Pro- and Antioxidant Activity of Selenomethionine: Preventative Measures against Metal-Mediated DNA Oxidation

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Pro- and Antioxidant Activity of Selenomethionine:
Preventative Measures against Metal-Mediated DNA Oxidation

A Thesis
Submitted to the Faculty
of
Rose-Hulman Institute of Technology

by

Austin Mroz

In Partial Fulfillment of the Requirements for the Degree
of
Master of Science in Chemistry

May 2017

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**Thesis Title**  Pro- and Antioxidant Activity of Selenomethionine: Preventative Measures against Metal-Mediated DNA Damage  

**DATE OF EXAM:**  April 20, 2017  

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<tr>
<th>Thesis Advisory Committee</th>
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<tr>
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PASSED  X  
FAILED  

1. Complete the table with the names of the examination committee members.
2. Specify the department for each member.
3. Confirm the passed status with an X.
DNA damage by reactive oxygen species (ROS) has been linked to several diseases. Antioxidants are one method of combating the effects of ROS; selenium-containing antioxidants have shown promise in alleviating in vitro DNA damage. This study aims to elucidate the effects of selenomethionine (SeMet) on DNA damage mediated by Cu(II), Fe(II), or Cr(III) metal ions. HPLC analysis was used to probe the oxidative effects of SeMet by quantifying the oxidative damage marker, 8-hydroxy-2’-deoxyguanosine. Prior literature indicates that the antioxidant activity of SeMet is potentially concentration dependent; however, HPLC results in this study indicate a metal-ion dependency. In order to better understand the metal-ion dependence, ITC studies were used to identify if metal coordination plays a role in the observed oxidative effects of SeMet. Cu(II) and Cr(III) ITC results indicate different degrees of metal coordination. This suggests that metal coordination to SeMet may play a role in the metal-ion dependence of the system. In order to more fully understand the metal-ion dependence, SeMet was modeled computationally to determine the most favorable metal binding site to the structure.

Keywords: chemistry, selenomethionine, metal-mediated DNA damage, oxidative damage
DEDICATION

For my mom and dad, Janet and Ron Mroz, who sparked and fostered my love for science, math, and engineering - providing me with the tools and opportunities to pursue my passions.
ACKNOWLEDGMENTS

There are several people without whom this thesis would not have been possible. I would like to thank my thesis advisor, Dr. Daniel Morris for his support, guidance, knowledge and unending patience. I would like to thank Lou Johnson, Lab Manager at Rose-Hulman Institute of Technology Department of Chemistry and Biochemistry, as well as Cyndi Erwin, Department Stockroom Manager. I am very grateful for the assistance of the members of my thesis committee: Drs. DeVasher, Hoffman, Poland, and White. I would like to specifically thank Dr. DeVasher and Dr. Poland for their assistance with the NMR studies, and Dr. Hoffman for her kindness and support in the graduate school process. Further, I would like to thank Drs. DeVasher, Hoffman, Poland, and Tilstra for being such strong female role models in the scientific community. I am also very grateful for the mentorship of Dr. White and his persistent enthusiasm for science. I would also like to thank two students for their support. First, Thomas Scarborough for sharing the results of his SeMet concentration study, which aided in the selection of concentrations used in the HPLC studies. Second, Steven Koos for teaching me how to use the ITC, and for sharing his baseline data. Finally, I would like to thank Dr. Mueller for introducing the idea of pursuing a master’s in chemistry, I would not be here if it weren’t for his assistance and willingness to help develop my plan of study.
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<th>Full Form</th>
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<tr>
<td>8-OH-dG</td>
<td>8-hydroxy-2’-deoxyguanosine</td>
</tr>
<tr>
<td>dG</td>
<td>2’-deoxyguanosine</td>
</tr>
<tr>
<td>DFT</td>
<td>Density Functional Theory</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>HF</td>
<td>Hartree-Fock</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance/Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest Occupied Molecular Orbital</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest Unoccupied Molecular Orbital</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>M</td>
<td>Metal</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SeCys</td>
<td>Selenocysteine</td>
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<tr>
<td>SeMet</td>
<td>Selenomethionine</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>ZPE</td>
<td>Zero Point Energy</td>
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LIST OF SYMBOLS

au  atomic units

eV  electron volts

ΔG  Gibbs Free Energy

ΔH  Enthalpy

J   joule

K   Kelvin

Ka  Binding Affinity Constant

mol mole

M   mole/liter (Molar concentration)

