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Intrinsic Buffer Hydroxyl Radical Dosimetry for Hydroxyl Radical Protein Footprinting

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INTRINSIC BUFFER HYDROXYL RADICAL DOSIMETRY FOR HYDROXYL RADICAL PROTEIN FOOTPRINTING

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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DEDICATION

For my grandmother, Nonna, whose boundless love and incredible work-ethic are my greatest inspiration.

And for my fiancé, Kass, whose support and love keep me going even through the most difficult of times.

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ABSTRACT

ADDISON ELOIT ROUSH: Intrinsic Buffer Hydroxyl Radical Dosimetry for Hydroxyl Radical Protein Footprinting

(Under the direction of Dr. Joshua S. Sharp)

Hydroxyl radical protein footprinting (HRPF) coupled to mass spectrometry is a powerful technique for the analysis of protein topography as it generates covalent mass labels that can survive downstream sample handling, and it is sensitive to the solvent accessibility of amino acid sidechains. Of the multiple platforms for HRPF, fast photochemical oxidation of proteins (FPOP) utilizes a pulsed 248 nm KrF excimer laser to label proteins by photolyzing hydrogen peroxide. FPOP is the most widely used HRPF platform because it labels proteins faster than unfolding can occur.

Variations in FPOP sample conditions make it difficult to compare results between experiments and labs. To compensate for this, reporter molecules, known as dosimeters, have been introduced to provide a metric for comparison. While several different molecules are currently in regular use, they all complicate FPOP by increasing the complexity of the sample environment and/or necessitating the addition of steps to the workflow. Here, the history of HRPF and FPOP are discussed in detail, and the development of a new dosimeter molecule, Tris(hydroxymethyl)aminomethane, is reported. This molecule is the first of its

kind in that it acts as both buffer and hydroxyl radical dosimeter simultaneously, thereby significantly simplifying FPOP sample preparation. Tris acts as a gain-of-absorbance optical dosimeter as it gains absorbance at 265 nm upon oxidation, and this absorbance gain correlates well to both protein oxidation and scavenging capacity of the FPOP sample. Tris is capable of being measured in real-time through the use of an inline dosimeter which facilitates rapid adjustment of experimental parameters. Finally, a potential mechanism for Tris oxidation via reaction with hydroxyl radical is presented.

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LIST OF ABBREVIATIONS

CID	Collision induced dissociation
CLMS	Covalent labeling mass spectrometry
Cryo-EM	Cryogenic electron microscopy
DTT	Dithiothreitol
ETD	Electron transfer dissociation
FPOP	Fast photochemical oxidation of proteins
GluB	Glu^{1}-fibrinopeptide B, human
HRPF	Hydroxyl radical protein footprinting
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
MES	2-(N-Morpholino)ethanesulfonic acid
MS	Mass spectrometry
$NaBH_4$	Sodium borohydride
NMR	Nuclear magnetic resonance spectroscopy
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1. FAST PHOTOCHEMICAL OXIDATION OF PROTEINS

I. Hydroxyl Radical Protein Footprinting: Early Innovations

Although it currently provides lower resolution than other structural biology techniques such as X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR), and cryogenic electron microscopy (cryo-EM), mass spectrometry (MS) has grown into the tool of choice for many proteomics applications due to its low sample-size requirement and tremendous flexibility in sample characteristics (homogeneity, size, dynamics, etc.).[1, 2] Solution-phase structural analysis of proteins by MS is typically divided into three groups: covalent labeling, chemical cross-linking, and hydrogen-deuterium exchange. Of the three techniques, covalent labeling is particularly useful for mapping interaction interfaces and protein surfaces, and it can be sensitive to changes in protein conformation.[3, 4] Covalent labeling operates on a footprinting platform wherein a reagent molecule is covalently bound to a protein and the labeling sites are determined via MS analysis. As ligands or binding partners are added and sample conditions are changed, the protein's footprint will change accordingly and can provide insight into biophysical changes in the protein's structure.

While many reagents can be used for covalent labeling mass spectrometry (CLMS), the hydroxyl radical is certainly one of the most common. Because it is similar in size to a molecule of water, CLMS using the hydroxyl radical provides a high-resolution assessment

of an amino acid side chain's solvent accessibility and is typically referred to as hydroxyl radical protein footprinting (HRPF).[4] Many platforms exist for performing HRPF with each varying in its method of radical generation. Some of the earliest HRPF experiments used a synchrotron X-ray beam to generate hydroxyl radicals by the radiolytic ionization of water.[5, 6] However, the technology necessary to perform these experiments is not readily available to most researchers. Following the introduction of HRPF via synchrotron radiolysis, two new methods were introduced by Sharp and coworkers which relied on chemical[7] and photolytic[8] production of hydroxyl radicals.

Chemical production of hydroxyl radicals was achieved using Fenton chemistry catalyzed by the reagent $NH_4Fe(SO_4)_2$. While the technique was capable of footprinting many amino acids which were highly solvent accessible, the timescale of the labeling reaction made it highly likely that secondary radical reactions would occur. Additionally, the method was not applicable to metal binding proteins, a very large class of proteins.[7] Contrastingly, photolytic radical production was much faster and used no metal catalyst, so it could be applied to a broader range of proteins. Radicals were produced when a sample containing hydrogen peroxide was exposed to rapid UV irradiation. As with the other HRPF techniques, photolytic oxidation was shown to be sensitive to the solvent accessible surface area of amino acids; however, though it occurred on a much faster timescale than chemical oxidation, it still labeled proteins slowly enough that unfolding due to oxidation could occur.[8] As photolytic oxidation for HRPF showed significant promise as a tool for protein structural analysis[9], a new method using a similar platform was soon developed.

