, the

expectation was that the DMTS peak area would remain constant over the course of the experiment.

C5 Search for products: GC-MS analysis of SPME headspace samples

This experiment was a parallel experiment to C4, except that a SPME fiber, rather than a gas syringe was used for collection of HS samples.[37] A set of replicate sample and control solutions were prepared in crimp sealed glass vials, and were continuously stirred.

The SPME fiber was conditioned for 30 min at 250 °C before each use. HS samples were gathered by SPME fiber from the two reaction vials at 30 and 105 minutes, respectively, following the initiation of reaction. HS samples were gathered by SPME fiber from the DMTS control, and the HbO_2 control at 70 and 90 min, respectively, following the start of the experiment. The measurement order for these samples is summarized in Table 11.

Table 11

Measurement order of GC-MS reaction and control samples

Measurement order	Sample name	Equilibrium time (min)	Incubation time (min)
1	PBS	90	15
2	Hb+DMTS	30	15
3	Hb control	70	15
4	Hb+DMTS	105	30

GCMS parameters for measurement. The injector, which was held at 250 ºC and run in splitless mode. The SPME fiber was kept in the injector for 7 min at the start of each

run. The oven temperature was held at 35 ºC for 3 min, ramped at 10 ºC/min to 150 ºC, ramped at 20 ºC/min to 220 ºC. Run Time was 26 min. The helium flow rate was 20 mL/min. The mass spectrometer scan range was 30 – 300 m/z. Trace Ion Detection was turned on, and the minimum threshold for peak recognition was set at 150.

CHAPTER III

Results and Discussion

A. Interactions of DMTS with blood

A1 Effect of blood storage on DMTS recoveries

In this first experiment, 5-day and 4-month old blood were identically spiked with DMTS. The chromatograms of samples gathered from the HS above these samples are shown in Figure 19. Prior to doping with DMTS, the color of the old blood was observed to be darker than that of the fresh. The retention times for the DMTS and DBDS peaks were 3.16 and 4.30 min respectively.

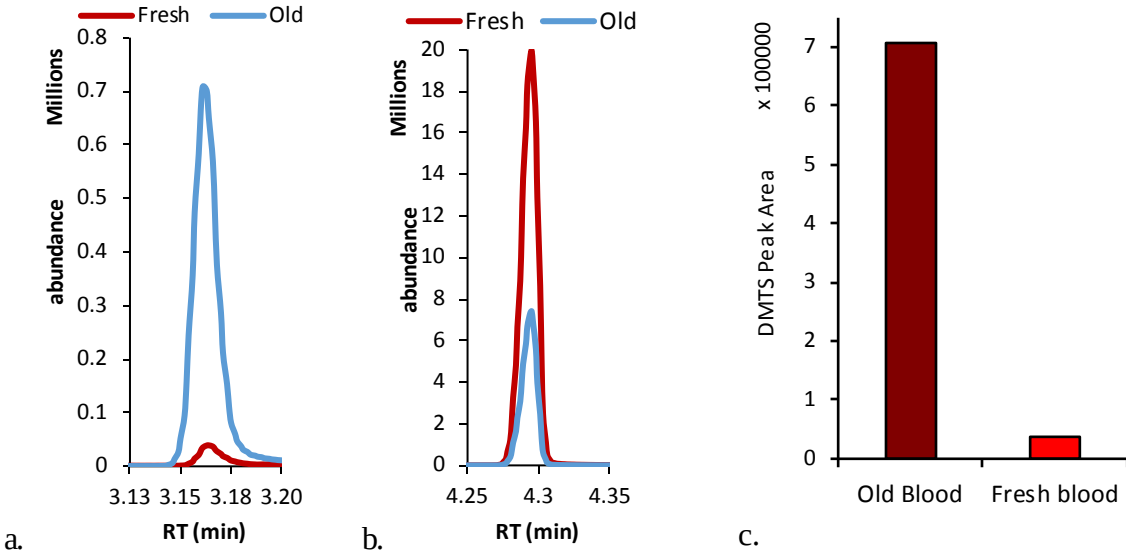


Figure 19. Chromatograms of DMTS-spiked fresh and aged blood. (a) DMTS and (b) DBDS peak area in in 5-day, and 4-month old blood. (c) DMTS peak areas in the chromatograms from samples gathered from the HS above old and fresh blood that had been spiked with identical concentrations of DMTS.

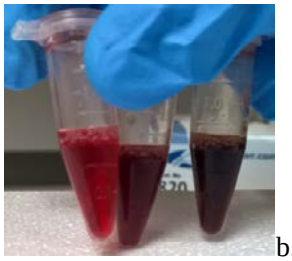
Based on the ratio of their DMTS peak areas, approximately twenty times more DMTS was recovered from the 4-month old blood, than from the 5-day old blood. When

the internal standard correction was made, the DMTS recovery in the 4-month old blood was fifty-six times (Figure 19) greater than in the 5-day old blood sample.

Sulfides, such as DMTS, can be oxidized to form sulfones, or reduced to form thiols. It is possible that the DMTS reaction mechanism in blood is due to the oxidation or reduction of DMTS by some components that are present in higher concentration in the 5-day old than in the 4-month old blood. Because the blood was observed to darken following DMTS addition, and because the oxidation of Hb to metHb is a well-known cause of darkening in blood, it was hypothesized that DMTS might be oxidizing Hb to metHb. It was further hypothesized that the rate of this reaction is likely to decrease as blood ages due to the gradual oxidation of Hb by oxygen in blood that accompanies aging.

A2 Comparing the effects of $NaNO_2$ and DMTS on blood spectra

To test the hypothesis that DMTS losses in blood are due to a redox reaction with Hb, the UV-vis absorption spectra of an unspiked control, and blood samples spiked respectively with DMTS, and known metHb former, $NaNO_2$, were collected and compared. As shown in Figure 20a, the color of the blood darkened noticeably following the addition of either $NaNO_2$ or DMTS.

a. 

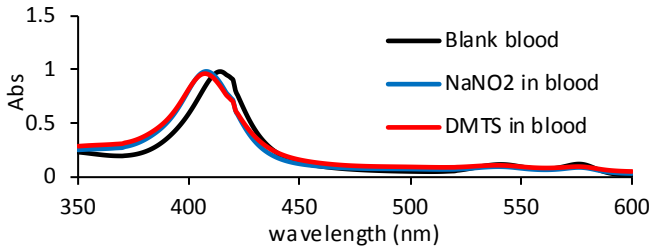
b. 

Figure 20. Comparison of color and spectra: $NaNO_2$ and DMTS treated blood. (a) Blood color change (left to right): untreated blood, $NaNO_2$ treated blood, and DMTS treated blood. (b) $NaNO_2$ and DMTS in blood absorption spectra.

The UV-vis absorption spectrum of the untreated blood (Figure 20b) exhibits the three characteristic peaks of HbO_2 centered respectively at 414, 541, and 576 nm.[50-53] When Hb is oxidized to MetHb, the following spectral changes are expected: a blue-shift of the Soret peak; the decrease of the Q bands at 541 nm and 576 nm in HbO_2 spectra; and the appearance of two new peaks at 500 and 630 nm, respectively.[50-53] The spectrum of the $NaNO_2$ treated blood shows both the expected blue-shift of the Soret peak, and the decrease in intensity of the Q bands in HbO_2. The fact that these peaks have not fully disappeared, and that the small new peaks expected at 500 and 630 nm are not clearly observed is most likely due to an incomplete reaction in which the Q bands in metHb are not yet large enough to be observed. The spectrum of the DMTS treated blood is almost identical to that of the $NaNO_2$ treated blood. Given that $NaNO_2$ is a known MetHb former, and the strong resemblance of the spectra obtained from DMTS and $NaNO_2$ treated blood, these UV-vis absorption experiments provide strong initial support for the hypothesis that DMTS degradation in blood is accompanied by the oxidation of Hb to MetHb.

A3 Probing the DMTS-to-Hb reaction ratio

In this experiment, UV-vis spectra were gathered from a sample of diluted blood between successive injections of DMTS, that were used to incrementally increase the DMTS to Hb ratio from 0 to 1 to 16 to 1. There was a waiting period between each DMTS addition to allow the Soret peak wavelength to stop shifting. The spectra collected at the end of each waiting period, with stabilized Soret peaks, were stored. The goal of the experiment was to obtain a preliminary estimate of the stoichiometric ratios of DMTS and Hb in the reaction. In carrying out this reaction for this purpose, the implicit assumption

was that the reaction was fast enough to be largely completed within a time frame of each waiting period.

After addition of DMTS, the solution became cloudier as the experiment proceeded. This was hypothesized to be due to the partial aggregation and degradation of Hb. The blood sample was diluted by DI water to lyse the blood cells. The DI water was likely not pH neutral, and since the isoelectric point (pI) of most proteins[54] is in the pH range of 4-6, it is likely that the charge repulsion between Hb was decreased due to an inadvertently lowered pH. A buffered solution would likely have aided in preventing aggregation.

The spectra that were collected following each addition of DMTS are shown in Figure 21.

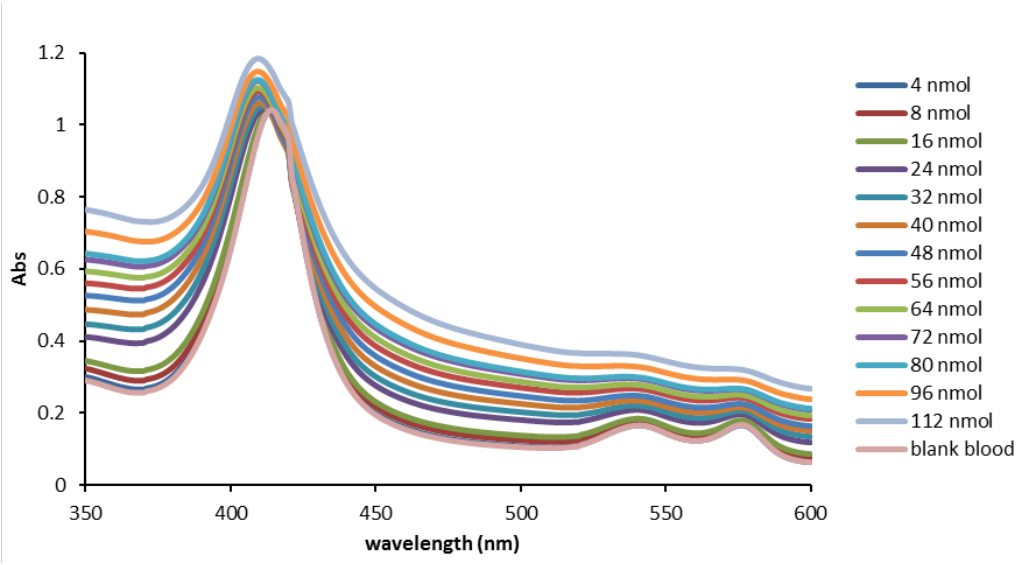


Figure 21. Absorption spectra of successive injection of DMTS in blood. The cumulative amount of DMTS added increases from 0 to 112 nmol with successive additions of DMTS. The amount of Hb in the sample was extimated to be 7 nmol (heme irons 28 nmol).

In this experiment, the Hb and the other blood cell components precipitation results in an increasing baseline due to Rayleigh scatter, and decreased Hb peak areas due to the loss of Hb. As expected for the formation of metHb, the Q band intensities at 541

nm and 576 nm decrease, and the Soret peak shifts from 414 to 409 nm as DMTS is added to the sample. The characteristic peaks of metHb in Q band did not fully appear. Stabilized peak wavelengths of the Soret band were extracted from the spectra shown in Figure 21, and plotted as a function of the cumulative amount of DMTS added to the diluted blood sample (Figure 22).

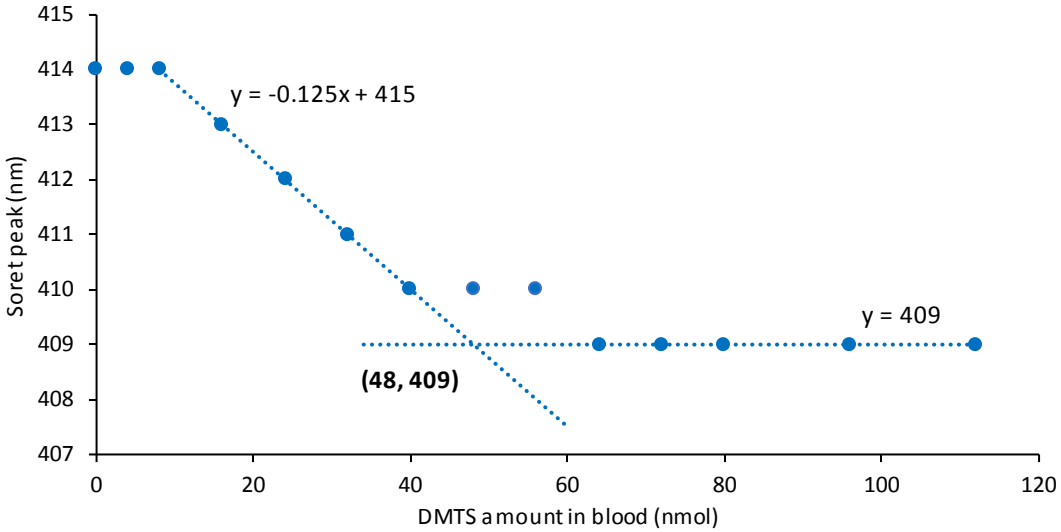


Figure 22. The Soret band shifts with increasing DMTS in blood.

It took around 4 h from the first injection of DMTS to the last injection. This was an early experiment with several challenges. First, the Hb was precipitating over the course of the reaction. Second, the criteria for stabilization of the Soret peak wavelength depended on an observation period of a few minutes. From later experiments of isolated Hb with DMTS, this period may not have been long enough for the complete reaction to occur. Nonetheless, the data is striking. The first two DMTS additions do not give rise to a blue shift of the Soret peak. This is likely due to slow reaction rates associated with the low DMTS concentrations present at that point. Additions 3-7 are accompanied by a linear shift

of the Soret peak to lower wavelengths. The anomalous break from the linear pattern that accompanies additions 8 and 9 is due to an instrumental limitation: the peak positions were obtained directly from the spectra, and in these scans intensities were only recorded at integer wavelength values. Following all subsequent additions (10-14) the Soret peak wavelength is stable at 409 nm. This data suggests that the reaction between DMTS and Hb ended between the 6^{th} and the 9^{th} addition of DMTS. To obtain a more quantitative estimate of the endpoint, the shifting and stabilized points in the curve were each independently fit to lines. The intersection of these lines indicates that the reaction ended when 48 nmol of DMTS had been added to the estimated 7 nmol of Hb (~28 nmol heme iron), suggesting a 1.7 to 1 reaction ratio between DMTS and heme iron. If one accounts for natural conversion of Hb to metHb that occurs upon blood storage, it is likely that the 7 nmol estimate of Hb is slightly high, and that the actual reaction ratio is closer to 2 to 1. In future experiments this was controlled by the use of unspiked control samples.

This result contradicts our initial predictions that DMTS would react with heme Fe(II) in either a 1 to 2, or 1 to 4 ratio. These predictions were based on the hypothesized reactions shown below.

$$CH_3SSSCH_3 + 2Fe^{2+}(HbO_2) + 2H^+ \rightarrow 2Fe^{3+}\,(\text{metHb}) + CH_3SSH + CH_3SH$$

or

$$CH_3SSSCH_3 + 4Fe^{2+}\,(HbO_2) + 4H^+ \rightarrow 4Fe^{3+}(\text{metHb}) + 2CH_3SH + H_2S$$

This contradiction is consistent with several interpretations: (a) the present analysis is correct and the initial hypothesis is wrong; (b) the analysis of the present experiment is faulty and the initial hypothesis is correct; or (c) that both the present analysis and the initial

hypothesis are faulty.

What factors might lead the present analysis to be correct, and the initial hypothesis to be wrong? It may be that DMTS oxidation of Hb is a multistep reaction. DMTS may bind to another component in blood cell, such as albumin or other enzyme first, then either portions of DMTS react with Hb or the degradation products of DMTS in blood reacts with Hb. This could lead to a more complex reaction sequence involving other intermediates in which the stoichiometry of DMTS to heme Fe(II) is in fact 2:1.

What factors might lead the present analysis to be faulty? If these measurements were carried out too quickly so that the reaction was still far from completion when the subsequent addition was made, it could lead to an overestimate of the DMTS heme Fe(II) reaction ratio. The possibility that DMTS was lost during the experiment due to evaporation was discarded because the boiling point of DMTS (170 ^{o}C) is higher than that of ethanol (78 ^{o}C), and thus evaporation is not expected to provide a viable loss mechanism for DMTS.

The results of this experiment suggest that DMTS partially oxidizes Hb in blood in around 4 h at an 8:1 ratio to Hb (2:1 to heme), contradicting our initial hypothesis of a simple reaction involving only DMTS and Hb. This experiment was complicated by Hb and the other blood cell components precipitation.

A4 Comparison of analyte (DMTS) and internal standard (DBDS) reactivity in blood

The kinetics of the reaction between DMTS and Hb in 26-day old sheep blood blood was studied by gathering SPME samples above the reaction mixture at 0, 5 and 30 min time points of the reaction. These SPME samples were analyzed by GCMS, and the resultant chromatograms are shown in Figure 23a.

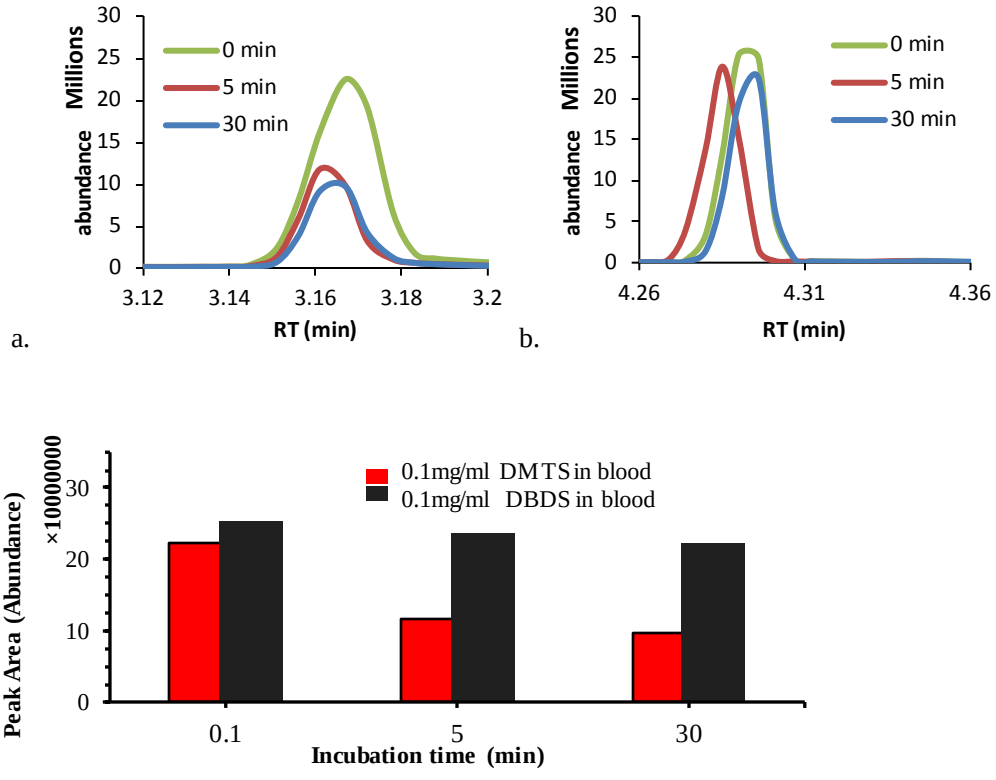


Figure 23. Comparison of DMTS and DMDS losses in spiked blood. DMTS concentration was 12 mM, Hb concentration was estimated to be 0.24 mM, the reaction ratio was 50:1. (a) DMTS peak and (b) DBDS peak in blood sample with different DMTS incubation time. (c) The peak area changes in DMTS peak and DBDS peak against time.