mL  milliliter

ΔS  Entropy

W   watt
1. INTRODUCTION

DNA damage by increased oxidative stress is becoming a growing concern in the healthcare industry. Oxidative stress is caused by several mechanisms, the most common of which is the production of reactive oxygen species (ROS) [1-8]. ROS are generated by both normal metabolic processes and abnormal physiological events, interacting adversely with DNA, lipids, and proteins [1, 4-6, 8-9]. Interactions between ROS and DNA cause, among other results, strand breakages, DNA cross-linking and mutations in synthesis [1, 4, 6, 8-10]. Under normal conditions, an individual cell experiences an average of 10,000 lesions per day [6, 11]. However, under increased ROS generation, or increased oxidative stress conditions, as many as 20,000 lesions occur per day [6, 11]. These DNA lesions lead to representative of mutations in DNA replication, which have been directly linked to an expanding number of diseases including cancer, Alzheimer’s disease, rheumatoid arthritis, pancreatitis, and types of asthma that are associated with increased inflammation [3-4, 6-8, 10, 12-13].

On a molecular level, there are several mechanisms by which ROS are generated. One of the most common mechanisms is ROS production as a result of interactions between endogenous hydrogen peroxide ($H_2O_2$) and metal ions [1-3, 8]. This is a natural physiological process with endogenous metal ions contributing to the slow degradation of nucleic acids [3, 9]. These interactions result in the formation of a hydroxyl radical and are commonly termed Fenton and Fenton-like reactions [3, 7-8, 10, 12, 14-15]:

\[ M^{n+} + H_2O_2 \rightarrow M^{(n-1)+} + HO_2^- + H^+ \]
\[
M^{(n-1)^+} + H_2O_2 \rightarrow M^{n^+} + HO^- + OH^-
\]

\[
HO^- \rightarrow H^+ + O_2^-
\]

\[
M^{n^+} + HO_2 \rightarrow M^{(n-1)^+} + H^+ + O_2
\]

Cu(II)-, Fe(II)- and Cr(III)-mediated hydrogen peroxide reduction mechanisms are the typical forms of \(\cdot OH\) generation by cellular mechanisms [2-8, 10, 14-18]:

\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + O_2^-
\]

\[
Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + H^+
\]

\[
Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + O_2^-
\]

\[
Cu^{2+} + H_2O_2 \rightarrow Cu^+ + O_2^- + H^+
\]

\[
Cr^{3+} + H_2O_2 \rightarrow Cr^{4+} + OH^- + O_2^-
\]

The hydroxyl radical is especially toxic due to its high reactivity and reported rate constants with many biological molecules [10].

The known deleterious activity of oxidative damage results in increased interest in assuaging the negative results. Cells possess two endogenous mechanisms for alleviating oxidative stress: (i) enzymatic and non-enzymatic antioxidants and (ii) DNA repair mechanisms [20-21]. Cells contain DNA repair pathways to manage DNA damage. However, under increased levels of oxidative DNA damage, these mechanisms might not enable the cell to continue function [20-21]. This study focuses on the role of extracellular antioxidants as the driving force behind the alleviation of DNA damage by ROS generation. There are three main mechanisms by which extracellular antioxidants are hypothesized to work: (i) radical scavenging (ii) prevention of radical formation by metal chelation and (iii) the formation of a metal-DNA-antioxidant adduct [1, 5, 21-23].
Radical Scavenging \[ A + \cdot OH \rightarrow A - OH \]

Metal Chelating \[ M^{n+} + mA \rightarrow M^{n+} - A_m \]

Adduct Forming \[ M^{n+} - DNA + A \rightarrow A - M^{n+} - DNA \]

where A denotes the antioxidant. Radical scavenging antioxidants work by reacting with ROS before other reactants have the opportunity to. Metal chelation antioxidants reduce oxidative damage by chelating the metal ions, thereby preventing them from reacting with the hydrogen peroxide and subsequently forming \( \cdot OH \). Lastly, antioxidants that work by forming adducts may prevent DNA damage either by preventing \( H_2O_2 \) from reacting with \( M^{n+} \) or by making \( M^{n+} \) unreactive toward \( H_2O_2 \) by shifting its redox potential. Several molecules and compounds have been identified as effective antioxidants—preventing and/or repairing oxidative DNA damage. Selenium (Se) and sulfur (S) compounds, specifically, are becoming increasingly prevalent in studies focusing on the alleviation of oxidative DNA damage by free radical generation [15, 24].

Se is a common trace element and an essential dietary component for many species [12, 16, 22, 23, 25]. The suggested daily intake of Se is 55 \( \mu \)g/day with the primary form being selenomethionine (SeMet), followed by selenocysteine (SeCys) [12, 23, 26]. These organic forms of Se are primarily ingested in the form of plants, which absorb it from the soil [12].