II. Fast Photochemical Oxidation of Proteins

This new technique is known as fast photochemical oxidation of proteins (FPOP), and it uses laser flash photolysis of hydrogen peroxide to generate hydroxyl radicals. Since its introduction by Hambly and Gross,[10] FPOP coupled with MS-based bottom-up proteomic methods has become a powerful technique for characterizing protein topography. FPOP relies on characterizing protein topography by measuring the apparent rate of reaction of amino acid side chains with diffuse hydroxyl radicals generated by laser flash photolysis of hydrogen peroxide. The apparent oxidation rate of each amino acid is dependent on both its inherent reactivity (which in turn is dependent on the sequence context[11, 12] and side-chain structure[13]) and the radical accessibility of the side chain.[12, 14-16] These labeling reactions produce covalently bound, stable modification products which are unaffected by down- stream sample handling,[17-19] and complete initial protein-radical chemistry on a low microsecond time scale that is faster than conformational changes can occur,[10, 17, 18] although secondary reactions can persist longer.[2, 20, 21]

III. FPOP Workflow

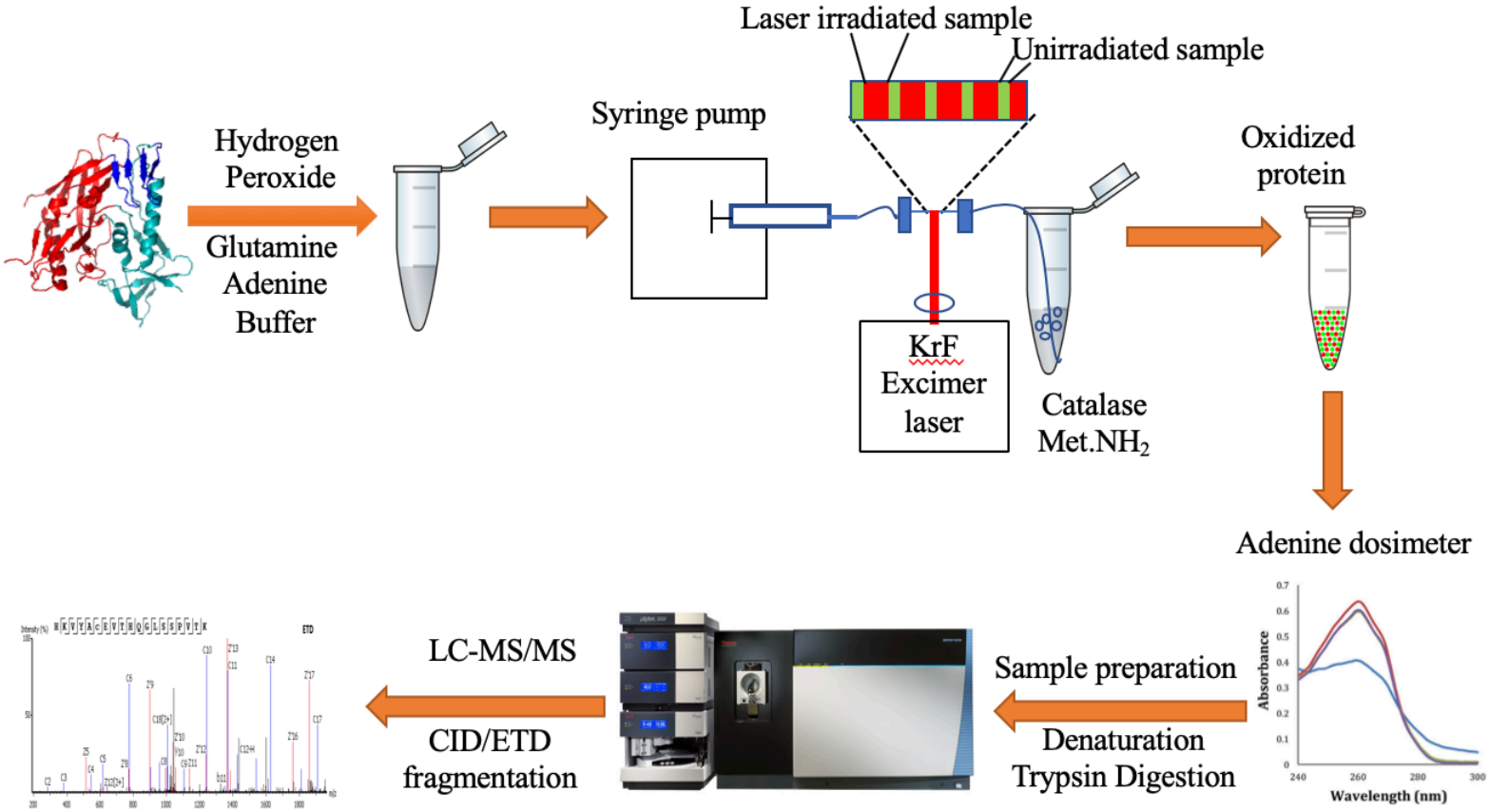
In order to oxidize a protein or peptide by FPOP, the sample is prepared in a buffer which is unreactive to hydroxyl radicals, often sodium phosphate, with glutamine or another radical scavenger typically included to control the lifetime of the radicals produced. FPOP samples also typically include a reporter molecule known as a dosimeter which will be discussed separately. Immediately before oxidation is to occur, hydrogen peroxide is

spiked into the sample. This is done to prevent unfolding due to oxidative stress prior to the bulk oxidation event which would result in inaccurate solvent accessibility data. FPOP oxidation is performed in a fused silica capillary passing perpendicular to the beam of a pulsed 248 nm (wavelength at which water and proteins are minimally absorbent)[17] KrF excimer laser. Flow rate is adjusted such that each bolus of sample is exposed only once, and an exclusion volume (unirradiated portion) is included between each bolus to further protect from this. Should a protein be exposed to the laser twice, it would have sufficient time between oxidation events to undergo conformational changes, and the footprint obtained would no longer be useful. Immediately after oxidation, the sample is deposited directly into a solution of methionine amide and catalase which stop the reaction by quenching excess radicals and secondary oxidants such as superoxides.[10, 17] Oxidized proteins can then be digested using trypsin or another protease to facilitate analysis by standard bottom-up proteomics tools. This workflow is further summarized graphically in **Figure 1**.