The absolute DMTS peak area ratio in chromatograms of samples with incubation time of 0, 5, and 30 min was 2.3:1.2:1, and the DBDS peak area ratio was 1.1:1.1:1, respectively. The internal standard-corrected DMTS peak area ratio was 2.1:1.1:1. DMTS signal from 5 min and 30 min incubation time samples were about two times lower than that of the 0 min incubation time sample, and there is only a small decreasing in both DMTS and DBDS peak area after 5 min. On the other hand, the peak of internal standard, DBDS, did not change much with incubation time. The results support that the DMTS reacts with blood, most likely with Hb. If the observation that Hb conversion to metHb is most rapid

in the first 5 min, proves reproducible, it might be explained in a number of ways: that the reaction is inherently fast; that the presence of an intermediate speeds up the reaction between DMTS and Hb, and that the rate decreases as this intermediate is used up; or that there is some sort of cooperative reaction effect, where the oxidation of one or more hemes, leads to structural changes in Hb that slow down subsequent oxidations. These are all potential areas for future studies.

A5 Kinetics of blood Hb oxidation in the presence of DMTS

In this experiment, the conversion of Hb to metHb in a dilute blood sample was monitored with UV-vis absorption spectroscopy as a function of time for six days following a single DMTS injection. Non-parameterized fitting of the absorption spectra was employed to determine the relative contributions of Hb and metHb to each spectrum. Parameterized fitting was employed to extract the Soret peak shift over the 6-day course of the experiment.

The first four-hour experiment with Ocean Optics spectrometer. The spectra collected over the first 4 h of the experiment are shown in Figure 24.

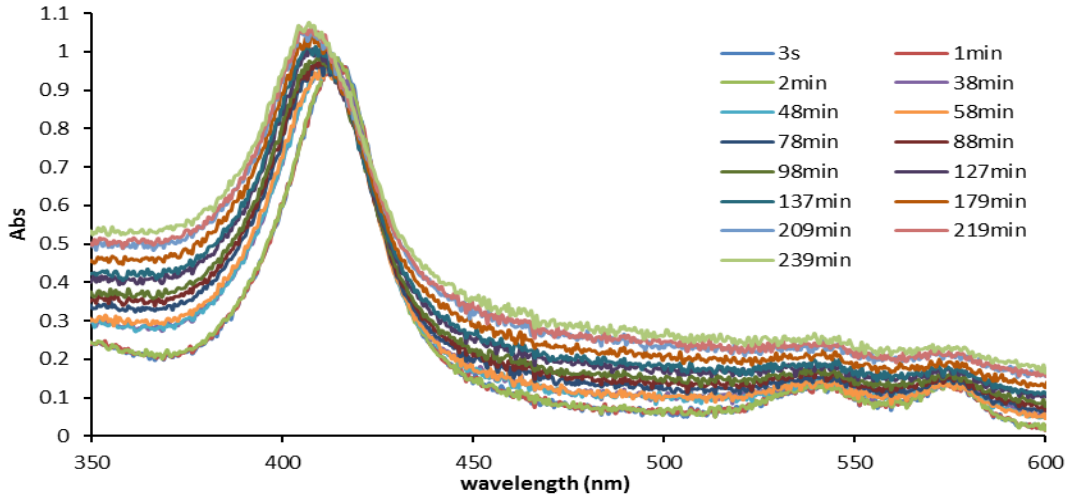


Figure 24. Absorption spectral changes of DMTS spiked blood with time. The Hb in blood was estimated to be 2.7 µM, DMTS was 39.6 µM, the molar ratio of DMTS to Hb is 14.7:1 The 4 h spectra shown in this figure were collected with the Ocean Optics UV-vis spectrometer.

As observed previously, the Soret peak blue-shifts, and the Q bands diminish as Hb is converted to metHb. The precipitation problem persists in this experiment, and is seen in the rising baseline and decreasing area of the Soret peak with time.

Non-parameterized fitting. The first analysis goal was to extract the fraction of Hb and metHb present in the solution at the different time points following the addition of DMTS to the blood. To extract this information, we assumed that HbO_2 and metHb were the only two species contributing to the spectra. We then further assumed that the first spectrum collected was a good representation of a HbO_2 spectrum, and that the last spectrum collected was a good approximation of a metHb spectrum. All the intermediate spectra S_n were then fit to a linear combination of the first (S_{HbO2}) and last (S_{metHb}) spectra as shown in Equation 20.

$$S_{n,fit} = m_1 * S_{HbO_2} + m_2 * S_{metHb} \qquad \text{Equation 20}$$

Where $m_1 = 1$, $m_2 = 0$ in the first spectrum, and $m_1 = 0$, $m_2 = 1$ in the last spectrum. The fits were found by changing the m_1 and m_2 values to minimize the sum of the squares of the deviations between the absorbances predicted by the fit, and the absorbances recorded in the spectrum being fit. The data were processed in Excel with the help of Solver add-in. The basic set-up of the Excel worksheet is shown in Table 12. The fitting quality was expressed by calculating the percent relative deviations between model and fit at each wavelength (Equation 21).

$$rel.dev.\% = \frac{\mathrm{A}_{model} - A_{raw}}{A_{raw}} * 100\% \qquad \text{Equation 21}$$

Table 12

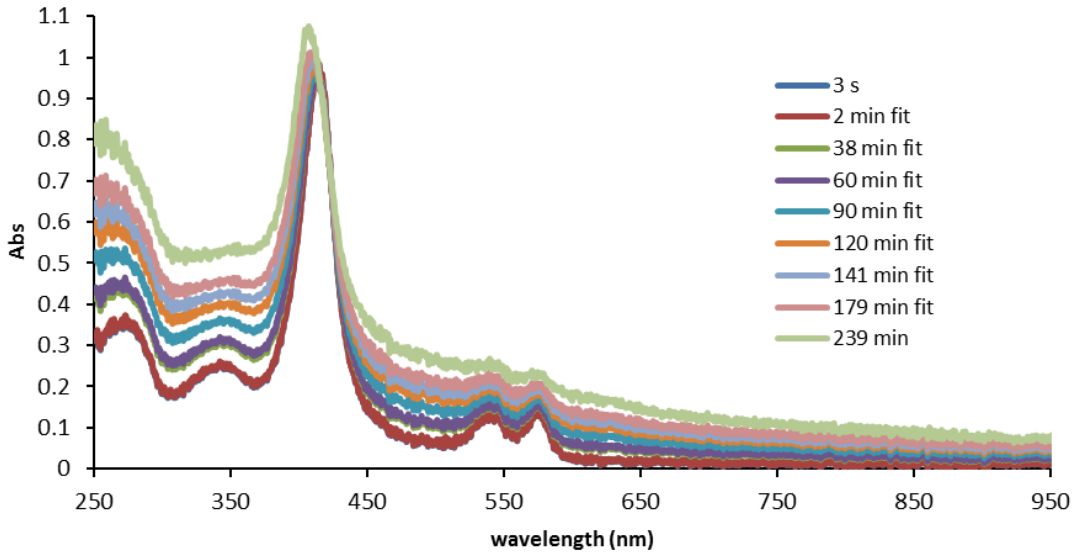
Summary of the non-parameterized fit worksheet for DMTS-blood experiment

Wavelength (nm)	HbO_2 Reference Spectrum (3s)	MetHb Reference Spectrum (239 min)	Intermediate Spectrum being fit	Fit Spectrum	Residuals	Dev. Sq.	Dev. Sq. sum	Fit parameters
250.39	0.321	0.791	0.326	0.325	0.33%	1.176E-6	0.064	m_1=0.9902
250.87	0.32	0.822	0.307	0.324	-5.60%	2.959E4		m_2=0.0089
⋮	⋮	⋮	⋮	⋮	⋮	⋮		
950.92	0.014	0.082	0.016	0.014	8.78%	1.975E-6		

Note: $Fit = m_1 * HbO_2 + m_2 * metHb, residuals = \frac{raw-fit}{raw} * 100\%, dev.sq. = (fit - raw)^2$

The fitting results and quality are shown in Figure 25.

a.



b.

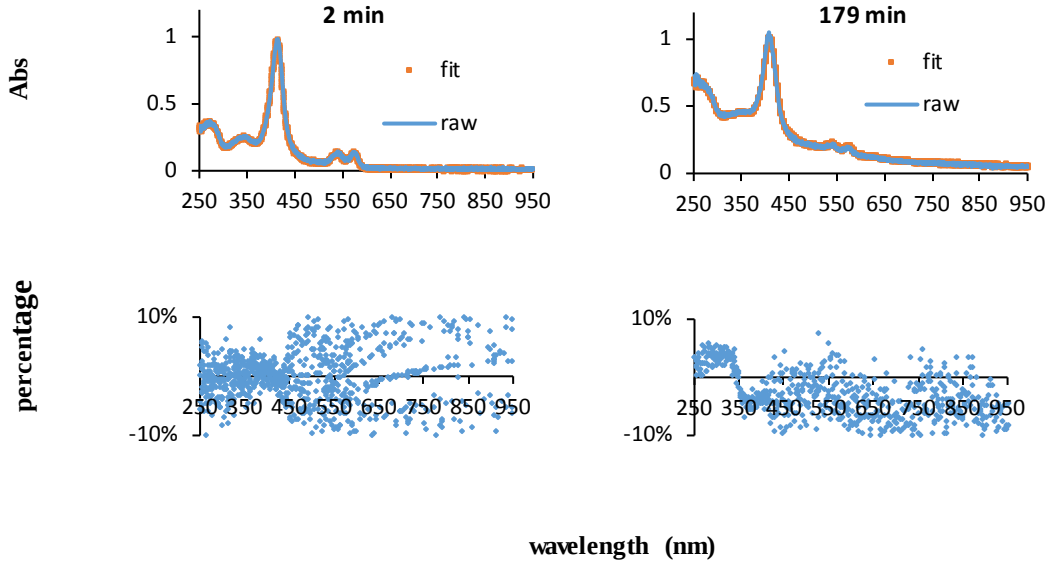


Figure 25. Non-parameterized fits to spectra from Hb-metHb mixtures. (a) Representative examples of the small but systematic blue shift of the Soret peak with time following the addition of DMTS to the fresh blood. (b) Representative fits to linear combinations of a metHb, and a Hb spectrum with residuals.

The fitting constant values m_1 and m_2 were interpreted as representing, respectively, the fractions of HbO_2 and MetHb present in the reaction mixture at the time that the spectrum was collected. Representative fitting constant values are shown in Table 13, and a plot of these constants that shows how the concentrations of HbO_2 and MetHb change with time is shown in Figure 26.

Table 13

HbO_2 and MetHb percentage in DMTS treated fresh blood with time

time (min)	m_1 (first spectrum)	m_2 (last spectrum)	sum (m_1+m_2)
2	0.990	0.009	0.999
38	0.778	0.209	0.987
60	0.740	0.242	0.982
90	0.574	0.409	0.982
120	0.433	0.551	0.983
141	0.350	0.640	0.990
179	0.250	0.745	0.995

Note: m_1 and m_2 are fitting parameters of the fraction of HbO_2 and metHb

The fitting residuals were generally less than 10% of the average signal levels, and were relatively randomly distributed. This observation, along with the fact that the sum of fitting fractions (m_1+m_2) shown in the last column of Table 13 were close to 1 gives confidence in the assumption that the observed changes in the spectra can be well described by two absorbers: HbO_2 and metHb. Two factors which may contribute to the small

systematic deviations in the residuals and to the subtle deviations of the fitting factor sum (m_1+m_2) from 1 are: (1) differences between the Hb precipitation rate that governs the growth in the background, and the DMTS Hb reaction rate which governs the spectral changes in the peaks; and (2) the fact that the first and last spectra used to represent HbO_2 and metHb had high proportions of those forms of Hb, but were not fully pure.

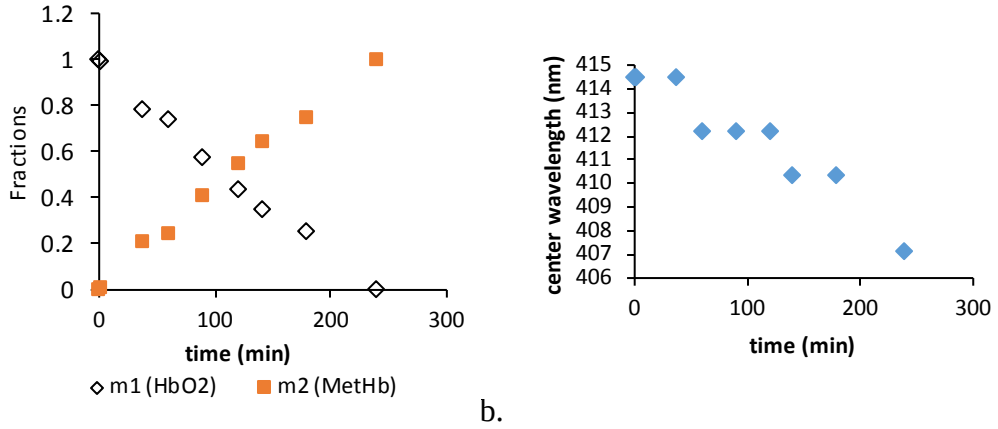


Figure 26. The metHb forming with time in DMTS spiked blood. (a) MetHb fraction growing and HbO_2 decreasing with time. (b) The Soret peak shifting with non-parameterized fits.

The data in Figure 26a indicates that the rate of metHb formation is nearly constant over the first 4 h of this experiment. The data in Figure 26b showing the wavelength shift of the Soret peak in this experiment out to six days, suggests that the rate is changing with time, but that the rate changes within this initial short 4 h time interval were small enough to be hidden in the noise. The under-sampling problem due to the choice of a 1nm step size is again apparent in the stepwise changing of the wavelength.

Parameterized fitting. The most obvious approach to overcoming under-sampling problem would be to repeat the experiment using a smaller step size between individual absorbance measurements. A second approach, which we used here, is to realize that even

though the wavelength of maximum absorbance may not be changing over a set of scans, the underlying peak is clearly shifting. By fitting the entire Soret peak with a Lorentzian model, we can obtain a much finer estimate of the peak wavelength in each spectrum.

To extract finer estimates of the peak wavelength in each spectrum, the background in each scan was fit to a third order polynomial (Equation 22).

$$A_{background}(\lambda) = a(\lambda-\lambda_0)^3 + b(\lambda-\lambda_0)^2 + c(\lambda-\lambda_0)^1 + d \quad \text{Equation 22}$$

This background was subtracted from the spectrum being fit. Then, the background-subtracted spectrum was fit as a sum of one Lorentzian, and four Gaussian peaks centered respectively near 414, 260, 320, 541, and 576 nm. The Soret peak was fitted with Lorentz equation shown in Equation 23.

$$A(\lambda) = H * \frac{\gamma^2}{\gamma^2+(\lambda-\lambda_0)^2} \quad \text{Equation 23}$$

A = absorbance, H = peak height, γ = standard deviation, λ_0 = center wavelength, Peak area was calculated by integrating Equation 23:

$$\int A(\lambda) = \int_{-\infty}^{+\infty} H * \frac{\gamma^2}{\gamma^2+(\lambda-\lambda_0)2} d\lambda \quad \text{Equation 24}$$

$$\text{Area} = H * \gamma * \pi \quad \text{Equation 25}$$

The remaining four peaks were fit with Gaussian equations:

$$A(\lambda) = H * e^{-\frac{1}{2}\left(\frac{\lambda-\lambda_0}{s}\right)^2} \quad \text{Equation 26}$$

Peak area was calculated by integrating the equation:

$$\int A(\mathrm{x}) = \int_{-\infty}^{+\infty} \mathrm{H} * e^{-\frac{1}{2}\left(\frac{\lambda-\lambda_0}{s}\right)^2} d\lambda \qquad \text{Equation 27}$$

$$\text{Area} = \frac{H * \lambda_0}{2\sqrt{2ln2} * 2\pi} = \frac{H * \lambda_0}{0.94} \qquad \text{Equation 28}$$

The overall fit model was

$$\frac{H_{Soret} \square^2}{\square^2 + (\lambda - \lambda_{\mathrm{Soret}})^2} + \mathrm{H}_{P1} * \mathrm{e}^{-\frac{1}{2}\left(\frac{\lambda-\lambda_{P1}}{s_{p1}}\right)^2} + \mathrm{H}_{P2} * \mathrm{e}^{-\frac{1}{2}\left(\frac{\lambda-\lambda_{P2}}{s_{p2}}\right)^2} + \mathrm{H}_{P3} * \mathrm{e}^{-\frac{1}{2}\left(\frac{\lambda-\lambda_{P3}}{s_{p3}}\right)^2} + \mathrm{H}_{P4} * \mathrm{e}^{-\frac{1}{2}\left(\frac{\lambda-\lambda_{P4}}{s_{p4}}\right)^2} + background \qquad \text{Equation 29}$$

The best fits for each background spectrum were found by changing the peak heights (H_{Soret}, H_{p1}, H_{p2}, H_{p3}, H_{p4}), peak center wavelengths (λ_{Soret}, λ_{p1}, λ_{p2}, λ_{p3}, λ_{p4}), and peak widths (Y_{Soret}, Ys_{p1}, Ys_{p2}, Ys_{p3}, Ys_{p4}) using the Microsoft Excel Solver add-in. The background fits were done by manually changing variables a, b, c, and d. Samples of individual peak, and background-subtracted spectrum fits are shown respectively, in Figure 27 and Figure 28.

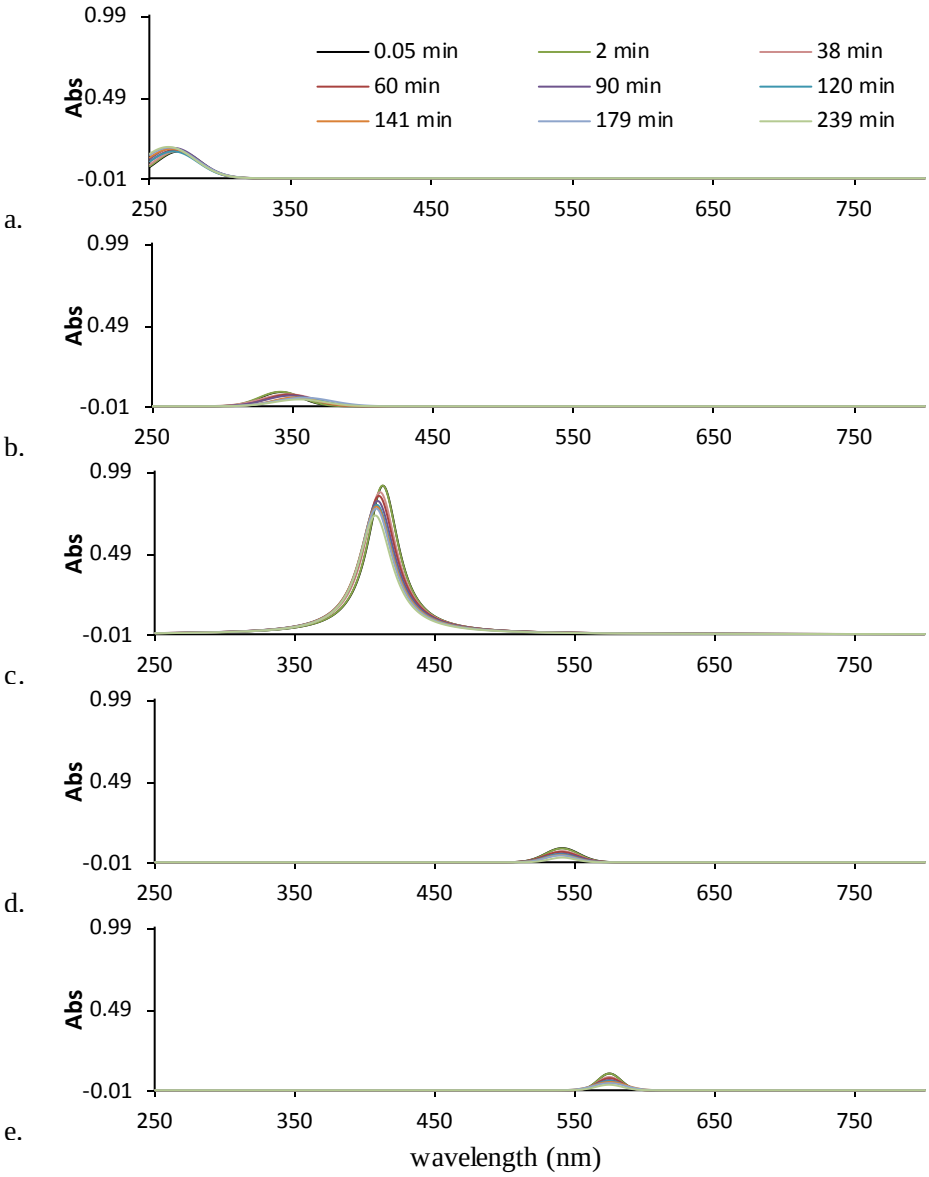


Figure 27. Parameterized fits to individual peaks from spectra of DMTS/blood. The spectra were collected for 4 h. (a)-(e) The parameterized fitted peaks at 260 nm, 340 nm, 414 nm, 541 nm, and 576 nm, respectively.