High concentrations of Se result in selenotoxicity or selenosis, and has been understood to be about 3200-5000 \( \mu \)g/day [12]. Under biological conditions selenotoxicity was characterized by Zainal et al. as an increase in ROS production – thereby implicating it as a pro oxidant [12, 27]. Further, selenotoxicity was manifested as several conditions including gastrointestinal disorders, liver cirrhosis, and neurological damage [23]. On the other hand, Se-deficiency has been correlated with an increased risk of Alzheimer’s, de-
pression, rheumatoid arthritis, pancreatitis, and endemic cardiomyopathy, among others [12, 15, 23]. These diseases are linked to increased oxidative stress. In this way, Se compounds are understood to work as both pro and antioxidants [12, 23, 28].

The scientific evidence of the importance of Se in biological systems, implicates Se as an especially promising, naturally-occurring oxidant. As stated previously, SeMet is one of the primary forms of dietary Se [12]. SeMet is non-specifically incorporated into proteins in place of methionine (Met) [12, 23, 29-32]. This non-specific incorporation likely accounts for the increased Se tissue levels with SeMet supplementation [12, 31, 33-34]. Further, Se-containing amino acids represent the least toxic form of Se compounds [35, 36]. Moreover, the higher tissue concentration of Se due to the non-specific incorporation of SeMet yields increased consideration of the biological role of SeMet in alleviating metal-mediated DNA oxidation. Several studies have reported the benefits of SeMet supplementation and its role as a potential chemotherapeutic agent [8, 24, 31-32, 37-40]. The previously reported antioxidant effects of Se-containing compounds, lower toxicity of Se-containing amino acids and non-specific incorporation in protein synthesis make SeMet a viable candidate for further study in alternative treatments for oxidative DNA damage.

This study aims to quantify the effects of SeMet on metal-mediated DNA damage and elucidate the potential mechanism(s) by which SeMet may exert its oxidative effects. High-Performance Liquid Chromatography (HPLC) studies were used to quantify the effects of SeMet on oxidative DNA damage. Isothermal Titration Calorimetry (ITC) studies were used to determine whether the oxidative effects of SeMet are due to metal coordination and to quantify the thermodynamics of these potential interactions. UV-vis studies were used to determine if metal coordination occurs in the Cu(II) and Cr(III)
systems. Computational studies were performed to better understand the experimental results.

Chapter 2 presents a detailed review of literature of previous studies on the oxidative effects of SeMet, as well as the potential mechanisms by which Cu(II), Fe(II), and Cr(III) interact with DNA and hydrogen peroxide.

Chapter 3 describes the system model. This section presents an overview of the experimental method and analysis — providing a macroscopic outline of the employed analysis techniques.

Chapter 4 presents the materials and experimental methods used to conduct this study. This section also provides the rationale behind the employed experimental method. Furthermore, Chapter 4 provides justification for the conditions that are employed in the experimental method and the application to the conclusions drawn from the results presented in Chapter 5. Chapter 5 presents the results, and subsequent analysis, of the experimental data.

Chapter 6 uses the results presented in Chapter 5 to present a discussion and establish experimental conclusions regarding the present study. The ideas presented in Chapter 6 frame the potential directions for future studies outlined in Chapter 7. Chapter 7 discusses alternative experiments focused on the identification of the effects of SeMet on metal-mediated oxidative DNA damage as well as the potential outcomes of those experiments in light of the present results.
2. REVIEW OF LITERATURE

Oxidative DNA damage has been correlated with the diagnosis of several neurodegenerative diseases in addition to rheumatoid arthritis, pancreatitis, and types of asthma that are associated with increased inflammation [3-4, 6-8, 10, 12-13, 41]. One of the most common causes of elevated levels of oxidative DNA damage is the production of reactive oxygen species (ROS) [1, 3-8, 15-16]. Generation of ROS typically results from a redox reaction between labile (non-protein-bound) metal ions and endogenous hydrogen peroxide ($H_2O_2$) [1, 3-5, 7-8, 10, 14-17, 37, 42]. There are two primary toxicity mechanisms for labile metal ions in biological systems: (i) the elevated levels of oxidative stress and subsequent damage by generation of ROS and (ii) direct interference with the DNA repair mechanisms for oxidative damage [43]. A one-electron, redox reaction with $H_2O_2$ [5] results in the oxidation of Fe(II), Cu(I), and Cr(III) ions to Fe(III) and Cu(II) and Cr(IV), respectively [37]. In this way, redox active metals react with endogenous hydrogen peroxide to generate ROS, which ultimately damages, not only DNA, but lipids, membranes, and proteins [1, 4-6, 8-9, 37, 44]. Oxidative DNA damage results in increased single and double strand breakage, DNA cross-linking, and altered DNA repair processes [1, 4, 6, 8-9, 37].