IV. Hydroxyl Radical Dosimetry

Structural characterization by FPOP typically depends on comparing protein footprints obtained under several conditions. However, alterations to hydroxyl radical scavenging capacity due to changes in buffer composition or the addition of some ligands and/or binding partners make it difficult to standardize results for comparison between experiments and between labs. To overcome this issue, several molecules have been introduced to the FPOP workflow which allow the effective hydroxyl radical concentration experienced by the analyte to be determined, thereby providing a metric with which

Figure 1. Fast Photochemical Oxidation of Proteins Workflow



Used with permission of Dr. Sandeep K. Misra.

experiments can be compared.[22-25] Each of these dosimeters competes with the analyte for hydroxyl radicals and experiences a change in its measurable properties proportional to the amount of radical present and not scavenged by other pathways.[2]

The UV-absorbent molecule adenine offers an easy option for radical dosimetry,[24, 26] and although it initially necessitated the introduction of additional steps to the FPOP workflow, the recent introduction of an inline UV spectrometer[19] negates this issue and allows hydroxyl radical production to be monitored in real time. Adjustments to peroxide concentration, laser fluence, and scavenging capacity can then be made as an experiment is performed to maintain a consistent level of oxidation across all samples.[2, 27]

Recently, while performing FPOP experiments in Tris buffer with the adenine dosimeter, members of the Sharp Laboratory observed adenine dosimetry readings that were inconsistent with protein oxidation and exhibited unexpected gain of absorbance behavior. Consequently, the properties of Tris under oxidative conditions have been investigated and are reported here.

CHAPTER 2: TRIS(HYDROXYMETHYL)AMINOMETHANE DOSIMETRY

I. Materials and Methods

All reagents used were of the highest purity available with no additional purification. Tris, myoglobin from equine skeletal muscle, human Glu[1] – fibrinopeptide B (GluB), and 2-(N-morpholino)ethanesulfonic acid (MES) hydrate were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Hydrogen peroxide (30%) was purchased from J.T. Baker (Phillipsburg, NJ). Sequencing grade modified trypsin was obtained from Promega (Madison, WI), and methionine amide was obtained from Bachem (Torrance, CA). The reductant dithiothreitol (DTT) was purchased from Soltec Ventures (Beverly, MA). LC/MS-grade formic acid and LC/MS-grade acetonitrile were purchased from Fisher Chemical (Fair Lawn, NJ).[2]

Previous experiments made use of 17 mM glutamine to limit the lifetime of the hydroxyl radicals produced during FPOP exposure.[10, 18] In order to maintain this same level of hydroxyl radical scavenging capacity in experiments using Tris, the second order rate equations for the reaction between glutamine and Tris were compared using the standard format shown in **Equation 1**.

$$v = k[A]^x[B]^y \qquad \textbf{Eq. 1}$$

[A] was taken to be the concentration of glutamine or Tris. In all cases, [B] was set equivalent to 200 mM hydroxyl radical based on the assumption that 100 mM hydrogen

peroxide would photolyze completely to yield two radicals per molecule of peroxide without a change in volume, thereby doubling the concentration of reagent. The partial orders of reaction (x & y) were assumed to be 1 because a unimolecular reaction in regards to both analytes is the simplest possible model for a second-order reaction between two different analytes and thus greatly simplifies calculations. Rate constants (k) were obtained from Buxton *et al*.[28] and are summarized in **Table 1**. The rate for reaction (v) with glutamine was calculated, and subsequently, the concentration of Tris required to maintain this rate was found to be 6.1 mM as shown in **Table 2**. Full calculations are provided in **Appendix 1**. Initially, the reaction rate constants were compared, and the required concentration of Tris was estimated to be only 8.5 mM. As substantial data had already been collected using this concentration, Tris was maintained at a concentration of 8.5 mM in all experiments reported here, but it should be decreased to 6.1 mM in the future so that new experiments more accurately replicate typical FPOP scavenging conditions.

Oxidation was achieved in all cases by exposing samples to the pulsed beam of a COMPex Pro 102 KrF excimer laser (Coherent Inc., Santa Clara, CA). The method for this oxidation is standard in the field[10] (for detailed explanation see **Chapter 1-III**). All experiments utilized offline dosimetry unless otherwise specified. This was achieved by measuring ultraviolet absorbance on a Thermo NanoDrop 2000c UV spectrophotometer with a 1 cm pathlength. When real-time inline dosimetry was required, ultraviolet absorbance was measured on the Pioneer series inline dosimeter from GenNext Technologies (Montara, CA).[19] After FPOP, all samples were deposited directly into a quench solution of 0.5 µg/µL catalase and 0.5 µg/µL methionine amide to reduce secondary oxidation products.

Table 1. Second-Order Rate Constants for Radical Scavengers

Molecule	**Rate Constant, *k* (L mol^{-1} s^{-1})**
Glutamine	5.4×10^8
Tris(hydroxymethyl)aminomethane	1.5×10^9

Rate constants are obtained from Buxton *et al*.

Table 2. Reaction Rates and Concentrations of Hydroxyl Radical Scavengers

Scavenger	**Concentration (mM)**	**Reaction Rate (M s^{-1})**
Glutamine	17	1.84×10^6
Tris	6.1	--

Calculations are summarized in **Appendix 1**.

Oxidized GluB and myoglobin were incubated at 90 ºC for 15 minutes in the presence of 5 mM DTT in order to denature them and reduce cysteine-cysteine disulfide bonds. The mixture was cooled at 4 ºC for 10 minutes. Sequencing grade modified trypsin was then added in a 1:20 w/w ratio, and the mixture was incubated at 37 ºC with slow rotation overnight to digest the oxidized samples into smaller peptides. The digestion was stopped by adding 0.1% formic acid, and the resultant peptides were analyzed by LC-MS/MS on an Orbitrap Fusion Tribid mass spectrometer (ThermoFisher Scientific). Myoglobin peptides were separated on an Acclaim PepMap 100 C18 nanocolumn (0.75 mm x 150 mm, 2 μm, Thermo Fisher Scientific). Elution was achieved using a binary gradient of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient began at 2% B and was increased to 35% B over 22 minutes, ramped to 95% B over 5 minutes, held at 95% B for 3 minutes, returned to 2% B over 3 minutes, and held at 2% B for 9 minutes to reequilibrate the column. Electrospray voltage was set to 2500 V, and ion transfer tube temperature was set to 300 ºC. Analytes were detected in positive ion mode, and the top eight peaks from MS^1 were fragmented by CID.[2]

The number of oxidation events per peptide were calculated using the method developed by Sharp *et al*., summarized here.[19] Peaks corresponding to unoxidized and oxidized peptides were first identified using Byonic version v3.6.0 (Protein Metrics, San Carlos, CA). The resulting selected ion chromatogram peaks were then integrated with a mass error of 7 ppm. Finally, oxidation events per peptide (n_{ox}) were calculated using **Equation 2** where I represents the integrated peak area of selected ions. $(+16)_{ox}$, $(+32)_{ox}$, and $(+48)_{ox}$ refer to the mass shift resulting from one, two, and three labeling events occurring on the given peptide. While the addition of hydroxyl groups should instead lead

to a mass shift of +17, +34, or +51, one hydrogen is typically lost for each hydroxyl group added thereby giving rise to the +16, +32, and +48 mass shifts. Each of these additions is weighted by the number of reactions a peptide would have to undergo in order to produce the given mass.