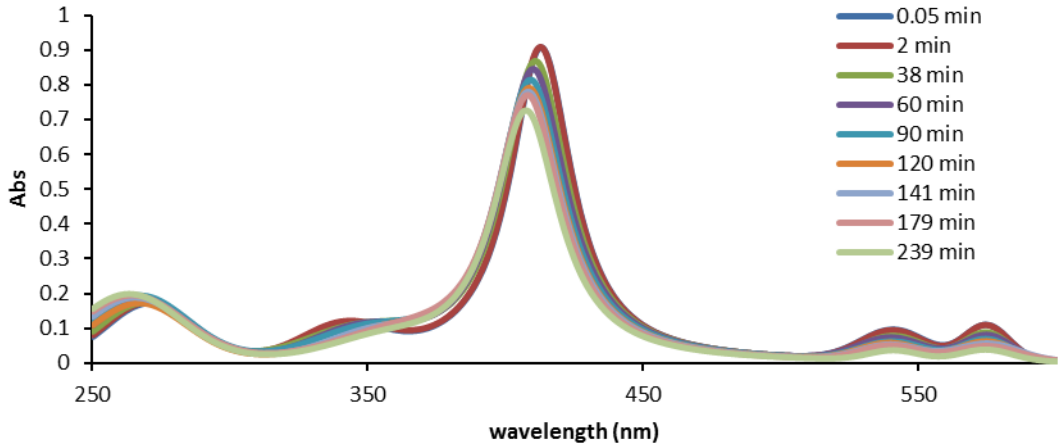


Figure 28. Parameterized-fittings whole spectra of DMTS/blood. The spectra were collected for 4 h.

The fitting quality examples are shown in Figure 29.

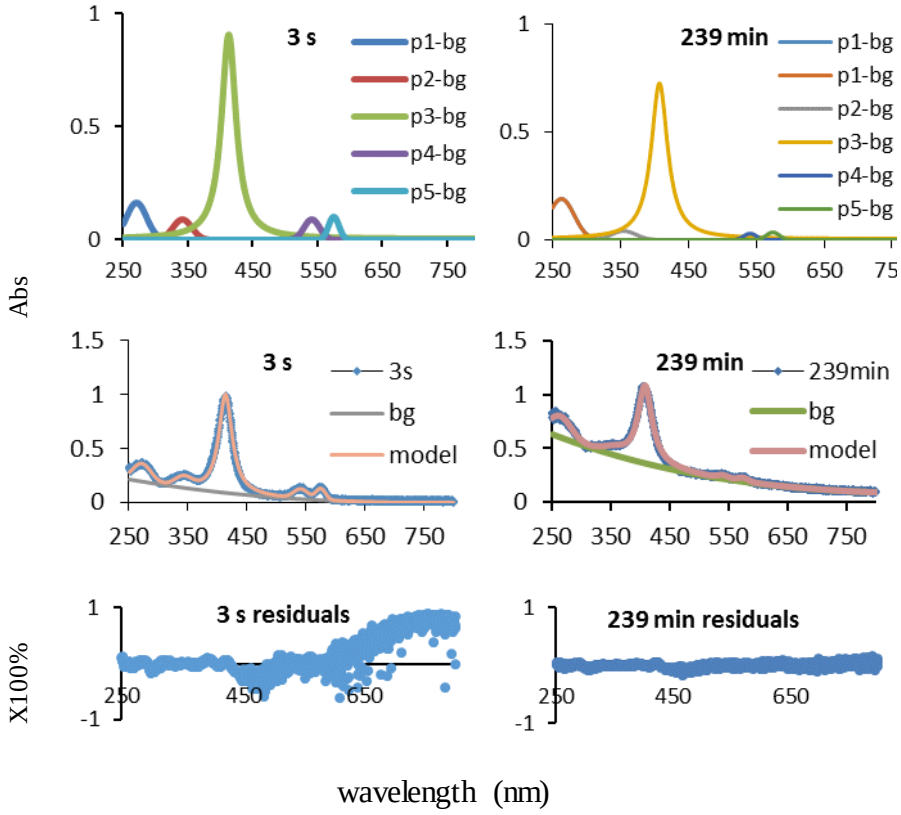


Figure 29. Parameterized fits quality to DMTS/blood reaction spectra.

Therefore, the center wavelength and peak area of each peak in interest (414 nm, 541 nm and 576 nm) can be found (Figure 30).

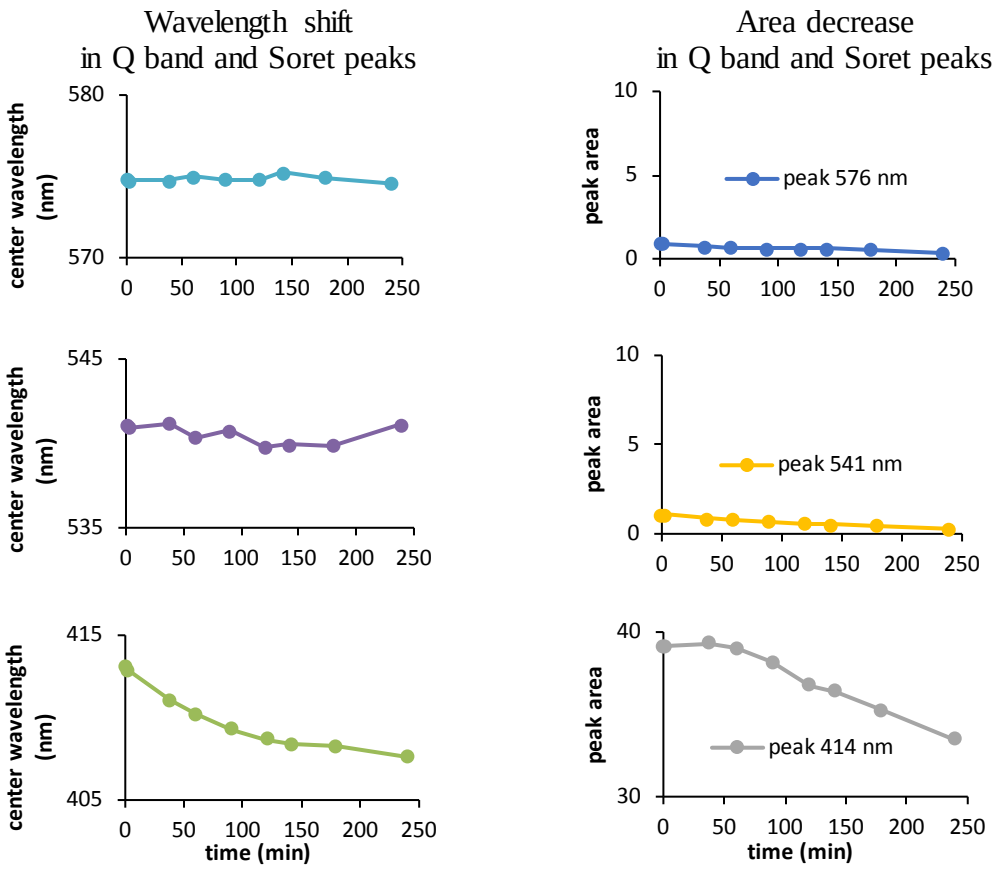


Figure 30. Peak shifts and peak area decreases of DMTS/Hb in 4 h.

The center wavelength of the Soret band shifts from 413.5 to 407 nm, while the center wavelengths of the Q bands remain the same, all of which is consistent with expectations for Hb oxidation. The peak area decreases by 14.3% in Soret band, and 74.4% and 61.5% in the two peaks in Q band. The decrease in Soret band peak area is due to the loss of hemoglobin by precipitation. The loss of peak area in the Q bands is assumed to be due to a combination of precipitation (14%) and metHb formation (48-60%) effects.

The continued 6-day monitoring with Jasco V 750. At the end of the first 4 h of reaction, the Soret peak wavelength was observed to remain stable at 408 nm for 30 min. At this point in the experiment, all samples were capped and stored overnight at room temperature. Subsequently, five additional UV-vis spectra were taken at the 14, 21.5, 48, 72 and 147 h marks with the Jasco UV-vis absorption spectrometer, and these are shown in Figure 31. Photographs of the same lysed blood solution taken at different times following the initial DMTS spike are shown in Figure 32.

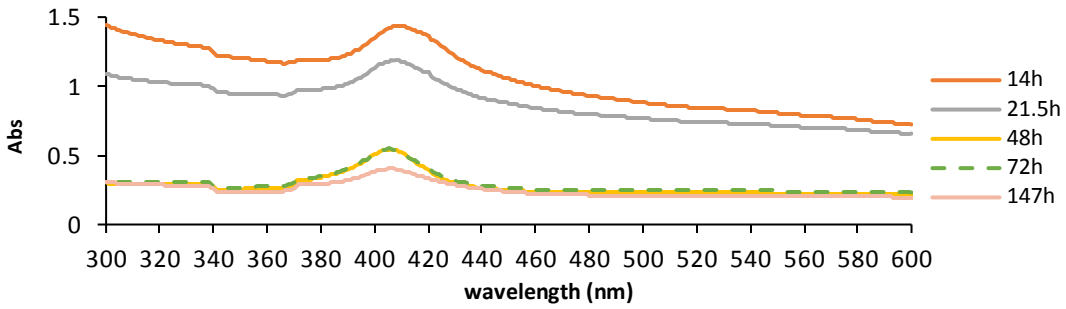


Figure 31. Raw absorption spectra of DMTS/blood reaction in 6 days. These scans were taken with the Jasco UV-vis spectrometer.

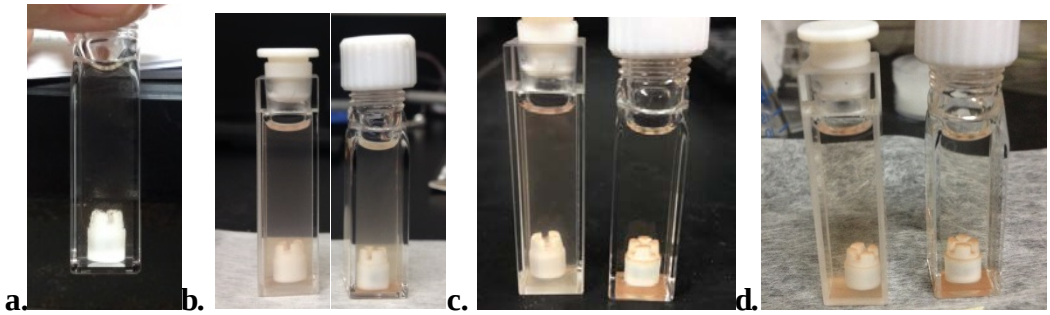
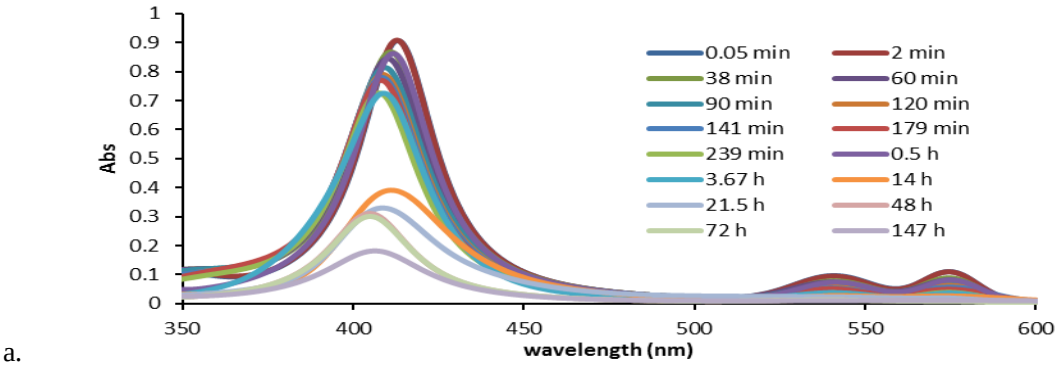


Figure 32. Photographs of blood solutions at different times post DMTS spike. a. DMTS/blood reaction mixture at 2.75 h. In b, c, and d, controls at 16, 44.5, and 68.5 h are shown on the left, and reaction mixtures at 19.5, 48, and 72 h are shown on the right.

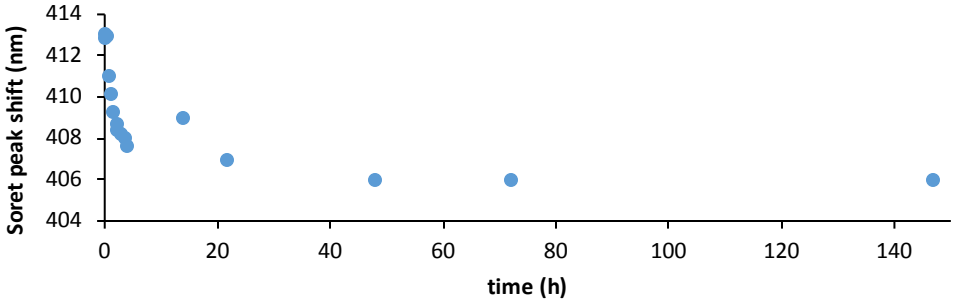
The DMTS treated sample became cloudy over the course of the first day and particles in solution is growing and aggregating, finally sediment to the bottom after 48 h

(Figure 32). Therefore, the background increased in the first 4 h and began to decrease from 14 h after the injection until the background decreased to almost the same level with the first spectrum. After the precipitate had sedimented, the particles on the bottom of the cuvette were red and the supernatant was clear, colorless and transparent. These observations suggest that the precipitate contained some Hb from solution and explain the peak area decrease observed in the Soret band. It is likely that if the solvent had been buffered to an appropriate pH, if additional heparin had been added with each dilution step, and if no ethanol had been used in the initial preparation of the DMTS, that this sedimenting problem would have been less severe. Similar gradual sedimenting of Hb was also seen in the control experiment.

Parameterized fitting. A subset of the spectra taken from the DMTS treated sample over the 6-day time course were fitted with Gaussian and Lorentzian equations and are plotted along with the fits from the first 4 h in Figure 33. Representative examples of fits and residuals are shown in Figure 34.



a.



b.

Figure 33. Parameterized-fittings of DMTS in blood spectra in 6 days. (a) Peak fitting of DMTS /blood in 6 days (with Ocean Optics and Jasco spectrometer). (b) Soret peak shift in 6 days.

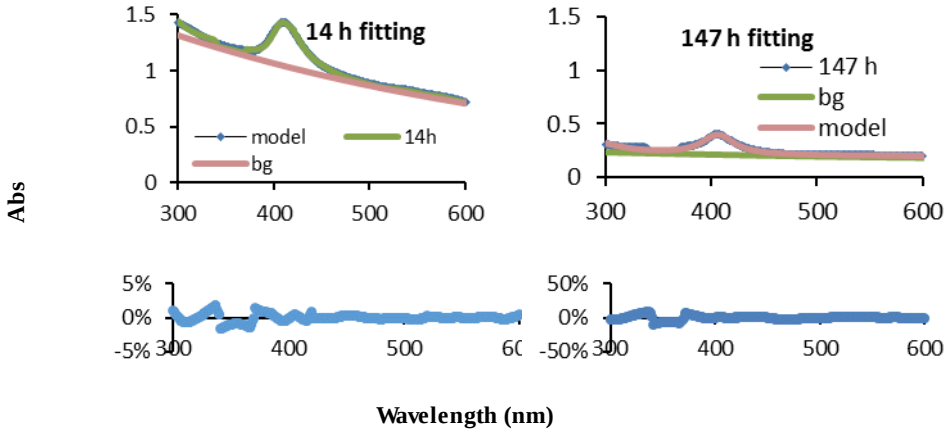


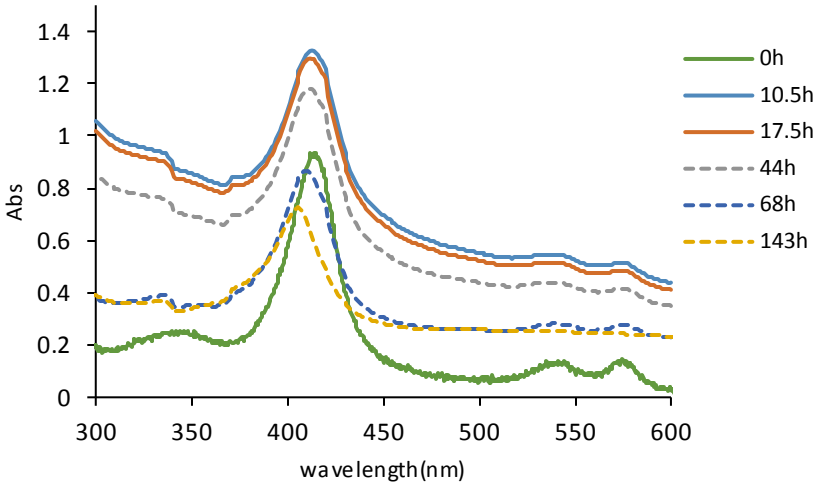
Figure 34. Qualities of parameterized fits to DMTS/blood reaction in 6 days.

The peaks area and peak height are not able to calculate since the peak lost with time and effect the total fittings quality especially after 4 h due to the complex precipitation problem, however the center wavelength shifting of the Soret peak in 6 days are found in Figure 33. In DMTS treated sample, the Soret peak shifted to 409 nm in the first 4 h, and continued shifting to 405 nm within the following 6 days.

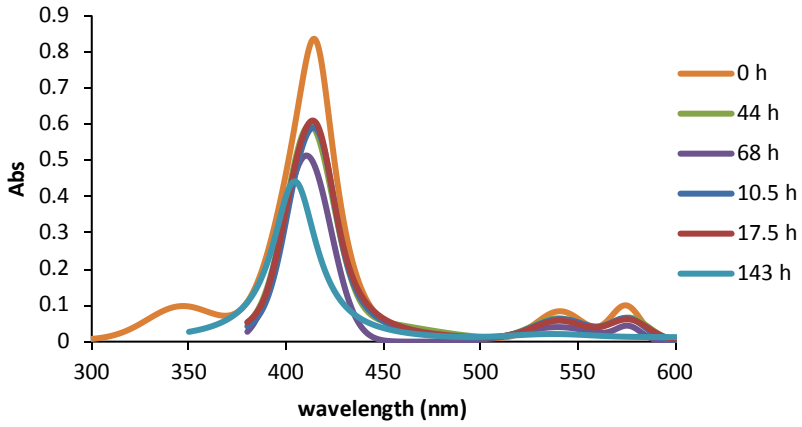
The reaction rate has a steep slope in the first 4 h and a gradual slope after 4 h. It was hypothesized that most of the DMTS and Hb was used up in the first 4 h and the concentration of both DMTS and Hb was low in the solution, therefore it took more time for the reaction to proceed.

Kinetics of blood Hb oxidation in the absence of DMTS (Control)

Spectral changes in blood were followed with UV-vis absorption spectroscopy as a function of time in the absence of DMTS. UV-vis absorption spectra were collected from the unspiked sample each day for 6 days. The data were fitted with parameterized fitting and are shown in Figure 35.



a.



b.

Figure 35. Parameterized fits to the absorption spectra of blood control. (a) Raw spectra of blood control solution in 6 days. (b) Parameterized fitted absorption spectra of blood control.

According to Figure 35a, the baseline increased until 10.5 h after preparation due to the precipitation, and then decreased to the level of that in the first spectrum. The Soret peak in the control experiment was also blue-shifted, indicating that the blood is also oxidized in the presence of air. The peak area of all peaks in both DMTS with blood and control experiment decreased with time. The Q bands completely disappeared at the end due to a combination of oxidation and precipitation.

Representative fits of the control spectra are shown in Figure 36.