One crucial cellular function is the maintenance of metal homeostasis [15]. Misregulation of this process results in elevated levels of labile metal ions, which are then able to participate in Fenton or Fenton-like reactions to produce hydroxyl radicals [15]. Excess labile Fe(II) and Cu(II) ions, specifically, have been correlated with hemochromatosis, anemia, diabetes, and cancer, as well as amyotrophic lateral sclerosis (ALS),
Wilson’s, Menke’s and Alzheimer’s diseases [15, 41, 45], further linking metal-mediated ROS production to increased DNA damage.

Two of the most commonly studied metal ions related to oxidative DNA damage and increased risk of disease are Cu(II) and Fe(II) ions [3-4, 10, 15, 17, 28, 46]. In comparing the Cu(II) and Fe(II) ions, the redox cycling of Cu results in the production of Cu(I) ions, which produce ROS 60 times faster than Fe(III) ions [3, 10, 15, 17]. Furthermore, in a study comparing the antioxidant activity of Se compounds against Cu(II)- and Fe(III)-mediated DNA damage, Aruoma et al. reported that Cu(II) ions caused a 14.3 % increase in the amount of DNA damage at pH 7.4 relative to Fe(II) using gas chromatography-mass spectrometry [4]. This study suggests that the Cu(II) oxidative DNA damage mechanism is mediated by reactions between Cu(II) ions that bind site specifically to DNA [4].

Several potential processes behind the biological activity of Cu(II) in regards to oxidative DNA damage have been proposed. One such process is the Fenton-like reaction of Cu(II) ions with endogenous $H_2O_2$, resulting in increased DNA fragmentation due to ROS generation [3-4, 8, 10, 17, 28]. Cu(II) ions may then bind to the lesioned sites and subsequently react with both ascorbic acid (which reduces $Cu^{2+}$ to $Cu^{1+}$) and $H_2O_2$ to produce additional $\cdot OH$ [4, 8]. The proposition of this method aims at explaining the increased ROS generation observed in Cu(II)-DNA systems over Fe(II) ions.

While the presentation of Cu(II) and Fe(II) ions in this light is not attractive, it should be noted that these metal ions are required to promote normal physiological function and are, therefore, an essential part of biological systems [10, 15, 46]. For example, Fe is crucial in several cellular processes including energy metabolism and oxygen transport [10, 42].
In addition to the deleterious effects of ROS generation by label metal ions, ROS such as superoxide radicals ($O_2^-$) and $H_2O_2$ species are byproducts of normal aerobic respiration in mammalian cells [4, 15], and have several beneficial effects on cell biology [37]. For example, ROS are an important safeguard against infection, through participation in several signaling pathways, as well as the mitogenic response [6]. One important distinction between normal and elevated physiological levels of hydroxyl radicals and endogenous $H_2O_2$ is the observed DNA strand breakage under elevated radical levels [4]. Therefore, it is the elevated levels of these byproducts that have detrimental effects on biological systems, calling for improved methods of decreasing and preventing ROS generation [5].

As stated previously, interaction between labile metal ions and DNA can result in DNA strand breakages [4-5, 15-17, 37]. This fragmentation is a direct result of the metal ions binding to the phosphate groups of the backbone and nucleotide bases [5, 8, 37]. When free radicals react with the guanine base specifically, 8-hydroxy-2-oxyguanosine (8-OH-G) is generated [53], Figure 2.1.

\[
\text{guanine (G)} \quad \xrightarrow{+\cdot OH} \quad \text{8-hydroxyguanine (8-OH-G)}
\]

**Figure 2.1:** 8-OH-G is a common indicator of oxidative DNA damage. This species is generated through the reaction between the guanine base of DNA and $\cdot OH$. 
The oxidized mononucleoside form of the guanine base, 8-OH-dG, is one of the most common measurable markers of oxidative DNA damage [37, 43] and was first recognized in 1993 [43]. The presence of 8-OH-dG is also indicative of strand breakage at the guanine base by ROS generation [8, 37]. Normal 8-OH-dG levels were reported by Aruoma et al. to be less than $1.02 \pm 0.09$ nmol/mg DNA [4].

The degree of oxidative damage can be quantified by comparing the levels of 8-OH-dG to undamaged dG [37]. Increased values of the ratio 8-OH-dG/dG indicates elevated levels of oxidative damage by radical generation [37]. Measurements of adenine base levels were used to assess the degree of non-radical-induced oxidative DNA damage [37]. Several methods have been employed to quantify the amount of 8-OH-dG in a system of damaged DNA, including LC/MS with selected-ion monitoring [4], fluorescence studies [37], and HPLC with UV Absorption detection [28, 37, 54].