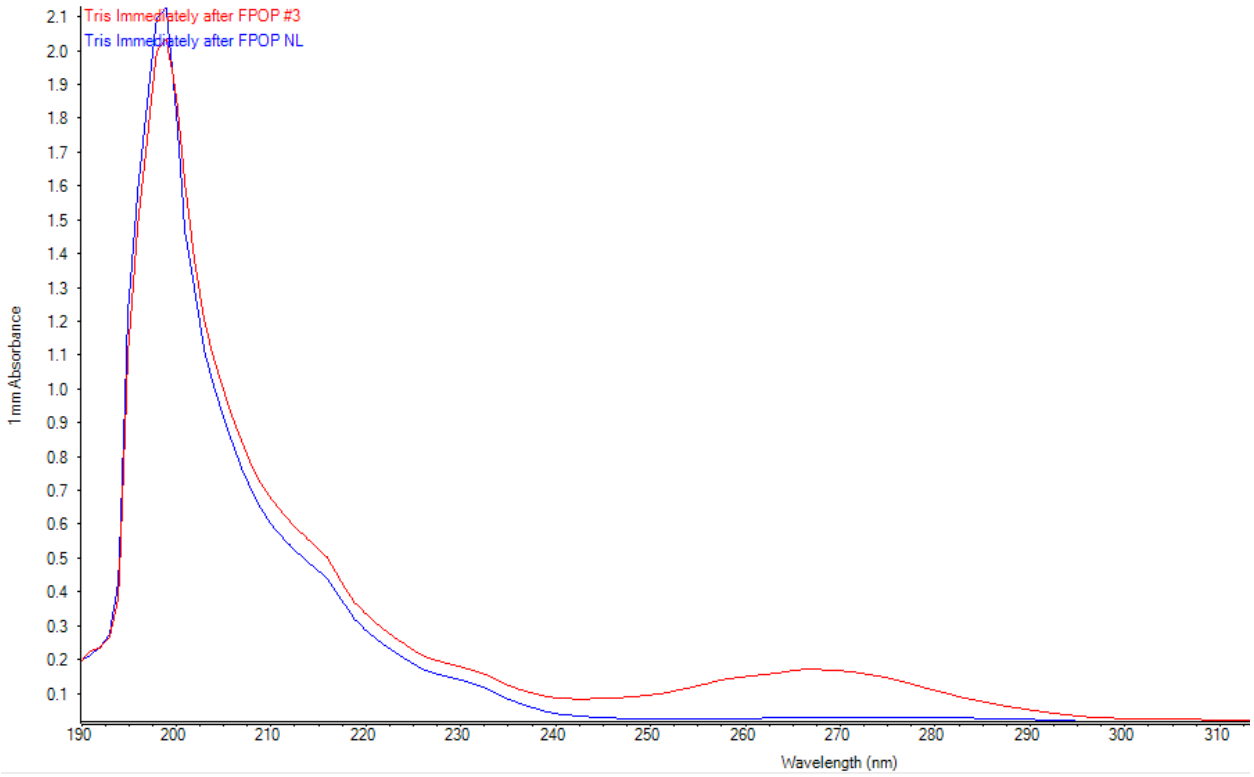
$$n_{ox} = \frac{[I(+16)_{ox}\times 1 + I(+32)_{ox}\times 2 + I(+48)_{ox}\times 3)]}{[I_{unox} + I(+16)_{ox} + I(+32)_{ox} + I(+48)_{ox})]} \qquad \textbf{Eq. 2}$$

II. Results and Discussion

Previous FPOP experiments performed in Tris buffer using the adenine dosimeter produced adenine absorbance readings that did not correlate to protein oxidation and demonstrated an unexpected gain-of-absorbance signal behavior. To investigate Tris buffer's role in this unexpected behavior, quadruplicate samples of Tris were oxidized by hydroxyl radicals produced by photolyzing hydrogen peroxide with an excimer laser. Additional Tris samples were combined with peroxide but not exposed to a laser pulse, so they remained unoxidized. UV absorbance spectra were obtained using a NanoDrop spectrophotometer, and a representative spectrum from each sample set is shown in **Figure 2**. When comparing the absorbance of oxidized and unoxidized Tris, it became evident that, while Tris is inherently absorbent in the short wavelength region of the ultraviolet spectrum (roughly 190-240 nm shown here), this absorbance changes minimally after oxidation by FPOP. In contrast, oxidized Tris shows substantial absorbance in the longer wavelength region from 250-310 nm with a maximum at 265 nm whereas unoxidized Tris is only minimally absorbing in this region.[2]