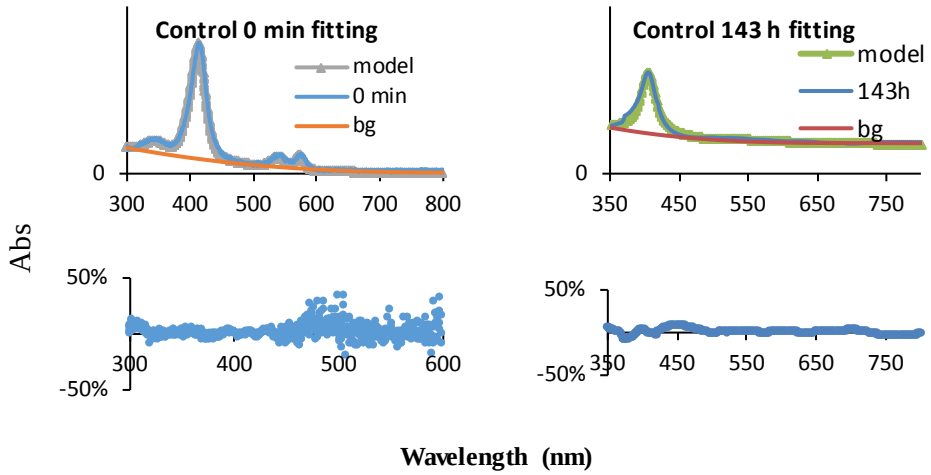


Figure 36. Examples of parameterized fitting quality of control experiment. The "bg" refers background, the model is the fitting model.

Reaction rate were compared between DMTS spiked blood and the blank blood according to the center peak wavelength shifting of Soret band.

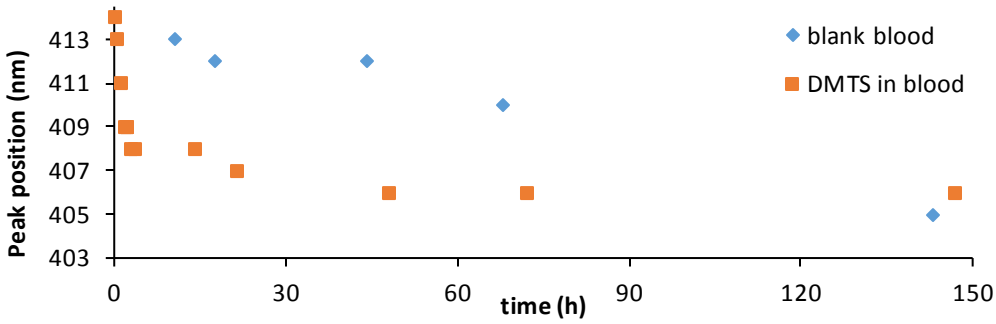


Figure 37. Soret peak shifts in DMTS spiked- and unspiked blood.

The Soret peak in control experiment blue-shifted at a slower rate due to the slow oxidation of Hb in the presence of dissolved air. (Figure 37) The rate of conversion to metHb was much faster in the presence of DMTS than in the blank sample to which no DMTS was added.

A6 Blood with DMTS and $NaNO_2$ *in vivo*

Absorption measurements after injection.

Per Figure 38, the center wavelength of the Soret peak shifting in each sample compared to the blank blood can be found by the highest absorbance near 414 nm. The center wavelength was estimated in Figure 38.

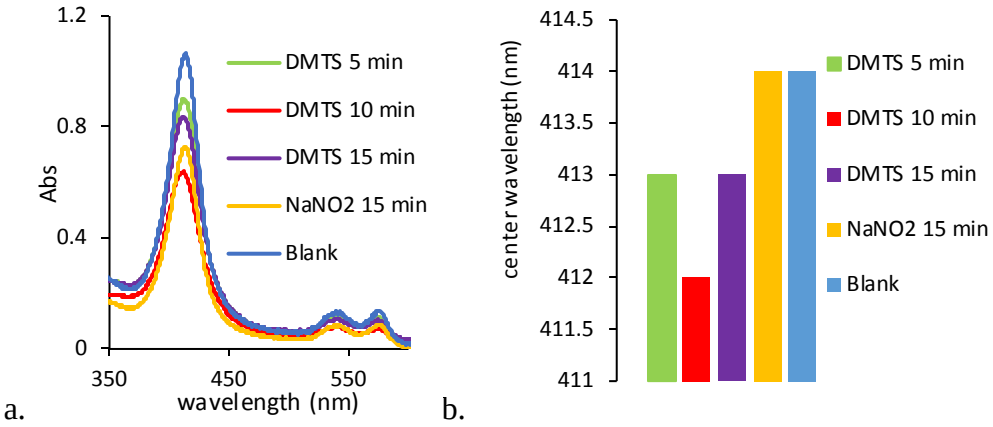


Figure 38. Hb spectra of blood drawn from DMTS and $NaNO_2$ treated mice. (a) Raw spectra of samples collected from mice injected by DMTS (50 mg/mL), $NaNO_2$ (9.4 mg/mL), and the mouse without any injection. The mice treated with DMTS were kept for 5, 10, and 15 min respectively. The blood samples were diluted to 0.125% with DI water. (b) The estimate center wavelength of the Soret peak in each diluted blood sample from rats.

Blood samples after kept in fridge for 18 h and then at room temperature for 4 h, and treated with additional DMTS/$NaNO_2$ in vitro.

The spectral changes following 22 h of storage are shown in Figure 39.

In the DMTS treated incubation time 5 min blood sample, the Soret peak in blank blood shifted 1 nm to 413 nm. After the first addition of DMTS (DMTS:Hb 11:1) after 22 h, the peak centered at 412 nm in the diluted sample after stored for 18 h, shifted 1 nm to 411 nm and stayed at the same position as more DMTS was added (DMTS:Hb 2563:1).

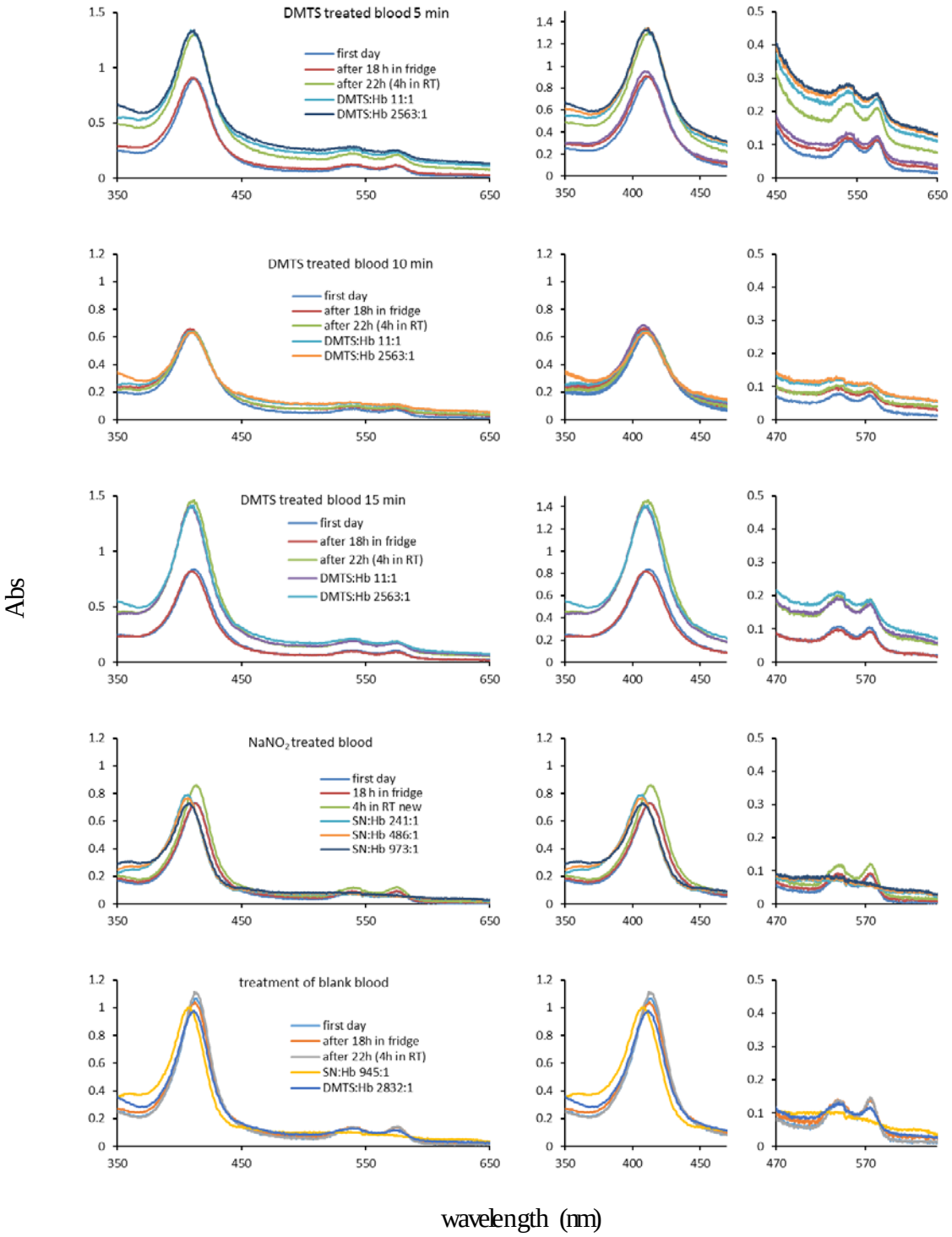


Figure 39. Spectra of blood samples post-treatment with DMTS or $NaNO_2$. Additional DMTS and $NaNO_2$ was added to the same blood sample stored in the fridge for 18 h and in room temperature for 4 h.

The center wavelength was estimated from the plotting. In the DMTS treated incubation time 10 min blood sample, the Soret peak in blank blood shifted 2 nm to 412 nm. After the first addition of DMTS (DMTS:Hb 11:1) after 22 h, the peak centered at 411 nm in the diluted sample after kept for 18 h, shifted 2 nm to 409 nm and stayed at the same position after more DMTS was added (DMTS:Hb 2563:1). In the DMTS treated incubation time 15 min blood sample, the Soret peak in blank blood shifted 2 nm to 412 nm. The peak shifting after addition of DMTS to each sample after kept 22 h is almost consisted with the shifting after injection of DMTS *in vivo* comparing to the peak position in blank blood.

The $NaNO_2$ treated blood sample from the rat did not shown any significant spectral changes indicating that $NaNO_2$ did not have much effect on HbO_2 at mole ratio 0.73:1. After further large mole ratio addition of $NaNO_2$ to the diluted blood sample *in vitro*, the Hb was converted to metHb completely (Figure 39).

The blank blood (control experiment) showed a good stability for a period of 22 h. After addition of $NaNO_2$ in a ratio of 945:1 to Hb, the Soret peak shifted from 414 nm to 406 nm, the Hb was converted to metHb immediately and completely. After addition of DMTS in a ratio of 2832 :1 to Hb, even though with larger mole ratio than $NaNO_2$ to Hb, the peak shifting is less, which is from 414 nm to 412 nm.

The first hypothesis is that DMTS reaction with Hb is reversible. DMTS can attach to heme iron and change the oxidation state of iron temporarily, but the bonding is reversible. The second hypothesis is that DMTS reaction of Hb is slow so that even excess addition of DMTS does not make much changes. However, the blood color changed after injection of DMTS *in vivo*, indicating the reaction happened quickly. With additional $NaNO_2$ and DMTS treatment to the diluted blood solution *in vitro*, the $NaNO_2$ can convert

Hb to metHb and the Soret peak in DMTS treated sample shifted. It was hypothesized that DMTS cannot reach the Hb as easily as $NaNO_2$, $NaNO_2$ will be a better metHb former.

In all samples, the diluted blood solution was easier to be oxidized by air than the concentrated blood solution.

B. Segue experiments (Moving from blood to Hb in PBS)

Introduction

To simplify the reaction matrix, and to test whether DMTS was capable of reacting with Hb in the absence of other factors in the blood, a series of experiments examining the direct reaction between DMTS and Hb were proposed. These experiments are discussed in section C of this chapter. These experiments required a source of purified Hb. Because Hb is standardly shipped as a mixture of Hb and metHb, it was necessary to: reduce metHb to Hb with dithionite (DT), isolate the reduced Hb from the DT; and assay the fractions collected from the reduction column for both Hb and residual DT.

Since ethanol is a known precipitation agent for proteins, we wanted to remove it from the experiments. In earlier experiments, ethanol had been used for the initial DMTS stock solutions. As part of the process of shifting to fully aqueous solutions, the solubility of DMTS in PBS was measured.

The results of developmental experiments associated with the reduction of metHb to Hb, and the solubility of DMTS in PBS are discussed in this section.

B1 Determining Hb in the reduced fractions

To determine whether the Hb reduction had been successful, a UV-vis absorption spectrum was taken of each fraction collected from the DT reducing column. The absorption spectrum of one of four Hb fractions collected in Eppendorf tubes following reduction by DT is shown in Figure 40.

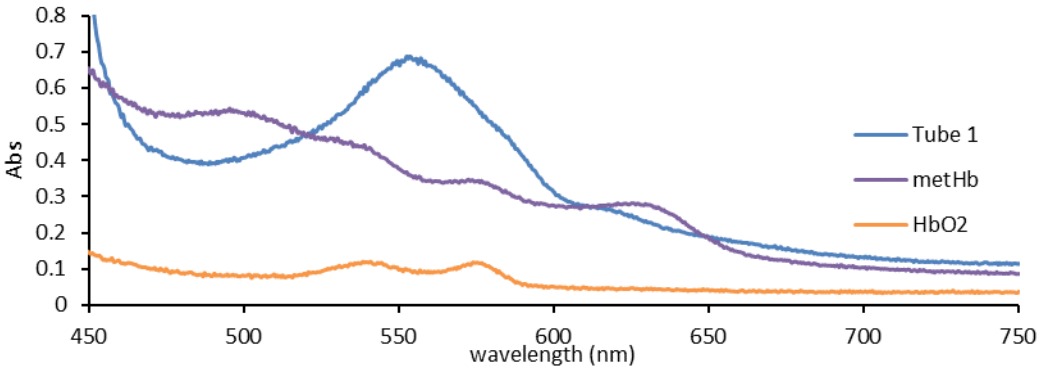


Figure 40. Absorption spectra showing the successful reduction of metHb. The spectrum collected from a Hb fraction taken directly after collection from PD10 reducing column. The characteristic peak at 554 nm is due to deoxyHb. For comparison purposes, metHb and HbO_2 spectra are also shown.

The two dominant features in the spectrum collected the first Eppendorf fraction (Tube 1), are the characteristic deoxyHb peak at 554 nm, and a small shoulder near 576 nm indicating the presence of some HbO_2. MetHb and HbO_2 spectra are shown for comparison purposes. The spectra of all the fractions were similar and the appearance of the 554 nm peak provided convincing evidence of the successful reduction of bovine metHb to deoxyHb. Based on the absorbance at 554 nm from each of these four spectra, and the molar absorptivity of deoxyHb ($\varepsilon_{554\ nm}$=13250 $M^{-1}cm^{-1}$), the deoxyHb concentrations in the four reduced fractions were calculated to be 0.206, 0.545, 0.488, and 0.141 mM respectively.

Once it was ascertained that the reduction had been successful, centrifugal

ultrafiltration was employed with the aim of removing any DT that had leaked from the column into the fractions. The centrifugal runs took ~70 min. After the Hb sample was redispersed following the first run, and then, the deoxyHb had been full oxygenated and converted to HbO_2. Subsequent determinations of HbO_2 concentration were based upon the absorbance at 576 nm, where the molar absorptivity is $\varepsilon_{576\,nm}$=15190 $M^{-1}\,cm^{-1}$.

B2 Ellman's reagent tests for thiols, and for DT in filtrate

Once good fractions of reduced HbO_2 had been obtained, preparations were made to look for the products of the hypothesized reaction with DMTS, and to probe the kinetics of the reaction between DMTS and HbO_2. The hypothesized reactions were given earlier and the first of these is reproduced below.

$$CH_3SSSCH_3 + 2Fe^{2+}(HbO_2) + 2H^+ \rightarrow 2Fe^{3+}\,(\text{metHb}) + CH_3SSH + CH_3SH$$

Ellman's reagent, DTNB, is commonly used to test for thiols. Because thiols are a product of the hypothesized reaction, we initially planned to add Ellman's reagent to reaction mixtures of DMTS and Hb, collect absorbance spectra, and analyze for spectral changes associated with the formation the yellow colored TNB^{2-} anion as a test for the appearance of product thiols.

Working toward this goal, a simple control experiment was run. DTNB was added directly to the reduced HbO_2 solutions following one, and two centrifugal ultrafiltration cycles. Upon addition of DTNB the color of HbO_2 solution turned from red to bright yellow (Figure 41a), indicating a strong reaction in the absence of DMTS.

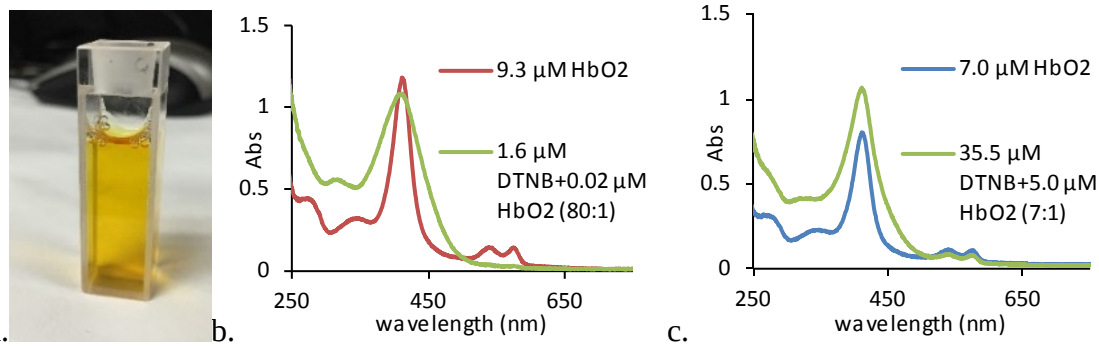


Figure 41. DTNB reaction with Hb. (a) picture of 300 µL 5 mM DTNB mixed with a 2 mL 9.32 µM HbO_2, (b) spectra of the DTNB and HbO_2 mixture mole ratio is 80 to 1, (c) spectra of HbO_2 solution after second centrifugal ultrafiltration run where the concentration is 7.0 µM and DTNB and HbO_2 mixture where DTNB was 35.5 µM and HbO_2 was 4.95 µM. The mole ratio of DTNB to HbO_2 is 7 to 1.

The spectra shown in Figure 41b and c, show the broadening due to the overlap of a new TNB^{2-} peak and the Hb Soret peak, and confirm that one or more of the components in the redispersed HbO_2 solutions was reducing the DTNB. Possible reductants include residual DT, iron(II) in HbO_2, or reducing groups of the globin. A set of related control experiments was carried out as summarized in Table 14. Three trials of each experiment were completed. The resulting spectra are displayed in Figure 42.

Table 14

Observation of DTNB, DMTS and DT control test.

	Trial 1	Trial 2	Trial 3
Step 1	DMTS+DT 1:1	DMTS+DTNB 1:1	DT+DTNB 1:1
Resulting Color	colorless	colorless	yellow
Step 2	DMTS+DT+DTNB 1:1:1	DMTS+DTNB+DT 1:1:1	DT+DTNB+DMTS 1:1:1
Resulting Color	yellow	yellow	yellow

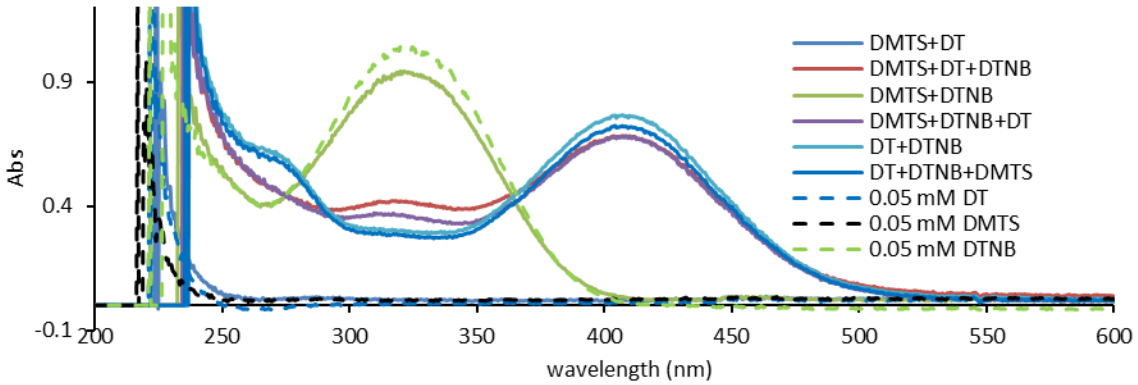


Figure 42. The DTNB control test with DMTS and DT. Final concentrations of all each ingredient in all solutions was 0.045 mM.