One way to mitigate oxidative DNA damage is the application of antioxidants to the system. Various methods are useful in determining the mechanism or mechanisms by which antioxidants alleviate oxidative DNA damage. Perron et al. employed gel electrophoresis to examine antioxidant effects of polyphenols on Cu(II)-mediated DNA damage [17]. Other specific methods used to identify the effects of antioxidants include mass spectrometry and cyclic voltammetry [15]. Specifically, electrospray ionization mass spectrometry (ESI-MS) was employed to identify the stoichiometry of coordination between Cu(I) and Fe(II) ions with antioxidants [15]. Electronic absorption spectroscopy is a common tool with which to identify coordination because it results in a shift in absorption maxima and/or hyperchromic or hypochromic effects [28, 37]. For example, examination of Fe(II) coordination has been measured in the UV region [37] and the $\lambda_{max}$ for Se-Fe(II) complexes falls between 311-389 nm [16]. Comparatively, Cr(III) ions
absorb in the visible region at 430 nm, thereby suggesting visible absorption studies as a viable option to determine the presence, and potential extent of metal coordination [37]. To identify the potential coordination complexes that are formed in the interaction between antioxidants and metal ions, $^1$H NMR spectroscopy has also been used [47-48].

Selenium (Se) and sulfur (S) compounds are becoming increasingly prevalent in studies focusing on the alleviation of oxidative DNA damage [16], and have demonstrated potential as effective antioxidants [12, 16, 22-23, 25]. Se compounds, specifically, have been reported to have protective effects in the Alzheimer’s pathogenesis by protecting against Cu and Fe toxicity [42]. Additionally, the Nutritional Prevention of Cancer (NPC) trial cited that 200 µg Se/day supplementation caused a 37% decrease in cancer risk [16]. Furthermore, several studies have illuminated the beneficial effects of Se supplementation, reporting a 50% decrease in cancer incidence with 200µg Se/day over a 4.5 year period [8, 24, 31-32, 37-40]. This is hypothesized to occur through the reduction of DNA fragmentation and chromosome breaks [38].

Se is a semi-metal [49], which acts as an essential biological nutrient that has been observed to have both antioxidant and prooxidant activity [23, 28, 37]. At concentrations of 0.01 mM and 1 mM, Se acts as an antioxidant, protecting cells against ROS activity [2, 47]. However, at higher concentrations, Se promotes ROS generation to the point of apoptosis and carcinogenesis [47]. Moreover, Se is an essential element in that it plays several important roles in biological function, including involvement in several selenoproteins and enzymes such as glutathione peroxidases and thioredoxin reductases. It also aids in protection against UV and ionizing radiation [38]. Glutathione peroxidases are a class of selenoenzymes that have motivated research into small-molecule Se compounds as potential methods of preventing ROS activity [16].
While Se as an element has proven to be beneficial in the prevention of diseases associated with oxidative DNA damage, the form of Se is crucial in the overarching biological effects [38]. Selenoproteins, specifically, have been shown to be more efficient in the alleviation of oxidative damage [38]. Likewise, Se that is not incorporated into a protein has been shown to be less effective at similar concentrations as protein-bound Se [38].

There are several hypothesized mechanisms of action for Se in the alleviation of oxidative DNA damage. First, due to the incorporation of Se in several important enzymes, Se has been hypothesized by Bera et al. to exert its chemopreventative effects by raising the level of repair enzyme activity [38]. This is proposed to be done by increasing the concentration of selenoproteins, which have been shown to decrease oxidative DNA damage [38]. Second, Se may work to curb the activity of ROS by targeting and removing them from the system (radical scavenging) [16, 38].

Several studies suggest that metal coordination is more important than ROS scavenging due to the disconnect between DNA damage mitigation and the oxidation potential of the Se compounds [16, 50-51]. This is despite reported radical scavenging activity of Se compounds [16, 23]. Mass spectrometry studies performed by Battin et al., proposed a 1:1 stoichiometry for the metal coordination between Se antioxidants and Cu(II) and Fe(II) ions [15]. Coordination is reported to occur between the Se atom, as well as the N and/or O atoms in the oragnoselenium compounds [16].