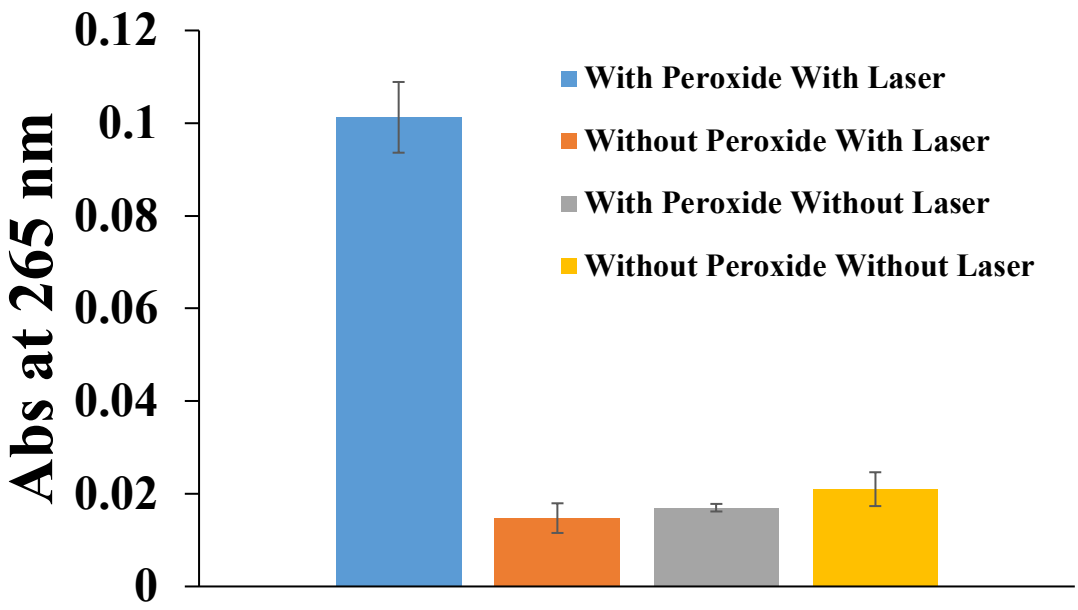
In order to better assess the source of Tris' absorbance behavior, quadruplicate samples of Tris were oxidized under four different conditions as shown in **Figure 3**. Each condition was one of four possible combinations of peroxide inclusion or exclusion and laser exposure or non-exposure with only samples receiving both peroxide inclusion and laser exposure having the necessary combination to produce hydroxyl radicals required for FPOP oxidation. In agreement with the expectations from **Figure 2**, all samples maintained a basal level of absorbance at 265 nm, but this absorbance significantly increased only upon exposure to hydroxyl radicals. Based on this, it was hypothesized that Tris buffer could serve as a hydroxyl radical dosimeter for FPOP reactions, but the ability of Tris absorbance to correlate to diverse reaction conditions was as yet unknown.

Figure 2. UV Absorbance Spectra of Tris in Oxidized and Unoxidized Forms



Red trace corresponds to 8.5 mM Tris oxidized by FPOP with 100 mM peroxide. Blue trace corresponds to 8.5 mM Tris combined with 100 mM peroxide but not exposed to a laser pulse.[1]

Figure 3. Tris UV Absorbance Gain Requires Hydroxyl Radical



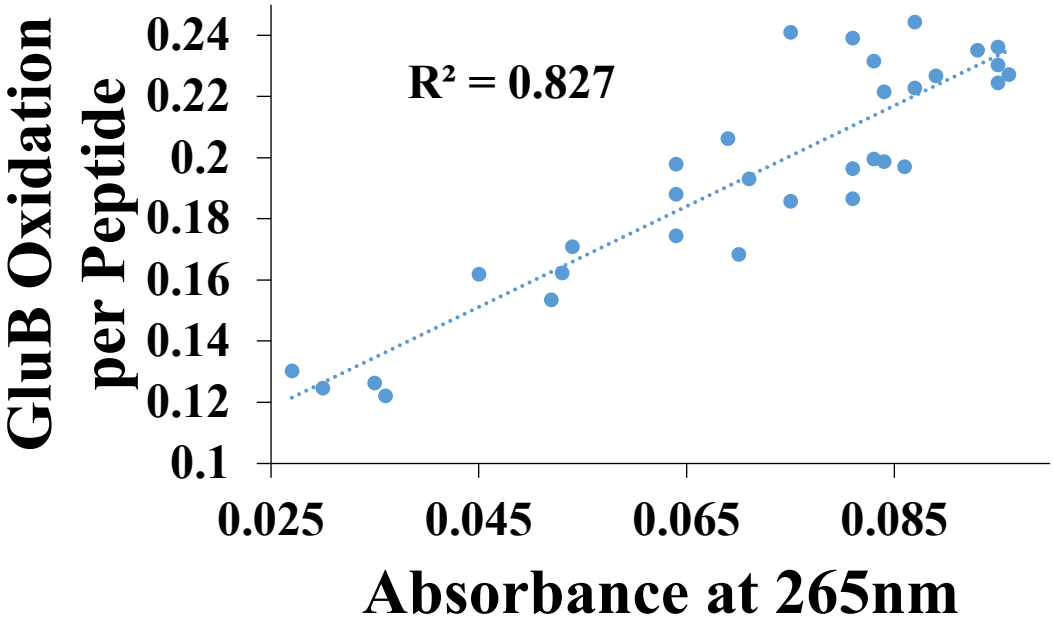
Absorbance of Tris at 265 nm as measured by NanoDrop after exposure to four sample conditions. Absorbance increases significantly only upon reaction with hydroxyl radicals generated by flash photolysis of hydrogen peroxide.[1]

To evaluate the correlation between Tris gain-of-absorbance and peptide oxidation, quadruplicate samples of Tris were again oxidized by FPOP but with the new addition of the model peptide GluB. Laser fluence was held steady at 10.23 mJ/mm^2 for all samples, and peroxide concentration was varied from 5-40 mM in 5 mM increments to generate increasing concentrations of radical. Typically, an increase in radical production corresponds to increased oxidation of analytes, so a direct, positive correlation was expected between Tris absorbance and GluB oxidation. As shown in **Figure 4**, Tris' absorbance gain at 265 nm does correlate both strongly and positively with the average oxidation per peptide of GluB.[2]

Abolhasani Khaje *et al.* suggest that the deviation seen in this correlation is likely due to an error in the mass spectral measurement of GluB oxidation rather than variation in the oxidation event itself.[29] Specifically, they showed that low signal intensity in the mass spectrum results in poor precision for FPOP workflows and can be ameliorated by increasing the amount of sample injected onto the LC column. This suggests that the correlation shown in **Figure 4** could have been improved by increasing the sample injection volume. However, as the R^2 value was 0.827, it was determined that the correlation shown sufficiently supported the hypothesis that Tris could act as a hydroxyl radical dosimeter, and work was continued to see if this held under additional conditions.