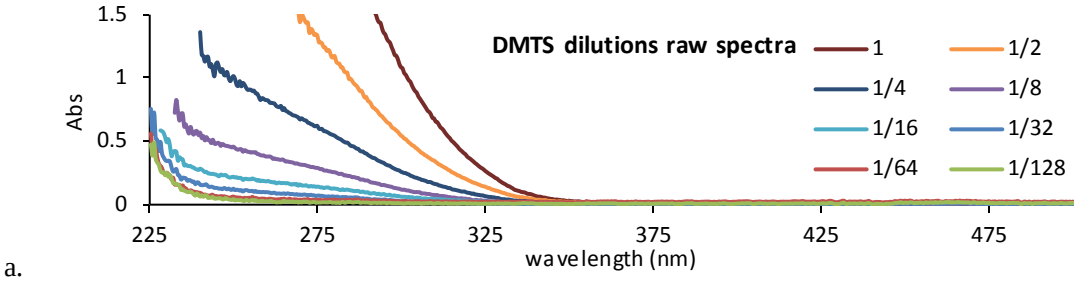
In Figure 42 the spectra from the DMTS/DT and DMTS/DTNB reaction mixtures did not differ appreciably from the sum the component spectra, indicating the absence of a rapid reaction. However, mixing of DTNB with DT always resulted in a visible change of the solutions color to yellow, and the appearance of the strong TNB^{2-} peak at 409 nm in the corresponding spectra. The color, and spectral changes provided convincing evidence that DT was reducing DTNB to produce TNB^{2-}.

Considering these experiments, it was realized that DTNB would provide an effective route for determining the extent of DT contamination following each centrifugal ultrafiltration cycle. Subsequently, instead of adding DTNB to the DMTS/HbO_2 reaction solutions to test for hypothetical product thiols, DTNB was added to the centrifugal ultrafiltration filtrate to monitor DT contamination levels.

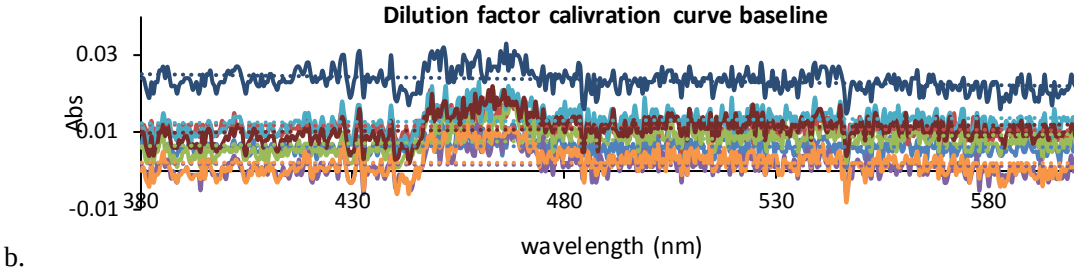
B3 DMTS loading capacity of PBS.

To help us move to fully aqueous DMTS solutions, and to help us determine an upper limit on DMTS concentrations for subsequent kinetics measurements, the saturated concentration of DMTS in PBS was determined using UV-vis spectroscopy. First a saturated solution of DMTS was prepared by mixing excess DMTS with PBS solution. The

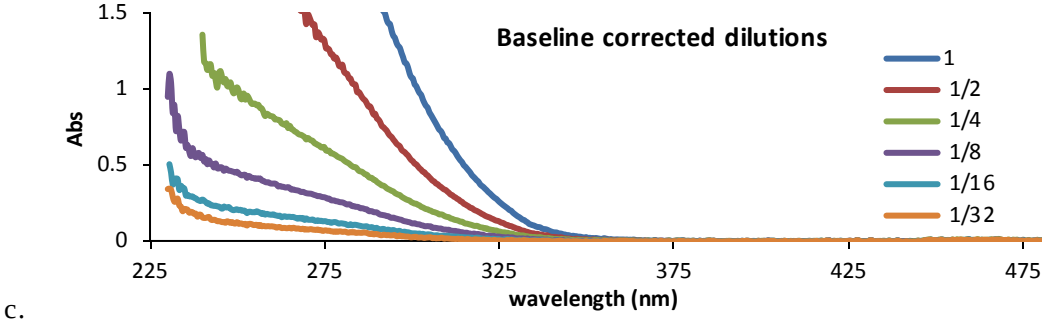
excess DMTS and aqueous layers were separated by centrifugation. Serial dilutions of the saturated aqueous phase were used to prepare solutions with concentrations that were respectively 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/28 the original saturated solution. Absorption spectra were collected from each of these solutions. These spectra are shown in Figure 43a. The absorbance values from 380 to 600 nm in each spectrum were averaged to estimate the small baseline offset present in each spectrum. The baseline- corrected spectra shown in Figure 43c were then calculated by subtraction of this offset.



a.



b.



c.

Figure 43. Absorption spectra of the dilutions from saturated DMTS solution. (a) The raw spectra of dilutions from saturated DMTS solution in PBS. (b)averaged baselines, and (c) baseline corrected spectra.

In these spectra, we see the tail of a UV absorbance peak, which increases systematically with DMTS concentration. Because the peak wavelength was not available, we selected a wavelength of 300.74 nm where the absorbance of the highest concentration solution was 1 to ensure that we remained in the linear range, and where the spectra still showed significant concentration dependent variations. We wanted to keep the higher concentration signals on scale because we were searching for the saturation limit. The absorbances from the 1/64 and 1/128 dilutions were below the limit of detection at 300.74

nm, and were removed from subsequent analysis. The plot of absorbance at 300.74 nm vs dilution factor is shown in Figure 44.

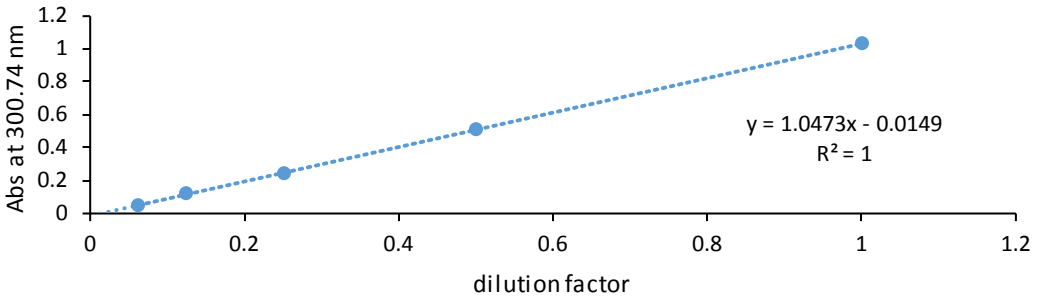


Figure 44. Calibration curve of the DMTS dilutions in fractions.

A linear least squares fit of the data in Figure 44 yielded the dilution factor calibration equation shown on the graph, which can be rewritten in terms of the actual absorbance $A_{300.74}$ and dilution factor D for each solution, as shown Equation 30.

$$A_{300.74} = 1.05\,D - 0.015 \qquad \text{Equation 30}$$

To assign absolute concentrations to the diluted solutions prepared from saturated DMTS, a set of standard PBS solutions were prepared having DMTS concentrations of 0.005, 0.05, 0.10, and 0.22 mg/mL. Baseline subtracted spectra for this experiment are shown in Figure 45.

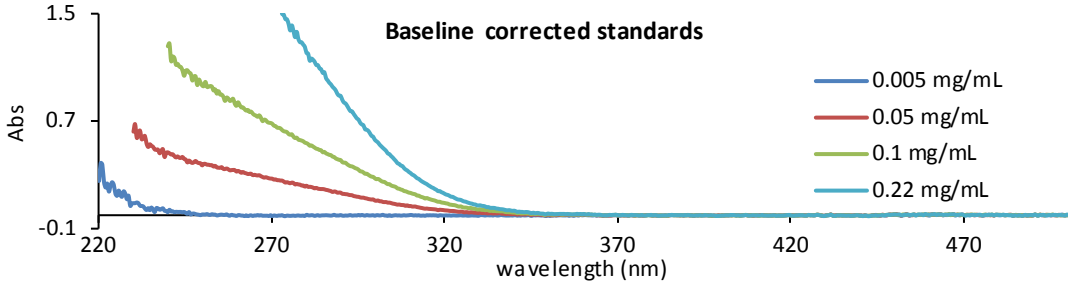


Figure 45. Absorption spectra of the standards DMTS solution in PBS. The baseline corrected spectra of DMTS standards.

The absorbance from the 0.005 mg/mL solution was discarded because it was below the detection limit, at the analysis wavelength of 300.74 nm. Each standard's measured absorbance at 300.74 nm was substituted into the dilution factor calibration equation (Equation 30), enabling the dilution factor $D_{standard}$ for that standard solution to be calculated. For a specific standard, D is factor by which a saturated stock solution would have to be diluted to obtain a concentration equal to that of the standard. (Equation 31)

$$C_{standard} = D_{standard} * C_{sat} \qquad \text{Equation 31}$$

Equation 31 can be rearranged as shown in to calculate the DMTS saturation concentration in PBS solution.

$$C_{sat} = \frac{C_{standard}}{D_{standard}} \qquad \text{Equation 32}$$

Using Equation 32, three estimates of the saturated concentration of DMTS in PBS were obtained, one from each standard solution. These are summarized in Table 15.

Table 15

The concentration of a saturated solution of DMTS in PBS

$C_{standard}$ (mg/mL)	$A_{300.74\,nm}$	$D_{standard}$	C_{sat} (mg/mL)
0.05	0.11	0.12	0.43
0.10	0.27	0.27	0.37
0.22	0.54	0.53	0.42

The average C_{sat} for DMTS in PBS solution was found to be 0.40 mg/mL (3.2 mM), and this value was used to calculate the concentration in each of the original diluted standards as shown in Equation 32. Substituting the actual concentration of each dilution into Equation 31, the final calibration curve (Figure 46) is $A = 2.59 * C_{actual} - 0.015$.

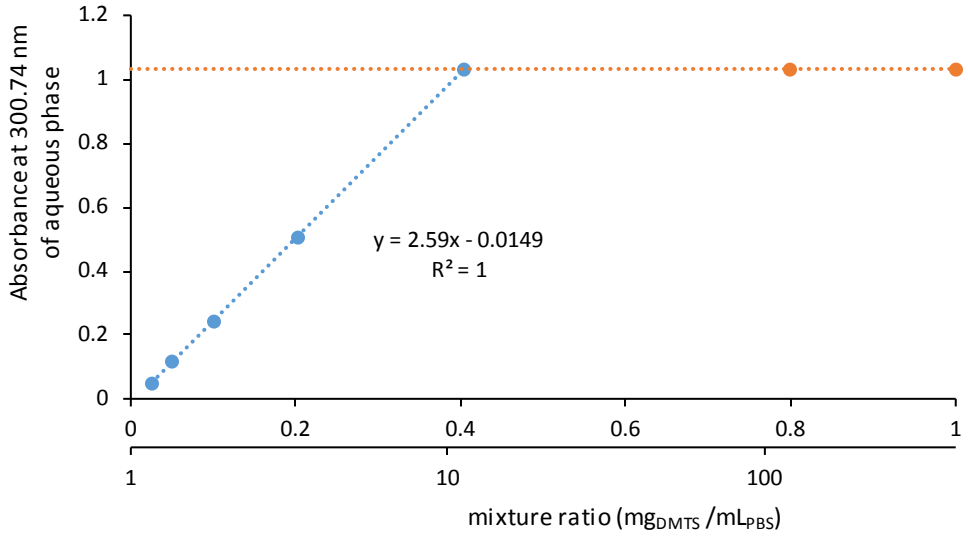


Figure 46. Calibration curve of DMTS in PBS at pH 7 room temperature. The upper, linear, x axis corresponds to the blue points which were obtained with solutions at or below the saturation limit. The lower log axis corresponds to the yellow points which were mixed at ratios that were too high to be miscible.

C. Interactions of DMTS with Hb

Introduction

Once good fractions of reduced HbO_2 had been obtained, preparations were made to look for the products of the hypothesized reaction with DMTS, and to probe the kinetics of the reaction between DMTS and HbO_2. The hypothesized reactions were given earlier and the first of these is reproduced below.

$$CH_3SSSCH_3 + 2Fe^{2+}(HbO_2) + 2H^+ \rightarrow 2Fe^{3+}\,(\text{metHb}) + CH_3SSH + CH_3SH$$

First, a series of experiments were conducted in which the conversion of HbO_2 to metHb was followed by UV-vis absorption spectrophotometry (C1). The goal of the first experiment described in C1 was to determine whether a low concentration of residual DT affected the rate of the reaction with DMTS. Because it was laborious to remove this DT, we wanted to check if it was necessary. The goal of the second experiment was to see if an excess of DT could reverse the effects of DMTS, by converting metHb back to Hb. In the third experiment the reaction between DMTS and Hb was initiated with different starting concentrations of Hb and DMTS, and the formation of metHb was followed by UV-vis spectroscopy. The goal was to collect a preliminary dataset that would enable us to begin characterizing the rate law for the reaction of DMTS with Hb.

Second (C2) HPLC-UV and UV-vis absorption spectrophotometry were employed to follow, respectively, the losses of HbO_2, and DMTS as they reacted with each other. The goal was to estimate the stoichiometric coefficients in the reaction of DMTS with HbO_2.

Third (C3), a control experiment was run to see if the presence of MeSH could be reliably detected with GC-MS. If so, the goal was to identify its GC retention time, and mass spectrum. MeSH is one of the hypothesized products of the reaction between DMTS and HbO_2.

The fourth (C4) and fifth (C5) experiments in this section used a gas syringe, and a SPME fiber respectively to collect samples from the headspace above a reaction mixture of DMTS and HbO_2 in PBS solution. The goal was to see if we could identify gaseous products like MeSH.

C1 Kinetics: Monitoring HbO_2 loss by absorption spectroscopy

Three experiments (identified as A, B, and C below) were conducted in which the conversion of HbO_2 to metHb was followed by UV-vis absorption spectrophotometry.

Experiment A (Experiment K1). UV-vis spectra were gathered as a function of time from a reaction mixture in which the initial concentrations of HbO_2, and DMTS were 3.2, and 640 μM respectively. The experiment was run with reduced hemoglobin samples gathered before and after using centrifugal ultrafiltration to separate the residual DT from the HbO_2. The Soret peak at each time point was fit to a linear combination of an HbO_2, and a metHb spectrum using the nonparameterized fitting approach summarized earlier in Equation 20. The fraction of metHb in each sample was then plotted as a function of time to obtain concentration isotherms (Figure 47) in the presence and absence of DT.

The metHb reaction isotherms in Figure 47 indicate that the reaction between DMTS and Hb proceeds faster in the presence of a small amount of residual DT, then in its absence. This, result surprised us. We had expected that the presence of DT would slow the oxidation of Hb to metHb, by either: (1) reducing DMTS and thus preventing it from reaction with HbO_2, or (2) by reducing the metHb formed by DMTS back to HbO_2. These experiments need to be repeated to test its validity. A hypothesis for the accelerated rate of oxidation in the presence of a small amount of DT is the following: (1) In this experiment, where [DMTS]> [HbO_2]>[metHb], DT would be expected to react with DMTS preferentially first because DMTS was present is such excess, and second because DMTS is a stronger oxidizing agent than metHb, (2) If DT reacts with DMTS, the products of the reduction would likely be MeSH and methyl hydrogen disulfide

(MeSSH). (3) The MeSSH product may be able to reach the heme more easily than DMTS because of its smaller size, and once in the pocket the thiol may prove more reactive than DMTS in oxidizing the heme iron. Thus, in situations where DT is added to HbO_2 in the presence of excess DMTS, it may speed up the oxidation of HbO_2 by DMTS, through formation of the more reactive MeSSH intermediate.

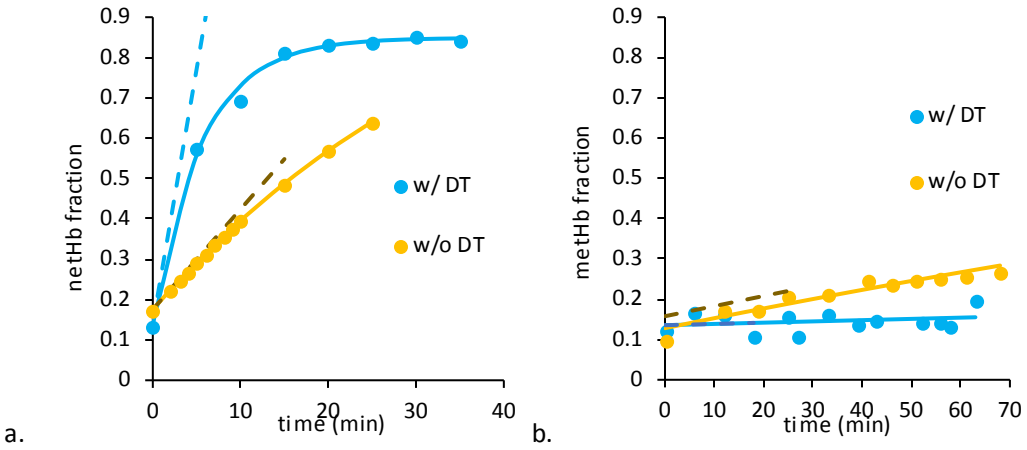


Figure 47 MetHb reaction isotherms in the presence and absence of DT. These reaction isotherms show the fraction of metHb present in (a) HbO_2/DMTS reaction mixtures and (b) Hb control samples as a function of time. Prior to the reaction DT had been removed from one sample by centrifugal ultrafiltration. In the other sample, no steps had been taken to remove residual DT.

The other hypothesis is the DMTS oxidize the Hb without attach to the heme. It was known that Hb oxidation can occur without any ligand bond to the heme iron which is known as outer sphere electron transfer. For molecules that larger than the access of the hydrophobic pocket is not easy to penetrate the protein moiety directly and bridge with the heme. It was hypothesized that the DMTS either transfer electron by electron jumping or it is cleaved by other reducing reagent first, such as DT or reductase in red blood cell, into smaller molecules, such as H_2S.

Experiment B. When DT is present in sufficient excess it is expected to reduce both DMTS and metHb. To test the reversibility of the reaction, 50 µL of 100 mg/mL (0.57 M) DT was added to metHb solution produced by reacting HbO_2 with DMTS. The mole ratio of DT to Hb was 3.6 to 1. In this case, as shown in Figure 48, the excess DT reduced the metHb back to HbO_2, reversing the effect of the prior DMTS treatment.

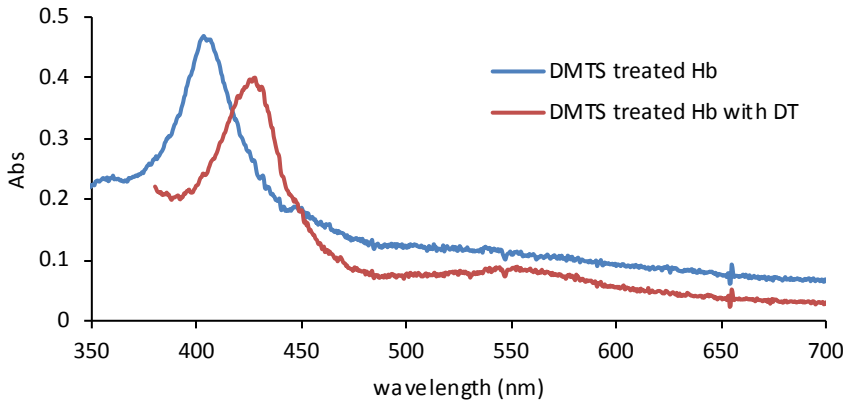


Figure 48. Reversibility of DMTS treated Hb by DT. The peak at 405 nm in metHb solution shifted to 430 nm after addition of DT, and a peak at 554 nm showed up, which is the peak of deoxyHb. The spectra showed that the Hb products of DMTS oxidation, metHb, can be converted to deoxyHb after addition of DT.