The specific Se compound structure, and subsequent oxidation state of Se [51], is important in determining the antioxidant effects of the compound. For example, compounds containing both amine and carboxylate functional groups show greater inhibition of oxidative damage by Cu(I) ions [15]. Furthermore, compounds containing two Se
atoms were suggested to be more effective than one Se atom in systems damaged by Fe(II) ions [15]. Hart et al. suggested that the degree of antioxidant activity was also dependent on the order of addition of the metal ions and the Se-containing antioxidants in studies involving Fe(II) [37]. They concluded that preincubation of Fe(II) ions with the Se-containing antioxidants resulted in a 66% decrease in the oxidative damage marker 8-OH-dG relative to no preincubation period [37]. This indicates that Se coordinates free Fe(II) ions better than those bound to DNA, which also suggests increased bond strength between DNA and the Fe(II) ions [37]. Hart et al. also suggested metal-coordination as the major mechanism behind Se alleviation of Cu(II) and Cr(III)-mediated DNA oxidation due to UV-vis studies [37]. UV-vis studies of the interactions between \(\text{SeO}_2\) and Cu(II) showed a decrease in total absorbance and a bathochromic shift from 677 nm to 699 nm as increased amounts of \(\text{SeO}_2\) were added to the system [37]. Correspondingly, the Cr(III)-\(\text{SeO}_2\) system resulted in an increase in total absorbance at 590 nm with a bathochromic shift from 585-588 nm [37]. This research group further found that Cu(II)-mediated DNA damage was alleviated by Se-containing antioxidants [37].

SeMet is one of the most common forms of biological Se [12, 26]. Battin et al. reported that SeMet inhibits >90% of oxidative DNA damage at a 1 mM concentration, and it inhibits 46% oxidative DNA damage at a 0.01 mM concentration [2, 16]. This is in contrast to SeCys, which decreases Cu(II)-mediated DNA damage by 50% at 0.1 mM [16]. In this system, Zimmerman et al. reported an \(IC_{50}\) value of 25±0.01 \(\mu\)M. In addition, SeMet has been proven to exert apoptotic effects on cancer cells, thereby inhibiting the growth and spread of cancer [28]. This wide range of effects is believed to be due to the non-specific incorporation of SeMet in protein synthesis.

In an electrochemical study performed by Zimmerman et al., SeMet did not appear
to undergo redox cycling [16]. These results suggest radical scavenging is not the primary mechanism of action for Se-containing antioxidants [16]. Complementary electronic structure studies performed by Zimmerman et al. revealed that the Se antioxidant ligands act as p-electron donors [16]. However, the highest occupied molecular orbital (HOMO) of a hydrated metal-SeMet complex was determined to correlate well with ROS scavenging capability, whereas the HOMO of free elemental Se do not [16], indicating the importance of organoselenium compounds over free Se.

Battin et al. employed ESI-MS to determine the stoichiometric binding ratio between SeMet and Cu(I) ions and found that SeMet binds with labile Cu(I) ions in 1:1, 1:2, and 1:3 ratios (Cu(I):SeMet) [15]. These ratios were correlated with mass-to-charge values of 261.9, 456.9, and 650.9 Da [15]. Using X-ray absorption spectroscopy, Zimmerman et al. determined that Cu(I) and selenium-containing amino acids coordinate in a trigonal planar geometry through the Se, N and O atoms [16]. With regards to Cu(II) ions, Zimmerman et al. used IR and Raman studies to characterize the geometry of (SeMet)$_2$Cu and (SeMet)$_2$Zn. These studies revealed that the metal ions coordinated with SeMet through the N, O, and carboxylic acid groups [16]. Cu(II) and Cu(I) are both relevant metal ions due to the redox cycling in the presence of hydrogen peroxide [5].

In the matter of Fe(II)-SeMet interactions, Battin et al. employed UV-vis studies to examine the potential metal coordination between Fe(II) and Se-containing compounds. Upon addition of Fe(II) to Se-containing compounds no spectral shifts were observed [15], suggesting that metal coordination is non-existent, or very weak [15]. However, ESI-MS studies performed by Battin, et al., suggest that Fe(II) coordinates SeMet in 1:2 and 1:3 (Fe(II):SeMet) ratios [15]. These ratios were correlated with mass-to-charge values of
448.9 and 643.9 Da. Zimmerman et al. also reported that organoselenium compounds, including SeMet and SeCys, coordinate to Fe(II) ions in a 1:1 and 2:1 ligand:metal ratio [16]. Fe(II) has been reported to bind through the N and O atoms, rather than the Se [16], which may be due to the hardness of Fe relative to Se. One potential explanation for the lack of metal coordination in Fe(II)-SeMet systems proposed by Zimmerman et al., is the inactive lone pairs on the Se atoms [16].

The effects of SeMet on Cr(III)-induced DNA damage has not been widely studied. However, oxidative DNA damage by Cr(III) ions is a large concern due to the ability of Cr(VI) ions to easily diffuse across cellular membranes, where they are subsequently reduced to Cr(III). Hart et al. reported that interactions between selenium-containing antioxidants and Cr(III) resulted in hypsochromic and bathochromic shifts in the UV-vis spectra, depending on the compound tested [37], suggesting metal coordination as a potential mechanism of action for the SeMet-Cr(III) system.