In some FPOP applications, the sample mixture may contain additional compounds, such as small-molecule drugs, that can scavenge hydroxyl radicals. In order for any potential dosimeter molecule to be useful, it is essential that it be able to respond reliably to these changes in chemical environment as well. To simulate this, quadruplicate samples

Figure 4. Abs_{265} of Tris Correlates to Peptide Oxidation



Tris absorbance at 265 nm correlates positively with average oxidation of GluB peptide.[1]

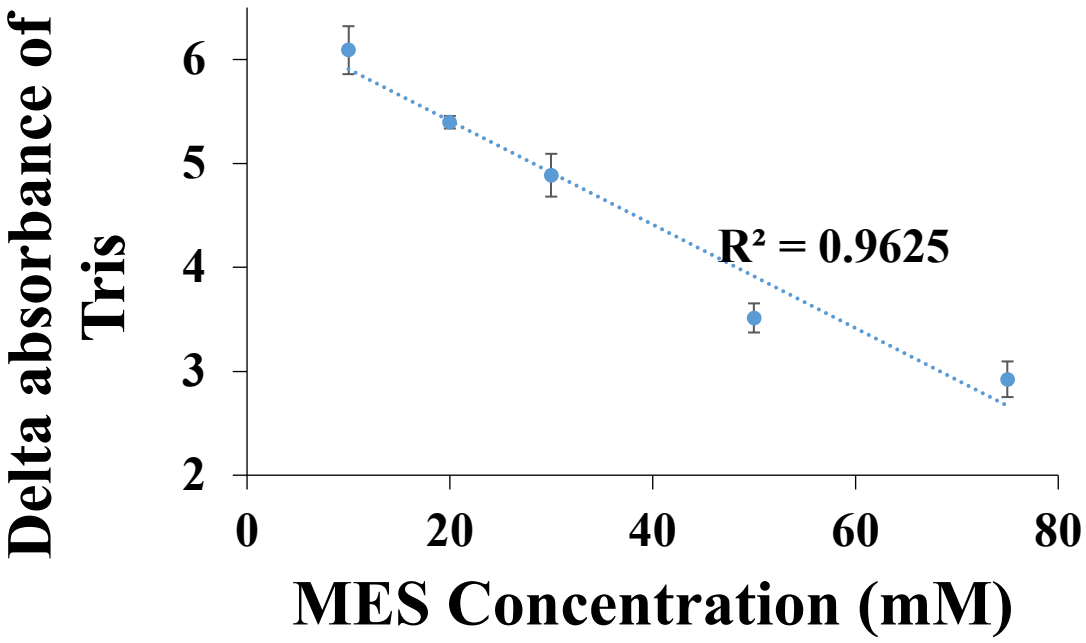
of Tris were oxidized by FPOP in the presence of MES buffer, a competing radical scavenger.

MES was added at concentrations of 10, 20, 30, 50, and 75 mM, and hydrogen peroxide was held at 100 mM for all samples. The laser fluence was also held constant so that all samples would receive an equivalent dose of hydroxyl radicals. Immediately after oxidation, the absorbance at 265 nm was measured using the Pioneer series inline dosimeter. By subtracting the average pre-oxidation baseline signal from the average signal post-oxidation, the ΔAbs_{265} was calculated for each sample. As seen in **Figure 5**, the ΔAbs_{265} of Tris correlates strongly ($R^2 = 0.9625$) with the concentration of scavenger present in the sample. As the concentration of scavenger is increased, the effective hydroxyl radical dosage experienced by other analytes decreases, and Tris experiences a proportional decrease in its gain-of-absorbance behavior.

This again supports the hypothesis that Tris could act as a potential hydroxyl radical dosimeter. However, it is important to note that both experiments used a simple sample mixture containing only Tris and one additional component. In most FPOP experiments, the sample contains several additional analytes, which compete with the dosimeter for radicals, so it is important to see that Tris maintains its dosimetry abilities under such complex conditions.[2]

In order to test the robustness of Tris acting as a radical dosimeter, a standard FPOP reaction containing myoglobin was carried out with buffer pH held at 8.0 to maintain myoglobin conformational stability. The reaction mixture was oxidized in the presence as well as the absence of MES buffer, and absorbance readings were obtained in real-time