As shown in Figure 49a, all initial spectra exhibited a small peak centered near 620 nm (Hb concentration is 3.2 µM, pH 6), which is characteristic of sulfhemoglobin (SHb).

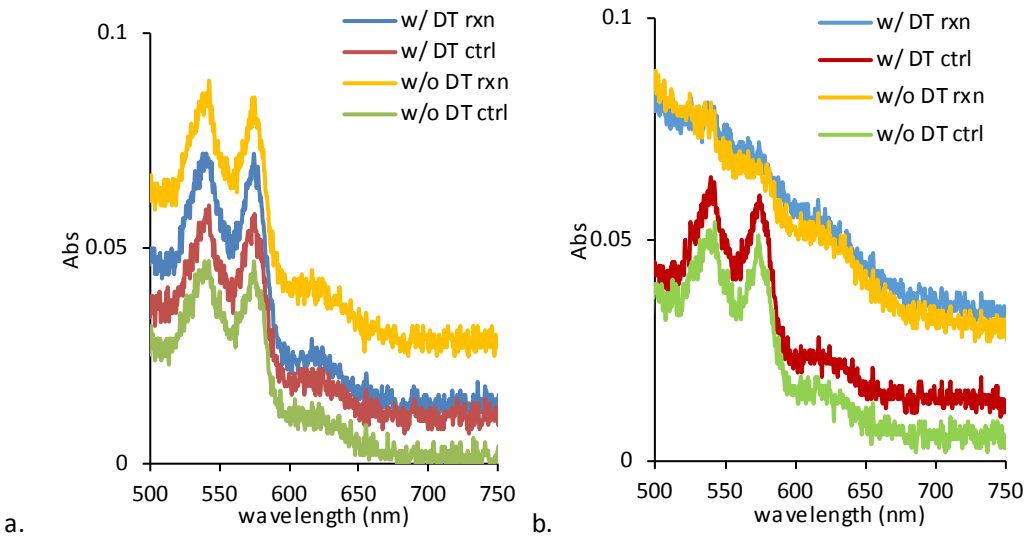


Figure 49. The Q band of all samples in DT effect on Hb/DMTS rate test. The first spectrum (a) and the spectra at 60 min (b) of all Hb/DMTS reaction and Hb control samples with and without DT are compared. The peak at around 620 nm in each sample, which is a characteristic peak of SHb,[21] was observed.

SHb is known to be formed by the reaction of H_2S with Hb in the presence of DT.[21] Because of its structural similarity to H_2S, MeSH may react similarly with Hb to form SHb. Upon reaction with DMTS, the center of the SHb peak redshifted slightly (Figure 49b) to ~625 nm. This shift is likely due to the formation of metHb, which exhibits a peak at 630 nm. Some sulfmethemoglobin (SmHb) is likely formed as well. The signature of SmHb formation would be the loss of the SHb peak, and the growth of a new SmHb peak at 717 nm. However, the molar absorptivity of the SmHb peak is twenty times less than that of the SHb peak, which was too low to be observed under the conditions of the experiments in Figure 49.

In addition to the small Q band peak at ~620 nm that is highlighted in Figure 49, the formation of a small amount of SHb is expected to cause the Soret peak to red shift. In the future, it would be helpful to run a reaction in which SHb is fully formed so that a

reference spectrum of SHb can be gathered, and added to the non-parameterized fitting model. This should then be oxidized to SmHb so that its spectra can also be added to the fitting model. Addition of these two spectra to the model would enable the small amounts of SHb and SmHb to be quantitatively determined from their subtle effects on the Hb spectra.

Experiment C (Experiment K2-K4). After determining that DT influenced the rate of the reaction between DMTS and HbO_2, every subsequent HbO_2 sample was cycled through numerous centrifugal ultrafiltration cycles. After each cycle DTNB was added to the filtrate, which reacts with DT to form yellow colored TNB^{2-}. If the filtrate turned yellow upon addition of DTNB, then another centrifugal ultrafiltration cycle was carried out. Once a filtrate was obtained that did not appear visibly yellow after the addition of DTNB, then a UV-vis spectrum was collected of the filtrate. Centrifugation cycles continued until no TNB^{2-} peak could be seen in the filtrate spectrum. At that point the redispersed HbO_2 was deemed sufficiently free of DT to be used in subsequent experiments.

Three additional DMTS HbO_2 reactions (K2, K3, and K4) were initiated. In combination with the corresponding data from experiment K1, the starting concentrations, and initial rates of metHb formation for these reactions are summarized in Table 16.

Table 16

Initial rates of DMTS/Hb reaction in kinetics experiments

Exp #	Mole ratio of DMTS:Hb	DMTS (µM)	Hb (µM)	Hb and DMTS initial rate	Hb control initial rate	Corrected initial rate
K1	206:1	640	3.2	w/ DT 0.1264	0.0003	0.1261
				w/o DT 0.0289	0.0025	0.0264
K2	166:1	800	5	0.0211	0.0033	0.0178
K3	79:1	800	10	0.0117	0.0034	0.0083
K4	116:1	600	5	0.0163	0.0157	0.0006

Spectral changes in each reaction mixture and its associated control were followed by UV-vis spectroscopy. Controls were equivalent to reaction mixtures, except that no DMTS was added to the controls. The controls were used to account for the much slower oxidation of Hb to metHb due to dissolved air. Representative reaction, and control spectra from experiment K3 are shown in Figure 50. The changes in the reaction spectra shown in Figure 50a correspond to the systematic oxidation of HbO_2 to metHb over the course of the reaction. As expected, the control samples in Figure 50b show much slower oxidation.

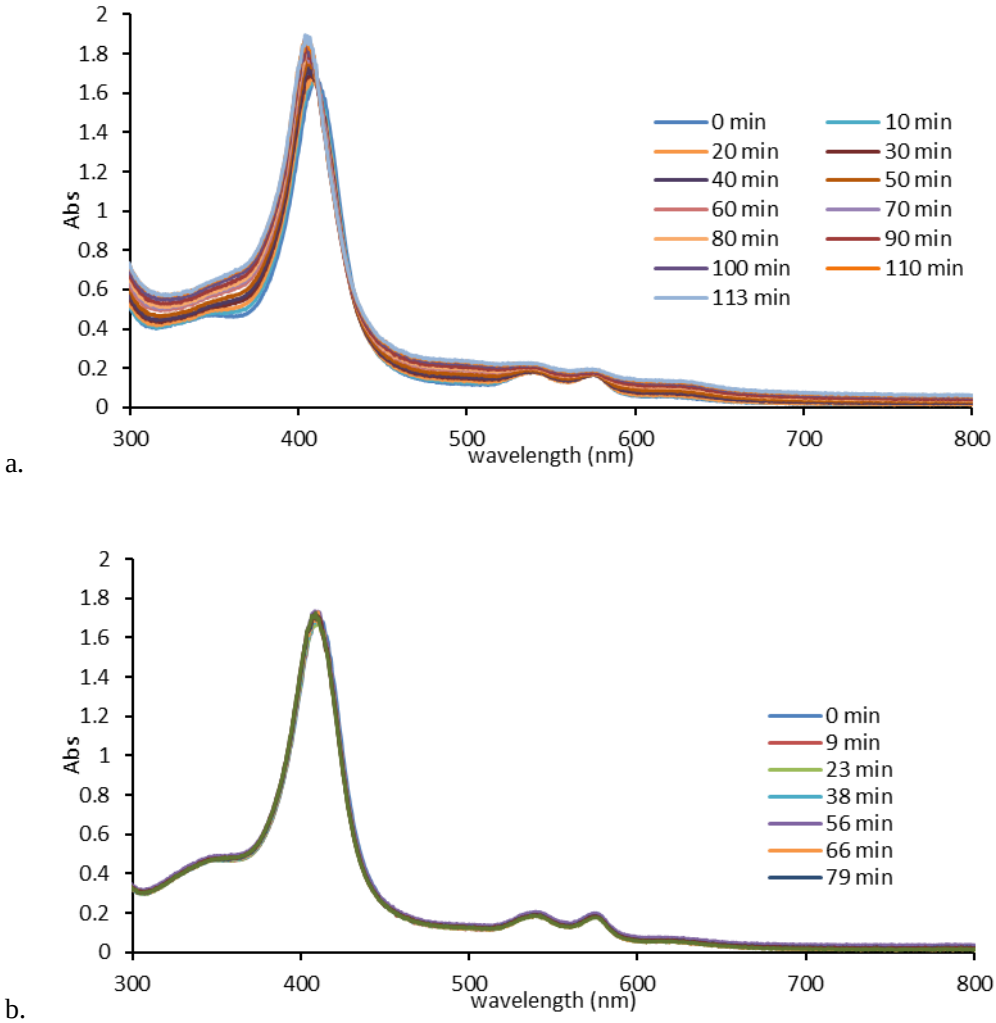


Figure 50. Absorption kinetic spectra examples of DMTS/Hb and Hb. The data were from experiment K3 (a) DMTS/Hb reaction and (b) Hb control sample. The DMTS concentration is 800 µM and HbO_2 concentration is 10 µM. The mole ratio of DMTS to Hb is 79:1.

The molar absorptivity of the Soret band is about 10-fold greater than the molar absorptivity of the Q bands, and each form of Hb gives rise to a corresponding shift in the Soret band.[29] Because of these properties the Soret region (370 to 470 nm) of each spectrum was fitted using the non-parameterized model, as the weighted sum of a reference HbO_2 and a metHb spectrum.

Figure 51 shows a representative fit to the spectrum collected 40 min after the addition of DMTS to reaction solution K3, along with the spectrum, residuals, and the two reference spectra used in the non-parameterized fitting model ($S_{Fit} = a \cdot S_{HbO2} + b \cdot S_{metHb}$).

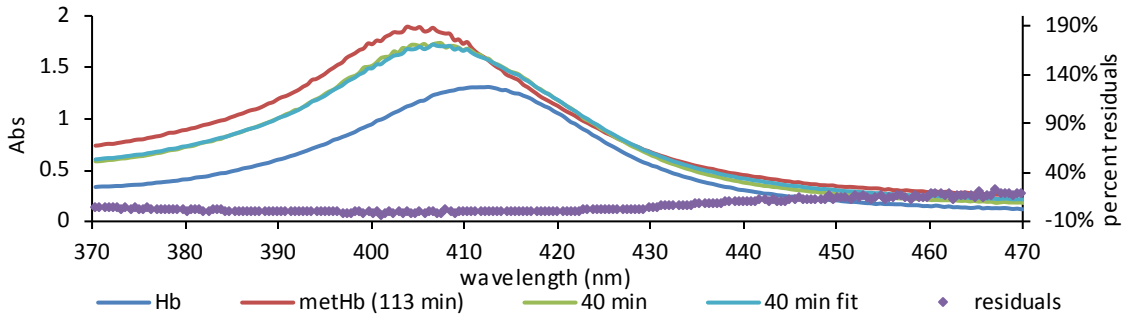


Figure 51. Fits examples to the spectra of Hb/DMTS reaction and Hb control. The spectrum was from experiment K3, Hb and DMTS reaction at 40min and the fitting residuals were plotted against the secondary axis, showing the fitting quality. The Hb spectrum used is from the Hb elutes from the column, the metHb spectrum used is the last spectrum collected at 113 min, which shows oxidized Hb spectrum features.

The HbO_2 reference spectrum, S_{HbO_2} (blue trace in Figure 51) was collected from reduced hemoglobin immediately after it passed through the DT reducing column, and scaled to match the size of the initial Soret peak in the reaction under consideration. The final spectrum collected from each DMTS reaction mixture (in which the Soret peak had shifted to around 405 nm), served as the metHb reference spectrum, S_{metHb}. This is shown as the red trace in Figure 51. New reference spectra were collected for each sub experiment (K2-K4), and then were held constant for the fitting of all reaction and control spectra within an individual sub experiment.

The reaction mixture spectrum is shown in green and the corresponding fit, S_{Fit}, in aqua. The fitting constants a and b, obtained for each spectrum, were interpreted as the

relative percentages of HbO_2 and metHb present at that time point in the reaction mixture. The control spectra were fit in the same manner.

The fraction of metHb, f_{metHb}, present in each reaction mixture at each time point, was calculated as shown in Equation 33.

$$f_{metHb} = b/(a+b) \qquad \text{Equation 33}$$

The metHb fractions calculated using Equation 33 were then plotted versus time to obtain an isotherm for metHb formation. In this manner, four reaction, and four control isotherms were generated. These are shown in Figure 52a and Figure 52b, respectively. Each metHb reaction and control isotherm was fit to an exponential rise function (Equation 34).

$$f_{metHb,t} = f_{metHb,0} * \left(1 - e^{-k*t}\right) + m_b \qquad \text{Equation 34}$$

Where $f_{b,t}$ and $f_{b,0}$ represent respectively, the fraction of metHb at time t, and the fraction of metHb at time 0 min. The fitting constants were k and m_b. These fits are shown superimposed upon the data in Figure 52.

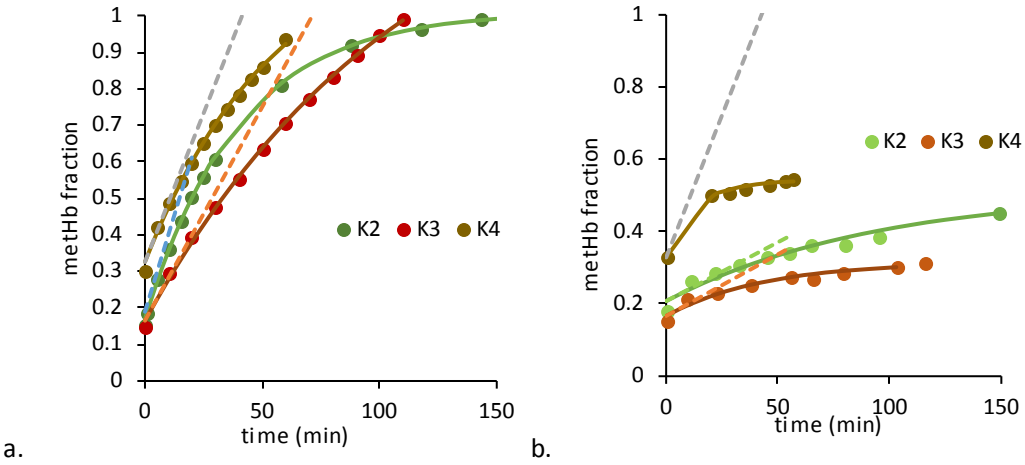


Figure 52. MetHb formation rate in each sample of kinetics exp. (a) metHb formation in DMTS/Hb reaction samples. (b) MetHb in Hb control samples. Dots are the fractions of metHb in each kinetics experiment. Solid lines are fits in the first order rate law to the fractions of metHb in each experiment. Dotted linea are the initial rate at time 0.

It was shown in the results, at time zero, the concentration of metHb was not exactly at 0, meaning some of the HbO_2 mixture was oxidized to metHb already. Experiment K4 has especially high concentration of metHb at initial of the reaction. Although not shown here, isotherms showing the loss of HbO_2 were also plotted, and fit using Equation 35.

$$f_{HbO2,t} = f_{HbO2,0} * e^{-k*t} + m_a \qquad \text{Equation 35}$$

A formula (Equation 36) for estimating the rate of reaction $R_{b,t}$ at time t was obtained by calculating the derivative of the fitting model. This was evaluated at time 0, as shown in Equation 37, to find the initial rate of each reaction at time zero.

$$R_{metHb,t} = f_{metHb,0} * k * e^{[-k*(t-t_0)]} \qquad \text{Equation 36}$$

$$R_{metHb,t=0} = f_{metHb,0} * k \qquad \text{Equation 37}$$

Based on the hypothesized reaction the form of the differential rate law shown in Equation 38 was proposed.

$$Rate = k * [DMTS]^a * [Hb]^b * [H^+]^c \quad \text{Equation 38}$$

Because the pH was held constant in these reactions, the hydronium term was incorporated into an effective rate constant, as shown in Equation 39.

$$Rate = k * [DMTS]^a * [Hb]^b \quad \text{Equation 39}$$

In this model k, a, and b represent, respectively, the effective rate constant, the order of the reaction with respect to DMTS, and the order of the reaction with respect to Hb.

Reaction order b was found by substituting initial rates, and initial concentrations (Table 16) for reactions 2 and 3, and then dividing the resulting rate laws as shown in Equation 40. This enabled an estimate of the order of the reaction with respect to Hb to be calculated as b = -1.10.

$$\frac{Rate\ 2}{Rate\ 3} = \frac{k * [DMTS]_2^a * [Hb]_2^b}{k * [DMTS]_3^a * [Hb]_3^b} = \frac{k * [800\ \mu M]^a * [5\ \mu M]^b}{k * [800\ \mu M]^a * [10\ \mu M]^b} \quad \text{Equation 40}$$

$$= \frac{0.0178}{0.0083}$$

$$b = -1.10$$

The process was repeated with data from reactions K2 and K4 as shown in Equation 41 to estimate the order of the reaction with respect to DMTS (a = 11.78).

$$\frac{Rate\ 2}{Rate\ 4} = \frac{k * [DMTS]_2^a * [Hb]_2^b}{k * [DMTS]_4^a * [Hb]_4^b} = \frac{k * [800\ \mu M]^a\ * [5\ \mu M]^b}{k * [600\ \mu M]^a\ * [5\ \mu M]^b} \qquad \text{Equation 41}$$

$$= \frac{0.0178}{0.0006}$$

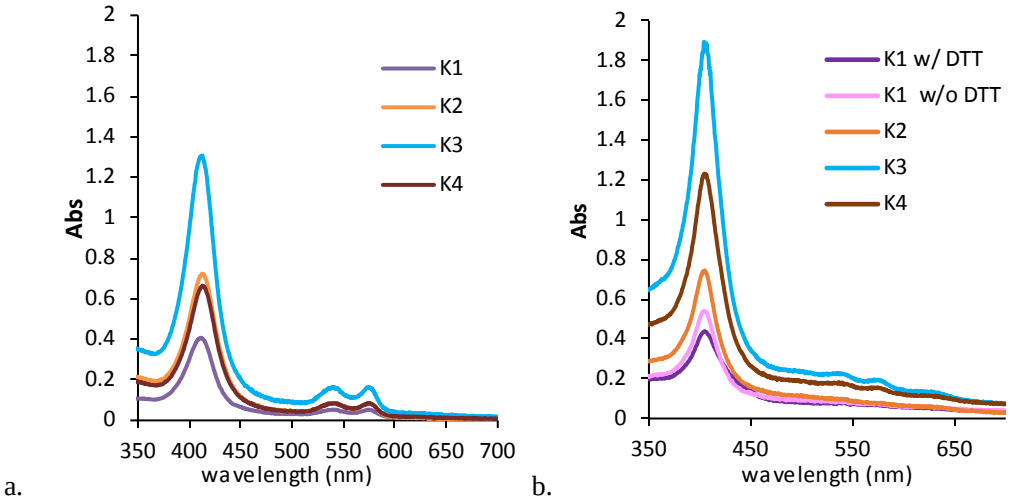
$$a = 11.78$$

The reaction orders, the corrected reaction rates, and initial concentrations were then substituted in for their corresponding variables in the rate law equation. The resulting equation was solved to find the rate constant K. This was repeated for each of the three reactions (K2, K3 and K4) used in this analysis. The average value of the rate constant was found to be $6.62 * 10^{-36}$. The effective rate law for the reaction of Hb and DMTS reaction obtained by these calculations is shown in Equation 42.

$$Rate = 6.62 * 10^{-36} * [DMTS]^{11.78} * [Hb]^{-1.1} \qquad \text{Equation 42}$$

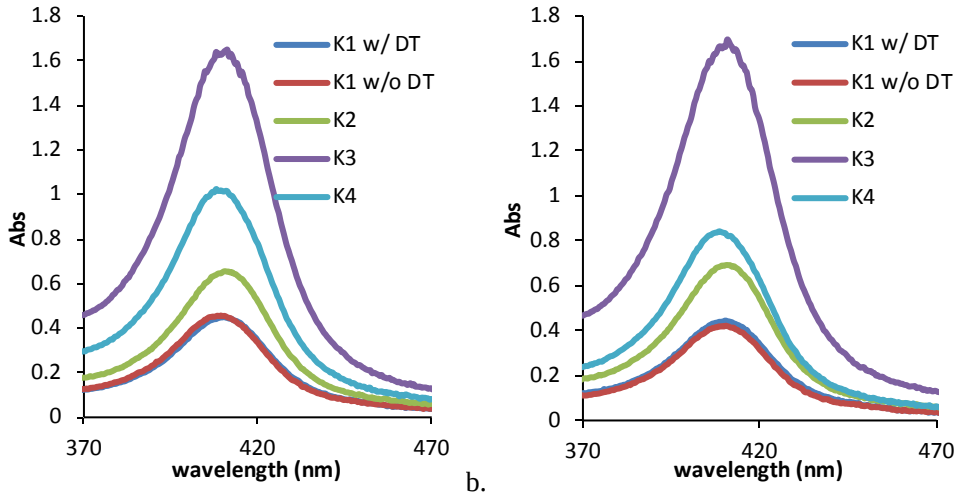
Unfortunately, this rate law is suspect because of the negative reaction order for Hb. In trying to understand what might have compromised the analysis, we revisited the initial reaction rates. The negative order for Hb arises because the increase in Hb concentration from reaction K2 to K3 is accompanied by a decrease in the initial reaction rate. From a viewpoint of fundamental kinetics, this does not make sense. Within the noise, the initial reaction rates of the control samples from the first three reactions without DT (0.0025, 0.0033 and 0.0034) are indistinguishable. However, the rate for the control associated with reaction K4 is five times faster, 0.0157, which is anomalous and suggests a contamination problem with this control. This experiment shows good proof of principle. However, the results suggest that the experiment was compromised by errors in analysis, and/or errors in experiment.