Ultimately, prior literature has implicated Se compounds (including SeMet) as radical scavengers, and metal chelators [5, 15-16, 37]. The present study seeks to further elucidate the direct effects and potential mechanisms of action of SeMet on Cu(II)-, Fe(II)-, and Cr(III)-mediated DNA damage.
3. DESCRIPTION OF MODEL

Four techniques were used to characterize the effects of SeMet on each of the metal-mediated DNA oxidation systems: HPLC, UV-vis, ITC, and computational studies. The HPLC studies were used to quantify the effect of SeMet on metal-mediated oxidative DNA damage and discern the role that metal ion coordination may play in its antioxidant behavior [37]. Oxidative DNA damage was assessed by measuring the oxidative damage marker, 8-hydroxy-2'-deoxyguanosine (8-OH-dG), and the undamaged guanosine marker (dG) [37]. To normalize the results, the amounts of oxidative DNA damage are presented as a ratio of nmol 8-OH-dG/dG. This accounts for potential discrepancies in DNA digestion between samples. The chromatograms were obtained at wavelengths 254 and 297 nm. The peak areas were calculated by bringing both sides of the peak to baseline. This was especially important for peaks chromatograms that did not exhibit ideal separation. Three concentrations of SeMet were examined to elucidate the potential concentration-dependent antioxidant behavior of SeMet (0.5 mM, 1 mM, and 2 mM). Reactions include a Control, in which $H_2O_2$ was added to a solution of DNA and $M^{n+}$ to observe maximum 8-OH-dG production ($DNA + M^{n+}$). Reactions in which DNA, $M^{n+}$ and SeMet were combined in different orders were also performed to discern the role of metal binding (Condition 1: $M^{n+} + DNA + SeMet$, Condition 2: $M^{n+} + SeMet$). Each of the concentration studies for each metal ion system was performed in a single set of samples. In a given set, 5 trials of the Control, 5 trials of Condition 1, 5 and trials of Condition 2 were performed. 5 trials of a DNA control (without $M^{n+}$ or SeMet) were also analyzed. Differences between each of the conditions and the control group indicate
the behavior of SeMet as a pro or antioxidant. Differences between the amount of DNA oxidation between Conditions 1 and 2 suggest that metal ion coordination may play a role in antioxidant activity.

UV-vis studies were performed for the Cu(II) and Cr(III) systems to provide a clearer indication of whether direct metal chelation occurred between SeMet and the metal ions in Tris buffer at pH 7.4. UV-vis absorption spectroscopy is a viable method to examine metal coordination system [5, 15, 37]. Should metal coordination occur, a decrease in absorbance at the maximum wavelength for each metal ion is expected to occur as the metal ion is coordinated with SeMet. Fe(II) was not examined in the UV-vis studies because it is readily oxidized to Fe(III).

Isothermal Titration Calorimetry (ITC) studies were used to quantify the thermodynamics of potential reactions between SeMet and metal ions in Tris buffer at pH 7.4. These studies were performed at three different mole ratios (SeMet:metal ion), 1:1, 2:1, and 3:1. These ratios were chosen based on previous mass spectrometry studies of Fe(II)-SeMet and Cu(II)-SeMet systems performed by Aruoma et al. and Battin et al. [4, 15]. ITC data can indicate whether metal coordination occurs and can provide stoichiometry data. ITC is a viable technique for transition metal complexes based on prior work performed by Johnson et al., who examine the efficacy of ITC analysis for metal-ligand binding interactions [46]. In the present study, 3.2 mM metal ion in Tris buffer was titrated into varying concentrations of SeMet (0.46 mM, 0.23 mM, and 0.153 mM) to investigate the potential 3:1, 2:1, and 1:1 (SeMet:M$^{n+}$) complexes. The presence of metal coordination would be reflected in both the shape of the curve, as well as the reported stoichiometry of the reaction [46]. The shape of the curve mirrors that of a pH titration curve and the equivalence point indicates the mole ratio required for coordina-
tion between SeMet and Cu(II) [46, 55]. Fe(II) was not examined using ITC because of
the inability to limit Fe(II) oxidation to Fe(III) in air.