Figure 5. Tris Dosimetry is Sensitive to Competing Radical Scavengers

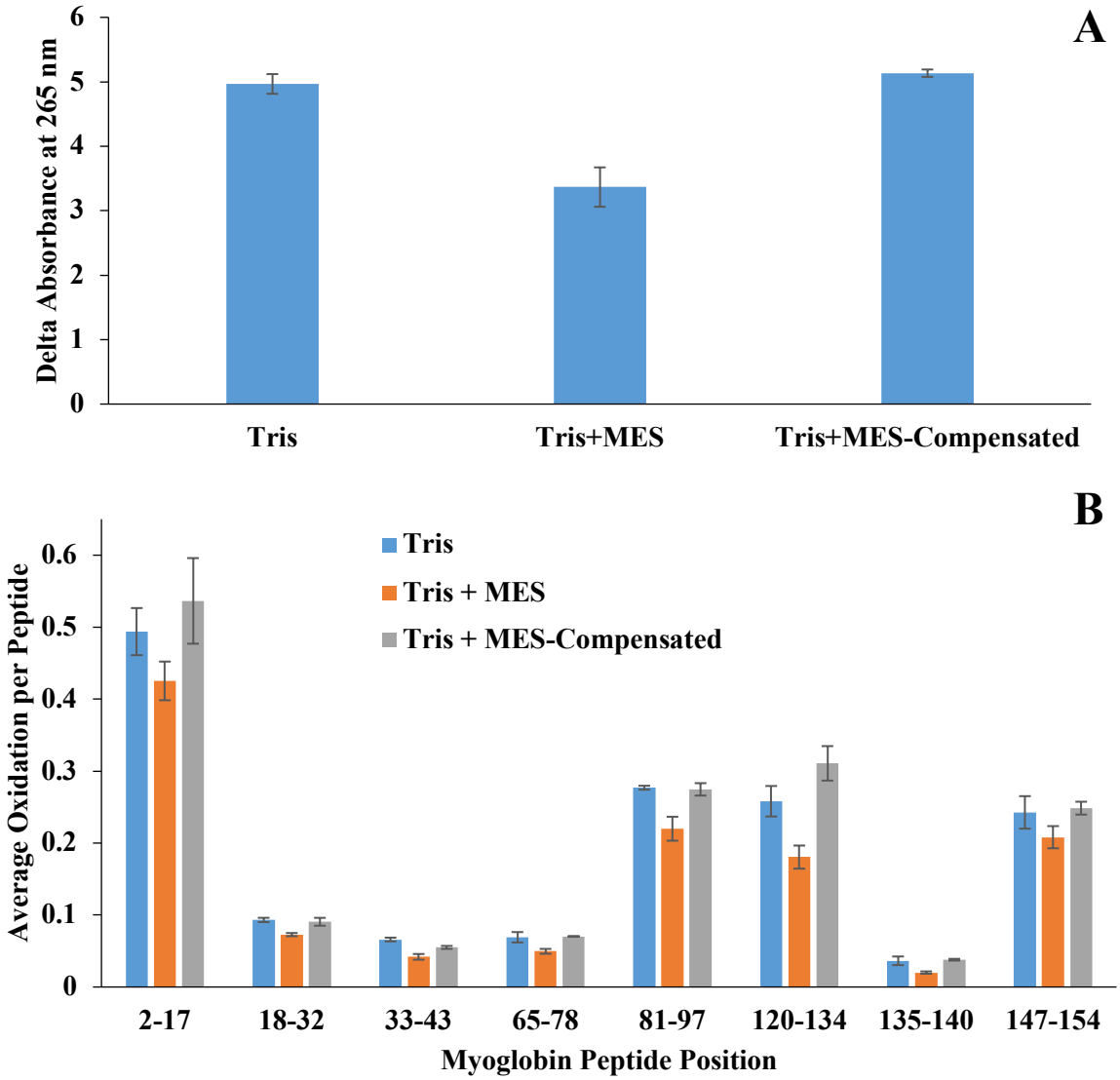


The gain in Tris absorbance decreases with increasing MES concentration.[1]

using inline dosimeter monitoring. First, myoglobin was oxidized and the ΔAbs_{265} readings were 4.97 ± 0.15 absorbance units, at a laser fluence of 11.66 mJ/mm^2. In the presence of 10 mM MES buffer, the ΔAbs_{265} decreased to 3.37 ± 0.30 absorbance units at 11.66 mJ/mm^2, reflecting scavenging by the MES buffer. In a separate experiment, laser fluence was increased to 15.30 mJ/mm^2 during the exposure of the myoglobin + MES sample to achieve a ΔAbs_{265} reading ≈ 4.97, identical to that of myoglobin without MES buffer (**Figure 6A**). When FPOP is performed in the Tris buffer alone, the peptides are more oxidized; when MES is also added to the mixture, a drop in the oxidation of all myoglobin peptides is observed. By compensating for the scavenging capacity of MES buffer using Tris as a dosimeter, the compensated oxidation of all myoglobin peptides in the presence of MES buffer is the same as in the samples without MES scavenger as shown in **Figure 6B**, demonstrating that Tris can act as a functional and practical radical dosimeter for scavenging compensation.[2, 27]

While it is clear from these results that the common buffer Tris can act as both an effective hydroxyl radical scavenger and dosimeter for FPOP applications, it is not clear how this new chromophore is formed. Based on the location of the UV absorbance maximum, it was first suspected that the chromophore was an aldehyde formed from the oxidation of an alcohol group as acetaldehyde is reported to have an absorbance maximum of 290 in the organic solvent cyclohexane.[30] While this does not directly overlap with the absorbance maximum seen in **Figure 2**, the increased polarity of water over cyclohexane stabilizes the *n* molecular orbital more greatly than it does the Π^* molecular orbital resulting in a hypsochromic shift (or blue shift) of the $n \rightarrow \Pi^*$ transition.[31] To test for the presence of an aldehyde, a Fehling test was used on a sample of oxidized Tris. This classic

Figure 6. Real-Time Compensation using Tris Dosimetry



(A) Tris absorbance change for myoglobin samples without MES scavenger, with 10 mM MES scavenger, and compensated conditions with 10 mM MES scavenger and increased laser influence to obtain a $\Delta Abs_{265} \approx 4.97$.
(B) (Blue) Peptide oxidation for myoglobin peptides in the absence of MES; (Orange) Peptide oxidation for myoglobin peptides in the presence of 10 mM MES; (Gray) Peptide oxidation for myoglobin peptides in the presence of 10 mM MES under compensating laser fluence conditions, using Tris as a dosimeter for radical compensation. No statistically significant differences were detected in peptide oxidation between no MES samples and with MES-containing samples compensated using Tris dosimetry.[1]

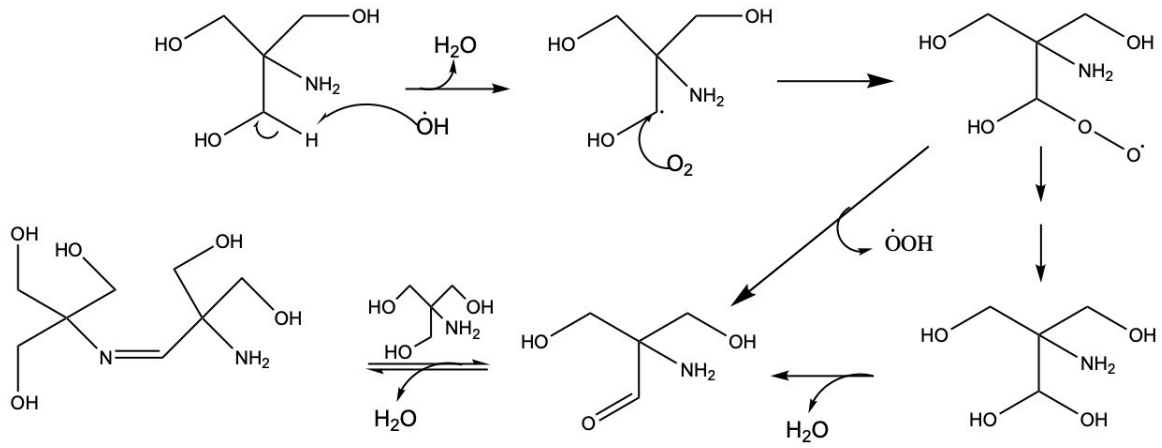
organic analytical method produces a red precipitate if an aldehyde is present and a green solution if an aldehyde is absent. When applied to Tris, the solution produced was a confusing blue which did not correlate with any standard Fehling test result.