When this experiment is repeated, it would be advantageous to refine the experiment. The same reference HbO_2 spectrum, and the same reference metHb spectrum should be used for fitting all data. In this experiment, as shown in Figure 53, the reference spectra were similar, but not identical from reaction to reaction. Experimental steps should be taken to mitigate the partial oxidation of the initial sample. These steps may include removing oxygen from the sample, raising the buffering pH from 6 to 7.4, and possibly using dialysis in place of centrifugal ultrafiltration. It would be advantageous to speed up the reactions by using higher initial concentrations of HbO_2. This would also enable the reaction progress to be followed with the Q bands, which are more selective than Soret bands, and which can be followed without exposing the sample to UV radiation. The Hb concentrations in each sample should be experimentally determined from spectra using the known molar absorptivities of the Q bands. As shown in Figure 54, the initial spectra for samples and their controls in this experiment were similar but not always identical. However, analysis is simpler if reaction mixtures and their controls contain equivalent concentrations of Hb. Thus, in future experiments, the initial absorbances of reaction samples and their controls should be equivalent, and this equivalency should be checked at the beginning of each experiment.



a. b.

Figure 53. The Hb and metHb used for fitting in each kinetics experiment. The 1, 2, 3, and 4 refers to kinetics experiments K1, 2, 3, and 4. (a) HbO_2 spectra for fitting in each experiment. In experiment K2 and K4, the Soret peak was at 413.5, which is almost HbO_2, in experiment K1 and K4, the Soret band blue shifted 1 nm to about 412 nm, which means it was partially oxidized. (b) MetHb spectra for fitting in each experiment. The Soret band of the metHb spectra used in experiment K1 and K4 were at 405 nm, which is 404 nm in experiment K2 and 406 nm in experiment K3.



a. b.

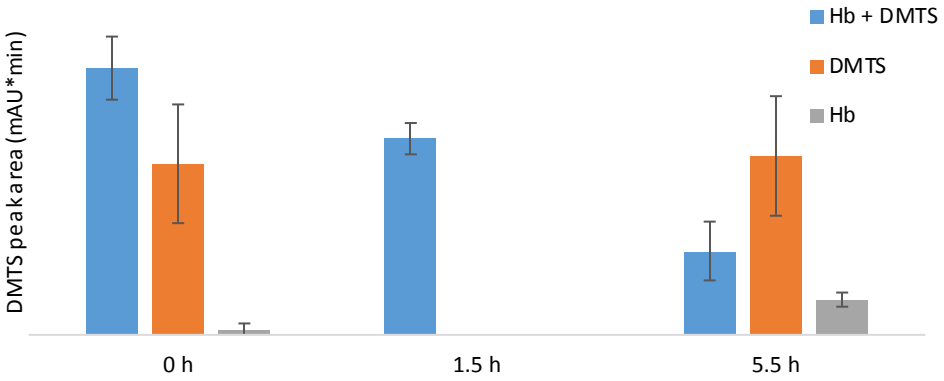
Figure 54. The first spectrum of DMTS/Hb in each experiment. The first spectrum was used to calculate the initial percentage of metHb and the rate of (a) DMTS/Hb and (b) Hb control in each kinetics experiment was different.

C2 Kinetics: Monitoring DMTS loss by HPLC

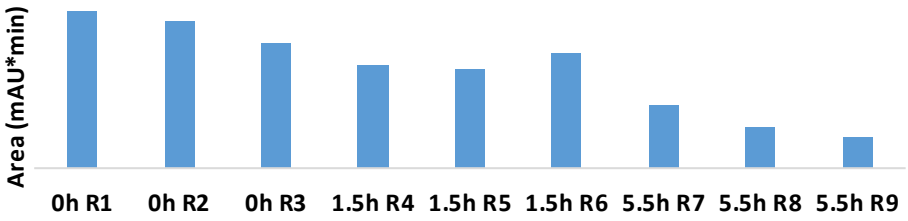
We have hypothesized that the stoichiometric ratio of DMTS to heme Fe is either 1:2, or 1:4. The stoichiometric reaction ratio can be calculated from simultaneous measurements of the change in HbO_2, and DMTS concentrations. Working toward this goal, HPLC analysis was tested as a method for following the loss of DMTS over the course of a reaction. A reaction of DMTS and Hb was initiated and samples were collected at the 0, 1.5, and 5.5-hour time points, prepared for analysis, and analyzed using HPLC-UV analysis. The DMTS peak areas in the chromatograms collected from the reaction mixture, the DMTS control solution, and the HbO_2 control solution are plotted versus sampling time in Figure 55a.

Control Experiments. The DMTS peak areas in the control solutions remained constant over the course of the experiment. The DMTS peak area from the Hb control is zero at the first two time points as expected, and increases slightly above zero in the last time point, suggesting a small amount of cross contamination at the last time point. With the exception of this contamination issue, the controls perform as expected in this experiment.

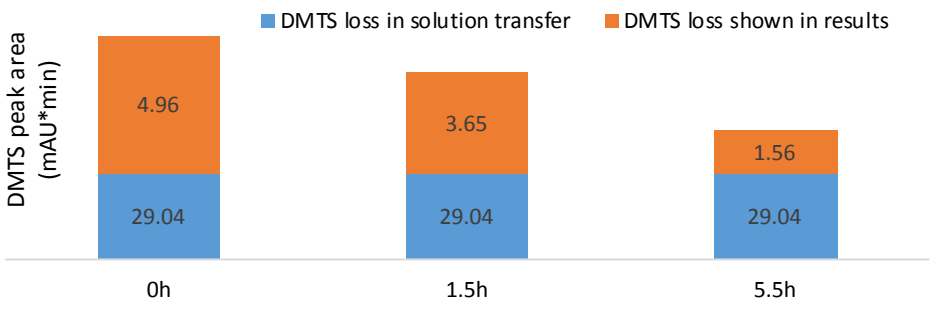
Reaction Experiment. The DMTS peak areas from the reaction mixture decrease with time, however, the decrease in peak area is greater than expected. The molar ratio of DMTS to Hb in this experiment was 10:1. The hypothesized reaction ratio between DMTS and heme Fe is either 1:2 or 1:4. Since there are four hemes per Hb, the hypothesized reaction ratio between DMTS and Hb is either 1/0.5 or 1/1. Thus, we expected the DMTS peak to decrease by 1/10 over the course of this reaction. Instead, as shown in Figure 55a, it is observed to decrease by a factor of 7/10.



a.



b.



c.

Figure 55. Peak area loss of DMTS in Hb and DMTS reaction mixture. The DMTS concentration is 50 µM, and the Hb concentration is 5 µM. (a) the DMTS peak area decreasing in reaction mixture, Hb control and DMTS solution. (b) The replicates of DMTS and Hb reaction mixture at each time point. They are measured by HPLC in the order from left to the right. The time difference between each sample is 20 min. (c) The hypothesized scheme of DMTS loss in reaction.

The greater than expected decrease in the DMTS peak area could be due to an error in our hypothesis. It could also be due to time dependent DMTS losses between extraction and analysis, or to transfer losses of DMTS.

DMTS losses between extraction and analysis. Figure 54b shows the three replicate peak areas for each sample in order of analysis. For the first and last time points there appears to be a systematic decrease in peak areas with wait time between sampling and analysis. The third replicate at 1.5 hours goes against this trend. There is a possibility that DMTS continues reacting upon storage, likely via oxidation by dissolved air. If upon further study, this proves to be this case, then it will be important to find ways of stabilizing the DMTS samples between sampling and HPLC-UV analysis.

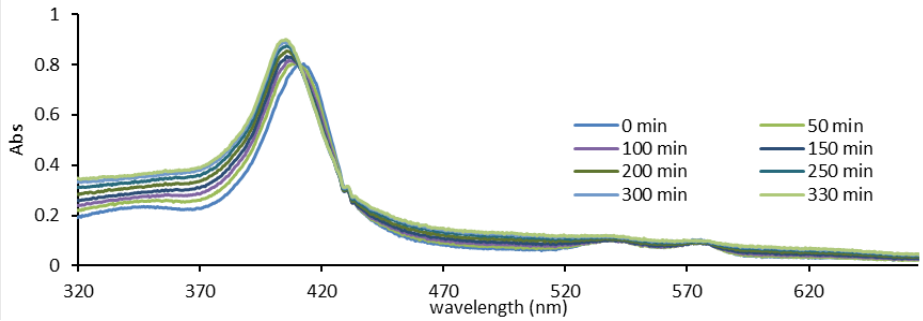
An equal absolute transfer loss from each sample, could also explain the observed area ratios. Assume that an equivalent amount of DMTS, is lost in the preparation of each sample for analysis, causing the area of each peak to decrease by X. Equation 43 and Equation 44 show how the expected final to initial final peak area ratio of 9/10 could be modified by a consistent loss of sample (X) to give the observed ratio of 1.56/4.96.

$$\text{Expected Ratio} = \frac{Final\ Peak\ Area}{Initial\ Peak\ Area} = \frac{9}{10} \qquad \text{Equation 43}$$

$$\text{Observed Ratio} = \frac{Final\ Peak\ Area}{Initial\ Peak\ Area} = \frac{9-X}{10-X} = \frac{1.56}{4.96} \qquad \text{Equation 44}$$

Equation 44 predicts a decrease in all peak areas of X=29.04, due to uniform sample loss. This would correspond to large losses in the sample preparation process, and thus is likely not the reason for these observations. Recovery controls can be incorporated into future experiments to test this hypothesis.

a.



b.

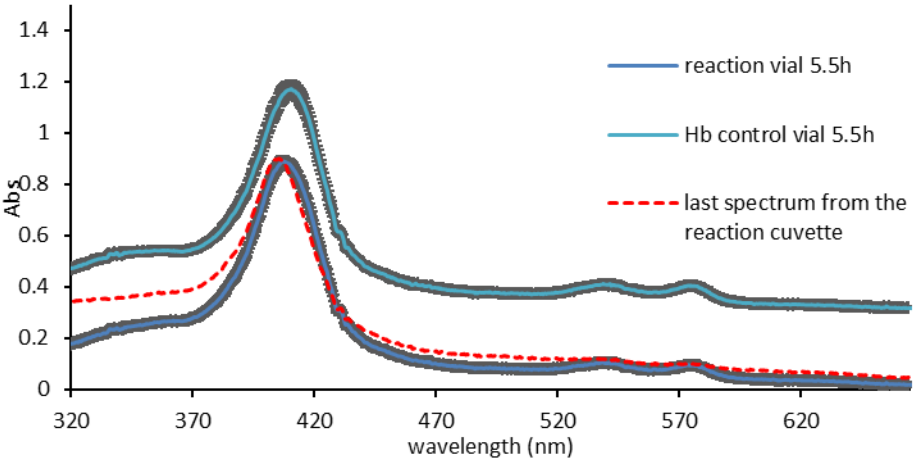


Figure 56. Absorption spectra of DMTS/Hb for monitoring reaction process. The DMTS was 50 µM and Hb was 5 µM. (a) The spectra of the reaction mixture in the glass cuvette monitoring the reaction process were collected from 0 h to 5.5 h. (b) Reaction mixture and Hb control solution in HPLC vials after measurement. The spectra of DMTS and Hb mixture was following in the 5.5 h, the red dot line is the last spectrum (5.5 h) from the reaction cuvette.

Prior to extraction, spectra of reacted samples and Hb controls were collected at the final time point (5.5 hours) from the reaction mixture and control. These are shown in Figure 56, along with the 5.5-hour spectrum from a parallel sample that was followed via UV-vis absorption spectroscopy over the same time period. What is most striking in Figure 56, is that the HbO_2 Q band peaks at 541 and 578 nm did not decrease as much, and the Soret peak did not shift as far in the reaction vials used for the HPLC samples, as in the parallel cuvette from which spectra were continuously collected. This suggests that

differences between the treatment of the parallel HPLC and UV-vis samples may be important. Specifically, the HPLC sample received a much smaller dose of UV radiation, and was more tightly sealed (screwed cap with Teflon septum, vs loose Teflon cuvette cover) than the UV-vis sample. It will be important to test for the effects of UV radiation, and seal quality on reaction rates in future experiments.

C3 Search for products: GC-MS analysis of MeSH standard (control)

The goal of the final three experiments in this thesis (C3, C4, and C5) was to search for the sulfur containing products of the reaction between DMTS and Hb. MeSH was hypothesized to be one of the products. The goal of experiment C3 was to detect the presence of standard MeSH with GC-MS, and to identify its GC retention time, and mass spectrum under the condition of our experiments. Because MeSH is toxic, these experiments were carried out with many safety precautions as described in chapter 2.

A SPME sample was gathered in the hood from the headspace above a 5 μL sample of 15% (w/w) aqueous sodium methyl thiolate solution. The resulting chromatogram is shown in Figure 57a. A Grob type injection was used in which the injector was run in splitless mode for ~0.8 min, before returning to a split mode. The peak widths correspond to the duration of the splitless period. Peaks for MeSH and dimethyl disulfide (DMDS) are observed a retention times of 1.62, and 2.31 min, respectively. They were identified by their mass spectra, with match qualities of 91 and 96% respectively. The unexpected peak DMDS is hypothesized to be the oxidation product formed by the oxidation of two MeSH molecules.

To see if we could reduce the extent of the reaction leading to the formation of DMDS, the experiment was repeated under slightly different conditions. The headspace

sampling vial was purged with Argon gas, and covered with Al foil prior to adding the sodium methyl thiolate solution and headspace sampling. Our goal was to reduce the partial pressure of oxygen, and the exposure to UV light with the hope that these would reduce the rate of conversion of MeSH to DMDS. Because we were concerned that these reactions might be favored by higher temperatures in the GC, the temperature program was altered so that the column reached 70 ºC in 4 minutes in the second test, as compared to 90 ºC in the same time in the first test. The chromatogram from the second experiment is shown in Figure 57b.

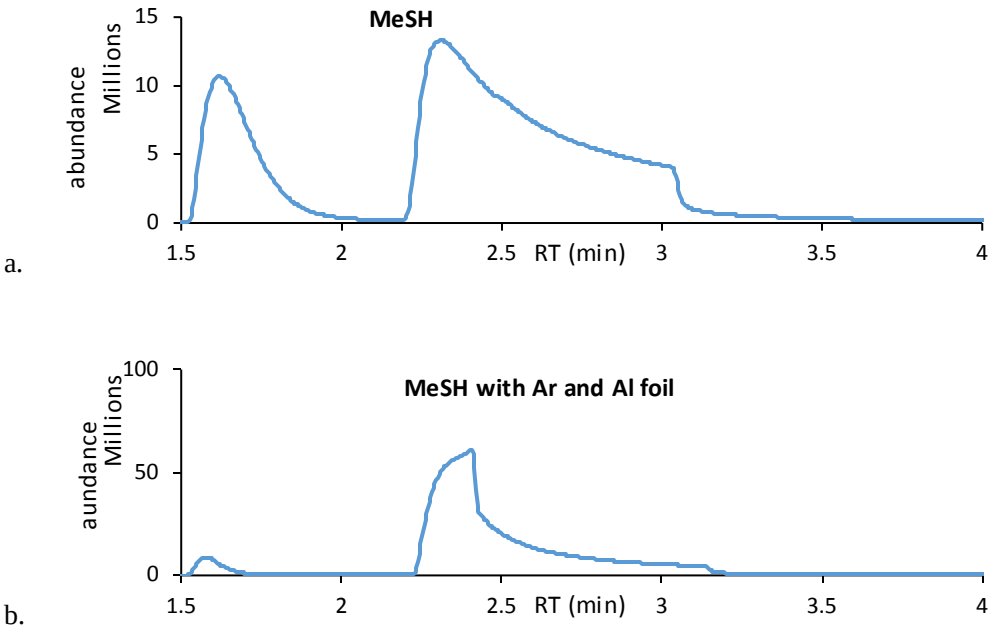


Figure 57. The chromatograms of HS above MeSNa solution (15% w/w). (a) the HS sample was taken from a 5 µL MeSNa in a 2 mL PTFE septum sealed vial, equilibrated for 15 min and then the SPME fiber injected and incubated for 15 min. (b) The same type of vial was washed with Ar gas prior to the solution transfer for 5 min at 20 mL/min rate, the vial was covered with Al foil, and the equilibrium time and fiber incubation time were shortened to 10 min.

The result is the opposite of what we had expected. The DMDS peak is much more prominent in the second, than in the first experiment. This may be due to ongoing oxidation

that was initiated as soon as the bottle of NaMeS solution was opened. A glove box was not available, and no precautions were initially taken to maintain an oxygen free atmosphere above the stock solution after it was opened. These preliminary chromatograms highlight the reactivity of MeSH, and thus the corresponding difficulty in detecting it. Since DMDS appears to be the dominant product, then it may be possible to use DMDS as a proxy for MeSH. Such an approach would require careful quantitation because DMDS is also a natural product of the disproportionation of DMTS.

C4 Search for products: GC-MS analysis of syringe headspace samples

To search for gaseous products of the reaction between DMTS and Hb, the headspace above reaction mixtures and controls were sampled with both a gas tight syringe and SPME fiber. The syringe was used to collect a sample that would mirror the headspace composition, at a cost of sensitivity. The SPME fiber was employed, because it can concentrate analyte, provided that the analyte partitions into the stationary phase on the fiber successfully.

Gas syringe sampling. Examples of the total ion chromatograms obtained with gas syringe samples are shown in Figure 58a. The DMTS peak can be seen at a retention time of 3.55 minutes. In these experiments, the mass range was extended to see small molecules. In the total ion chromatogram, this results in large signals from the air that leaks through the septum. These large air signals mask the peaks of other early eluting compounds like MeSH and DMDS. Using ion extracted chromatograms it is possible to remove most of the air signal from the spectrum, as shown in Figure 58b. The peak in the extracted ion chromatogram that is shown is a DMDS peak. This sample was

collected from the headspace of a 600 mM DMTS control solution. In this chromatogram, the DMDS likely arises from the disproportionation of DMTS.

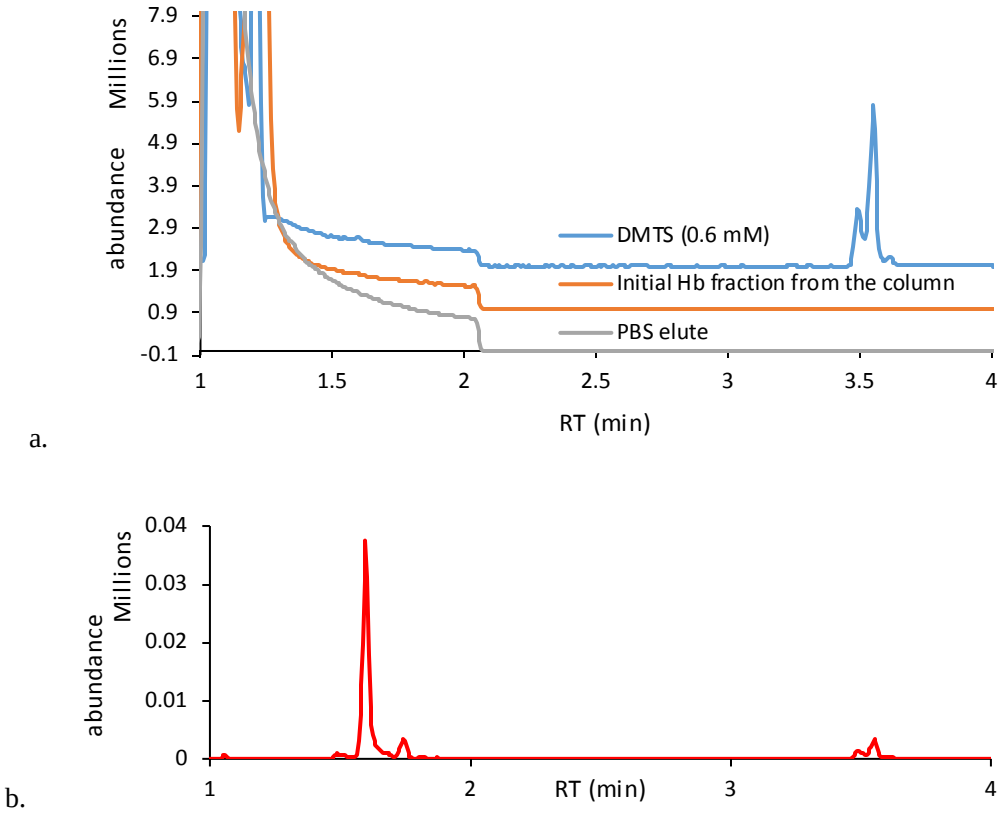


Figure 58. Chromatograms of HS above DMTS and control solutions. (a) The HS samples were collected from the 0.6 mM DMTS solution in PBS, the initial Hb fraction from the column, and the first PBS elute from the column. (b) The extracted ion chromatogram was found at m/z 94, which is the peak of DMDS, the disproportionation product of DMTS. The match quality is 97.

The gas syringe was used to collect samples from the HS above the DMTS and Hb reaction solution. However, no new peaks were observed in these experiments. This may be due to an absence of the proposed gaseous products, to a collection efficiency that is too low, or to reactions that prevent the analytes from partitioning into the headspace.

The gas syringe was also used to test the vial sealing quality by collecting HS samples above the DMTS and Hb reaction mixture at regular time intervals and injected

into a continuously running GCMS. The chromatogram (Figure 59) collected from this experiment shows 11 peaks corresponding to 11 replicate injections.

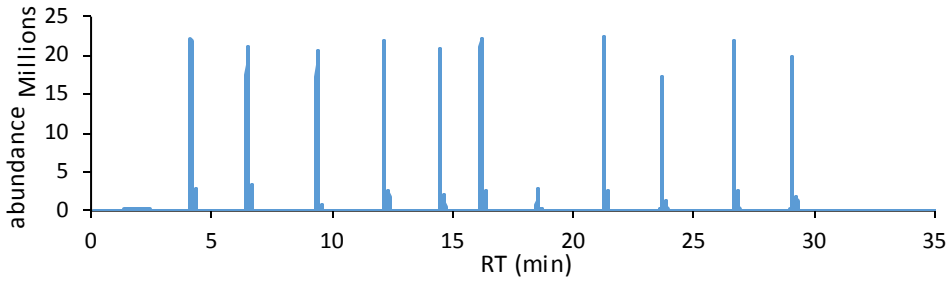


Figure 59. Chromatograms of multiple HS samples of HbO_2/DMTS reaction. DMTS concentration was 0.6 mM and Hb concentration was 5 μM. The HS was collected and injected to GC import every 2.5-3 min.

The variation in the heights of the 11 DMTS peaks provides an estimate of the reproducibility of the experiment. Each injection was sampled from a different reaction vial. The peaks were relatively stable for the first 16 min of reaction, and less after. The DMTS was in large excess and so peak areas were not expected to reduce by much over the course of the experiment.

C5 Search for products: GC-MS analysis of SPME headspace samples

Following up on the gas syringe experiments, experiments with a SPME fiber were carried out. Because the SPME fiber has the potential to concentrate analyte, we were hopeful that it might enable us to find low concentration reaction products that were not detected in the syringe samples. This was the case. DMTS, and its disproportionation products DMDS, and dimethyl tetrasulfide were all detected in SPME samples gathered from the HS above a reaction mixture of Hb and DMTS. Additionally, a new peak, identified by mass spectral library match as 2,4-dithiapentane was found to be uniquely present in the sample gathered from the headspace of the Hb/DMTS reaction mixture.

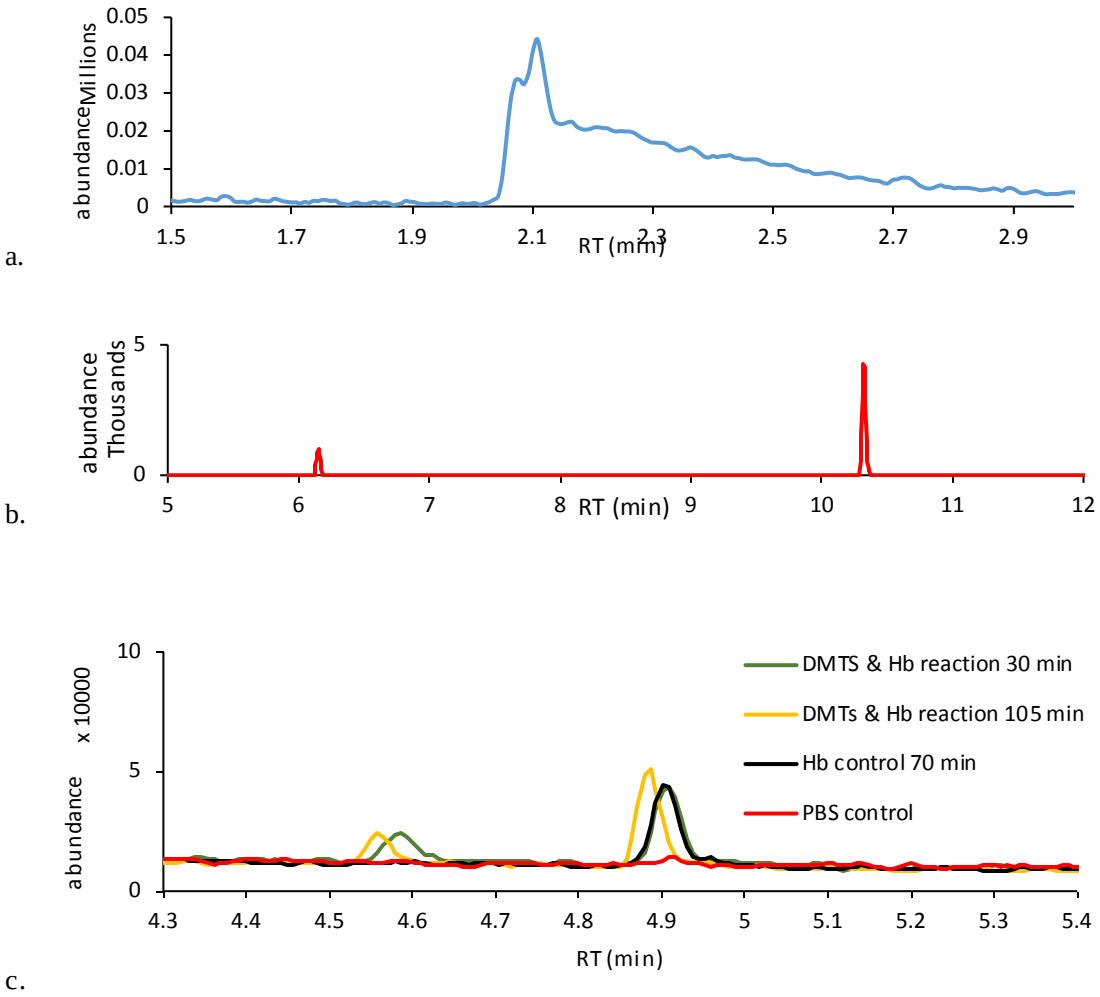


Figure 60. Chromatograms of in HS above DMTS and Hb reaction by SPME. (a) the total ion chromatogram showing the presence of a weak DMDS peak. (b) An extracted ion chromatogram (m/z 158, dimethyl tetrasulfide) of the HS above the reaction sample after 30 min collected by SPME, the peak at around 10.3 min is verified as dimethyl tetrasulfide, the peak at 6.2 min is DMTS. (c) The TIC of reaction samples, Hb and PBS controls. A 2,4-dithiapentane peak is observed to be uniquely present in the reaction samples.

In Figure 60c, the peak at 4.6 min was identified as 2,4- dithiapentane, by comparison to the Agilent mass spectral library with a quality factor of 80 and 87 for the 30 and 105 min samples, respectively. The peak at 4.9 min was identified as 1-[(2-methylpheny) thioxomethyl]-piperidine, was present in all of the Hb containing controls

and sample. Because this is a sulfur containing molecule, it may be a product of a DT side reaction.

In the end, these headspace sampling experiments were unable to detect the hypothesized products of MeSH or H_2S. This may because these are not the reaction products, or because they reacted prior to escaping the solution as gases, or that they reacted to form DMDS which was detected, which can only be seen by careful quantitative differentiation from the DMDS produced by disproportionation. Much was learned about headspace sampling, and the handling of reactive samples, that will inform experiments going forward.

CHAPTER IV

Conclusion

I. The reactions initiated by the addition of DMTS to both whole isolated blood and Hb were studied. The blood was first observed to darken as increasing amounts of DMTS were added. The DMTS was found less likely to react with darker aged blood than in lighter fresh blood. The UV-vis absorption spectrum of DMTS treated blood closely matched that of the $NaNO_2$ treated blood. GC-MS experiments monitoring the HS above DMTS treated blood showed DMTS peaks whose areas dropped systematically with time. Together these experiments provided strong initial support for the hypothesis that DMTS reacts with Hb to form MetHb in blood. An experiment involving successive additions of DMTS to isolated blood was carried out in a first attempt to determine the relative stoichiometry with which DMTS reacts with HbO_2 in blood. The stoichiometric mole ratio of DMTS/Hb (DMTS/heme) was found to be 8/1 (2/1), which was much larger than the DMTS/Hb ratios of 1/1 or 2/1 predicted by direct redox reaction. It is likely that the measured ratio overestimated the actual ratio due to a hysteresis between addition and reaction. The waiting periods in this experiment were short compared to the reaction times observed in subsequent experiments.

II. The changes in the Soret peak upon reaction of isolated blood with DMTS were followed spectrophotometrically. To test the hypothesis that DMTS was oxidizing HbO_2 to metHb, an attempt was made to fit each spectrum in the time sequence as a simple linear combination of an HbO_2, and a metHb spectrum. The fits showed high fidelity to the spectra, and provided a successful way of quantifying the proportions of HbO_2 and metHb present in the reaction mixture at different time points. To see if *in vitro* effects would be

mirrored *in vivo*, blood was drawn at systematic time intervals from rats who had been given intramuscular injections of either DMTS or $NaNO_2$ at therapeutic doses. Some conversion of Hb to metHb was observed in the blood drawn from the DMTS injected Rats where the ratio of injected DMTS to rat Hb was estimated to be 9 to 1. MetHb levels in the $NaNO_2$ treated rats were indistinguishable from those present in control samples. This observation was consistent with the lower dose of $NaNO_2$ (0.73 mol $NaNO_2$/mole rat Hb). The small extent of metHb formation in the blood of rats following injections of DMTS and $NaNO_2$ is consistent with the short time allowed between injection and blood draws; the expected delay between the intramuscular injection and full uptake into the circulatory system; the presence of metHb reductase in the blood, which reconverts metHb back to HbO_2; and the distribution of these antidotes from the circulatory system into other organs. Nonetheless, the successful observation of a spectral shift in the Soret band of blood drawn from rats following a DMTS injection, shows that the DMTS induced oxidation of HbO_2 occurs *in vivo*. The presence of this *in vivo* oxidation, and the corresponding spectral shift in the Soret band may one day provide an indirect avenue for noninvasively following the distribution of DMTS in animals by diffuse reflectance spectroscopy.

III. To see if DMTS would react directly with HbO_2 in the absence of other factors found in the blood, a series of experiments were carried out in which DMTS was reacted with isolated HbO_2 in PBS buffered solutions.

Because isolated Hb oxidizes readily, it is shipped predominantly in the metHb form. A key contribution of this work was the successful adaptation of: an established method for reducing metHb to HbO_2 with DT; a centrifugal ultrafiltration method for removing residual DT from HbO_2 solutions; and a DTNB assay for monitoring the extent

of DT removal from the filtrate. Additionally, the solubility of DMTS in PBS was experimentally determined to be 3.2 mM. A small amount of SHb was detected in both DMTS treated and untreated reduced Hb samples, and is likely formed through reaction with DT, and possibly also with DMTS.

Once this preliminary work had been successfully implemented, a series of experiments were carried out in which DMTS was mixed with isolated HbO_2 in PBS buffered solutions. A key result of these experiments was that addition of DMTS led to the successful oxidation of HbO_2 to metHb, even though other components of the blood matrix were missing. Residual DT accelerated the reaction rate of DMTS with HbO_2, leading to two explanatory hypotheses, the first being that DT may mediate the reaction as a catalyst. The second is that DT may reduce DMTS to MeSH and MeSSH. MeSSH may travel into the Fe center of the Hb more readily than DMTS because of its smaller size, and once there, MeSSH may oxidize the heme more rapidly than DMTS because of the higher reactivity of thiols than sulfides. Although DT is not present *in vivo*, other reducing agents and reducing enzymes are present in blood such as glutathiones and reductases. It is possible that these play a similar role in accelerating the reaction of DMTS with blood. The loss of reducing agents over time as blood is stored, may explain the decrease in reactivity with DMTS that was observed in aged versus fresh blood samples.

IV. HPLC-UV experiments monitoring the HS above a reaction mixture of DMTS and HbO_2 in PBS buffer showed DMTS peaks whose areas dropped systematically with time, whereas the area of the DMTS peaks in the control samples remained unchanged within the error of the experiment. A GC-MS search was launched to search for gaseous products of DMTS that such as MeSH and H_2S that are hypothesized to form when DMTS

oxidizes Hb. MeSH, and its oxidation product DMDS were successfully detected from a NaMeS control solution, however the MeSH signal was small relative to the DMDS signal, and both the MeSH and DMDS peaks varied considerably in amplitude between the two control experiments. This is likely due to the high reactivity of MeSH.

After successfully detecting MeSH in the control experiment, samples were gathered using gas syringe and SPME from the headspace above DMTS, HbO_2 reaction solutions. Although, no MeSH was observed, two possible products of MeSH were detected: DMDS, and 2,4-dithiapentane. DMDS was also detected in the HS above a DMTS solution without Hb, where it arises naturally as a disproportionation product. Accurate quantitation is required to determine whether the reaction of DMTS with Hb significantly adds to the pool of DMDS generated by the disproportionation of DMTS. This careful quantitation will be important for future experiments. In contrast, 2,4-dithiapentane was found uniquely in the samples gathered from the HS above DMTS and Hb reaction mixtures. 2,4-dithiapentane is hypothesized to be a reaction product between MeSH and formaldehyde-based polymeric materials in septa or plastic caps. These experiments strongly suggest that MeSH is a product of the oxidation of HbO_2 to metHb, but that it is highly reactive, and thus continued refinement of experimental methods will be required to obtain a set of data that establishes its presence and activity more conclusively.

The work presented in this thesis has presented an exploratory roadmap of the interactions of DMTS with HbO_2; has introduced important new methodologies for handling Hb, and safe analysis of hazardous gas phase products; and greatly expanded our group's understanding of the known spectroscopy of Hb.

This work suggests numerous experimental directions for the future. The priority is to develop a robust method for following DMTS concentrations simultaneously with Hb to obtain an accurate estimate of their stoichiometric reaction ratio. This can be done by SPME headspace sampling and will require many samples to obtain good statistics.

Methods of isolating samples from the air need to be implemented to enable reactions to be carried out under anaerobic conditions. Improved methods are needed for detecting the products of the DMTS oxidation of HbO_2. The products should be detected again in the HS above the Hb and DMTS reaction and appropriate controls with the Carboxen/PDMS SPME fiber, which has a higher selectivity for sulfur compounds. A more sensitive detector for sulfur compounds based on chemiluminescence or a pulsed flame photometry would be advantageous.

Experiments to probe the relative rate of HbO_2 oxidation in fresh isolated blood vs PBS solutions of HbO_2 would help to determine if there are factors in the blood which accelerate the oxidation of Hb by DMTS. These reactions should be conducted in the presence and absence of cyanide to determine how that might alter the extent to which they occur. In these experiments, it would be advantageous to use higher Hb concentrations to speed up reactions, and to follow spectral changes in the Q band rather than in the Soret band. Following the formation of metHb via the Q band spectroscopy has two advantages: it can be done using visible rather than UV light, and the spectra of the various forms of Hb (HbO_2, metHb, SHb, SmHb, and choleglobin) are more clearly resolved in this portion of the spectrum. The kinetic model used to analyze the data should be improved by gathering improved initial rate data.

Following up on the DT experiment positive controls should be run to test whether biologically relevant reducing and oxidizing agents will accelerate, decelerate or have no effect on the reaction of DMTS with Hb. Controls should also be run with heme in the absence of the globin, to understand how removal of the protein changes the nature of the reaction.

Finally, an attempt should be made to conclusively determine whether H_2S is formed as a product of the reaction between DMTS and Hb. H_2S is an important biological signaling molecule.[55, 56] In small doses, it may provide a complementary mode of CN antagonism, and may provide additional brain protection. In higher doses, it becomes toxic, and thus might be deleterious.[57-59] Determining whether H_2S is formed in this reaction, and if so, the level at which it is formed is a critically important scientific question for future exploration.

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Publications

Kiss L, Holmes S, Chou C, **Dong X**, Ross J, Brown D, Mendenhall B, Roy RJ, Coronado V, Stephens E, Thompson D, Petrikovics I. Analytical method development and validation for a novel cyanide antidote: detection from blood and brain. *Manuscript is under review in Toxicol. Res. (Camb).*

Huang X, Chen Y, Lin Z, Ren X, Song Y, Xu Z, **Dong X**, Li X, Hu C, Wang B. "Zn-BTC MOFs with active metal sites synthesized via structural-directing approach for highly efficient carbon conversion" Chem. Commun., **2014**,*50*, 2624-2627

Presentations and Posters

Kiss L, Chou CE, Mendenhall BA, Crews SR, Coronado V, Lowry J, **Dong X**, Roy RJ, Kaur M, Alam Md, Hossain SMd, Thompson DE, Petrikovics I. Analytical method developments for the cyanide antidote CAX1 for solubility, pharmacokinetics and blood brain-barrier penetration characterization. The 55th Annual Meeting of Society of Toxicology. March 13-17, 2016. New Orleans, LA.

Dong X, Barcza T, Kiss L, Roy RJ, Petrikovics I, Thompson DE. Effect of blood and an oxidation agent on a novel cyanide antidote. The 2015 Combined Southwest Region Meeting and the Southeastern Regional Meeting of the American Chemical Society. November 4-7, 2015. Memphis, TN.

Ross J, Chou CE, Brown D, **Dong X**, Coronado V, Mendenhall B, Roy RJ, Kiss L, Thompson DE, Petrikovics I. Method Development and Optimization for Detection of Cyanide Antidote Sulfur Donor X (SDX) by Use of Gas Chromatography-Mass

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Dong X, Kiss L, Holmes S, Hossain SMd, Ross J, Kaur M, Roy RJ, Brown D, Alam Md, Tuladhar P, Petrikovics I, Thompson DE. Improved method for a cyanide antidote detection by gas chromatography mass spectrometry and surface enhanced Raman spectroscopy. March 18, 2014, 18th Annual Graduate Research Exchange Program, SHSU, Huntsville, TX.

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