While this did not disprove the presence of an aldehyde, further discussion of potential reaction mechanisms led to the proposal that the newly formed chromophore could instead be an imine produced when the initially formed aldehyde condensed with the amine nitrogen of a second Tris molecule. Due to the high concentration of Tris relative to most other analytes and the presence of three potentially reactive alcohol groups on each molecule, it was hypothesized that this reaction could continue past the initial aldehyde production step to result in multiple aldehydes on the same molecule reacting with other Tris molecules to form a complex polymer. As imine condensation is reversible in aqueous conditions, the bond is commonly reduced using sodium borohydride ($NaBH_4$).[32] When excess $NaBH_4$ was added to an aqueous sample of oxidized Tris, the solution thickened to a gel-like consistency while samples of unoxidized Tris treated in the same manner remained unchanged. This agrees with the idea that a large polymer is formed upon Tris oxidation via the formation of an imine bond which can be reduced to prevent hydrolysis.

Based on these two observations, the proposed scheme for the reaction of Tris buffer with hydroxyl radical shown in **Figure 7** was drafted. Briefly, hydroxyl radicals generated by laser photolyzing hydrogen peroxide are thought to abstract a hydrogen from a C-H bond from Tris and produce a secondary carbon radical. This radical can then react with oxygen dissolved in the sample to produce a peroxy radical. A geminal diol can then be formed by one of several different pathways. Water is then spontaneously lost to form an aldehyde.

Alternatively, a hydroperoxyl radical can be lost to directly produce the aldehyde. The aldehyde of one Tris molecule can then condense with the amine of another Tris molecule to form an imine. While not shown in the scheme, this reaction is thought to repeat to form a lengthy and potentially branched polymer.[33, 34]

Figure 7. Proposed Scheme for the Oxidation of Tris by Hydroxyl Radical

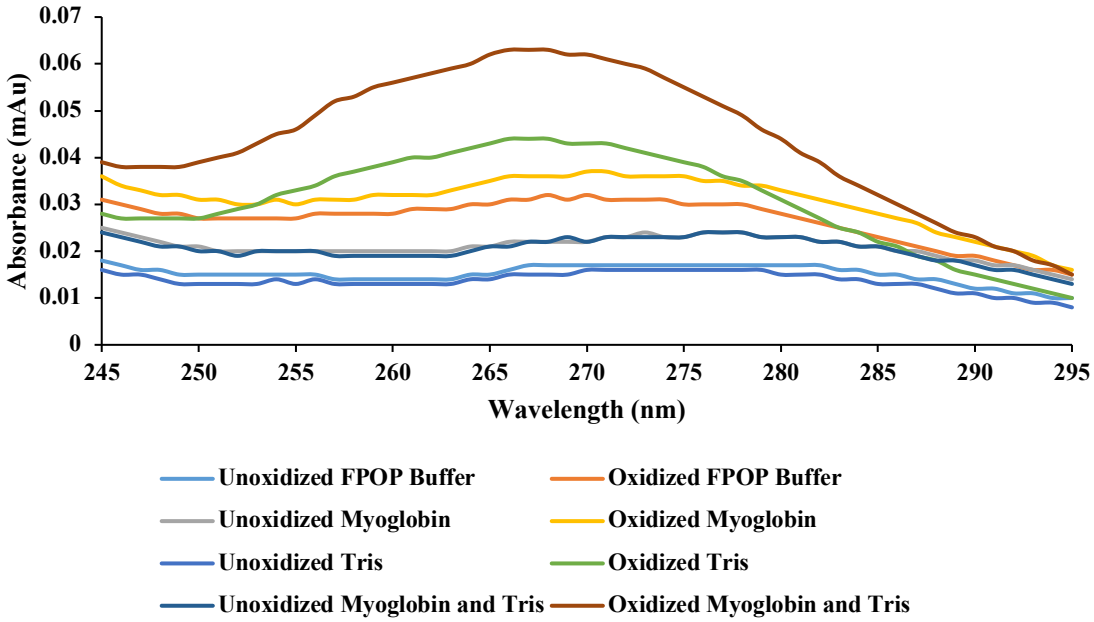


III. Conclusion

It has been clearly demonstrated that the common buffer Tris(hydroxymethyl)-aminomethane can be used as a highly effective hydroxyl radical dosimeter for FPOP experiments. Increases in Tris absorbance correlate strongly with peptide oxidation (R^2 = 0.827) and scavenging capacity (R^2 = 0.9625), and the absorbance loss resulting from increase scavenging capacity can be compensated in real time to maintain consistent protein footprints. This new chromophore is suspected to be the result of formation of a *gem*-diol followed by water elimination resulting in aldehyde and/or imine formation,[33, 34] with a proposed scheme as shown in **Figure 7**.

Several characteristics of Tris suggest that it may be a favorable replacement for adenine dosimeter in many FPOP applications. Because the molecule is UV active in the same range as adenine, no modifications to current measurement technologies are required for its adoption. As shown in **Figure 8**, Tris is the major contributor to absorbance change after laser exposure, so there is little interference from proteins or other buffer components. Tris also eliminates the need for the background scavenger glutamine, thereby simplifying sample preparation. Furthermore, the use of Tris instead of adenine will allow for the application of FPOP to nucleoside and nucleotide binding proteins (a very large category of proteins) without concern about dosimeter interference in protein structure.[2]

Figure 8. 265 nm Absorbance Contribution of FPOP Sample Components



pH was held at 8.01 for all samples, and 17 mM glutamine was used to maintain scavenging capacity in samples not containing Tris. Tris concentration was 8.5 mM, and myoglobin concentration was 5 µM. Oxidation was performed in 100 mM peroxide for all samples.[1]

APPENDIX

Appendix 1. Calculation of Reaction Rates for Hydroxyl Radical Scavengers

Glutamine:

$$v_g = (5.4 \times 10^8 \text{ L mol}^{-1} \text{ s}^{-1})(17 \times 10^{-3} \text{ M})(200 \times 10^{-3} \text{ M})$$

$$v_g = 1.84 \times 10^6 \text{ M s}^{-1}$$

Tris Concentration Required:

$$(1.84 \times 10^6 \text{ M s}^{-1}) = (1.5 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1})[\text{Tris}](200 \times 10^{-3} \text{ M})$$

$$[\text{Tris}] = \frac{(1.84 \times 10^6 \text{ M s}^{-1})}{(1.5 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1})(200 \times 10^{-3} \text{ M})}$$

$$[\text{Tris}] = 6.1 \times 10^{-3} \text{ M} = 6.1 \text{ mM}$$

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