Computational studies were performed to further understand the experimental re-
results. SeMet was first characterized individually using several solvation models. Second,
the metal ions were associated with two different binding sites on SeMet, the amine and
Se, and the carboxylate group. These binding sites were identified based on prior litera-
ture examining SeMet coordination complexes [15, 28-29]. Mehandzhiyski et al. identified
this as a viable computational method for examining potential interactions between metal
ions and ligands [56].
4. EXPERIMENTAL METHODS

4.1 Overview

High pressure/performance liquid chromatography (HPLC) was used to quantify the amount of oxidative damage for reactions involving DNA, metal ions, and $H_2O_2$ in the presence and absence of SeMet. SeMet was obtained from Acros Organics (99+%; L(+)-Selenomethionin, 99+, 3211-75-5). Calf thymus DNA (Deoxyribonucleic acid sodium salt from calf thymus, Type 1: fibers, D1501-1G) was purchased from Sigma-Aldrich® [37]. Three enzymes were used to conduct this study: nuclease $P_1$ from *Penicillium citrinium*, alkaline phosphatase from bovine intestinal mucosa (in buffered aqueous glycerol solution, ≥ 5, 500 DEA units/mg protein, A2356-10KU), and catalase from bovine liver (crystalline suspension in water containing -0.1% thymol, 28 mg protein/units, C30-1G). Each of the enzymes was purchased from Sigma® Life Science. All materials were used without any further purification.

ITC studies were used to determine whether metal-SeMet interactions occur—and if they do, to quantify the thermodynamic properties of the interactions between metal ions and SeMet in solution. As such, the reaction being characterized is:

$$SeMet + M^{n+} \xrightleftharpoons{K_a}{K_d} SeMet - M^{n+}$$

where $K_a$ is the association constant, describing the binding of SeMet to the metal ion. $K_d$ is the dissociation constant representing the release of SeMet from the metal ion. These constants describe the equilibrium in the metal-SeMet reaction. $K_a$ and $K_d$ are
defined as [64],

\[ K_a = \frac{[\text{SeMet} - M^{n+}]}{[\text{SeMet}] [M^{n+}]} \quad (1) \]

\[ K_d = \frac{[\text{SeMet}][M^{n+}]}{[\text{SeMet} - M^{n+}]} \quad (2) \]

Therefore, by definition,

\[ K_d = \frac{1}{K_a} \quad (3) \]

\( K_d \) describes the affinity of the metal ion for SeMet. Therefore, lower values of \( K_d \) are related to increased affinity of SeMet for the metal ion.

ITC measures the changes in heat when metal ions are titrated into SeMet samples. Several different parameters were calculated from this technique, including the binding affinity constant \( (K_a) \), the binding enthalpy \( (\delta H_o) \), and the binding stoichiometry \( (n) \). These values were used to determine the efficacy of SeMet as an antioxidant by metal coordination.

The binding association constant, \( K_a \), can be determined using Gibb’s free energy,

\[ \Delta G = RT \ln(K_a) \quad (4) \]

where \( \Delta G \) represents Gibb’s free energy, \( R \) is the universal gas constant, \( T \) is temperature, and \( K_a \) is the binding constant. \( \Delta G \) can be calculated from several key characteristics of the reaction using,

\[ \Delta G = \Delta H - T \Delta S \quad (5) \]

where \( \Delta G \) is Gibb’s free energy exchange, \( \Delta H \) is the change in enthalpy, \( T \) is the temperature, and \( \Delta S \) is the change in entropy of the reaction.

Several computational studies were performed using Spartan ‘16 from Wavefunc-
tion Inc. [57]. These studies were designed to characterize the interactions between SeMet and each of the metal ions: Cu(II), Cr(III), and Fe(II). DFT B3LYP/6-31G*, DFT ωB97X-D/6-31G*, HF/3-21G, and Semi-Empirical/PM3 and PM6 equilibrium geometry computations were performed to obtain total energies and HOMO-LUMO Band gaps for each of the association complexes.

4.1 Description of Experimental Procedures for HPLC Studies with Cu(II), Fe(II), and Cr(III)

As presented in the Description of Model Section, each metal-mediated system was examined at three different SeMet concentrations, 0.5 mM, 1 mM, and 2 mM. These concentrations reflect the total amount of SeMet in each reaction mixture. Each reaction mixture was prepared to have a total volume of 500 µL and Millipore-grade ultrapure water (resistivity of 18 MΩ at 25°C) was used to bring all samples to the total volume.

Reactions were classified as a control, Condition 1 and Condition 2 and are presented in Table 4.1.

**Table 4.1:** Each SeMet concentration study was composed of a control reaction and reactions described as Condition 1 and Condition 2 to identify the effects of Se-Met on metal-mediated oxidative DNA damage. In this table, $M^{2+}$ is used to represent each of the metal ions (Cu(II), Fe(II), and Cr(III)) in solution. The preincubation period was neglected during the Fe(II) trials due to the fast air oxidation of Fe(II) to the oxidized form, Fe(III).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reaction Scheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$DNA + M^{2+} + \text{preincubation} + H_2O_2$</td>
</tr>
<tr>
<td>1</td>
<td>$DNA + M^{2+} + \text{preincubation} + \text{SeMet} + H_2O_2$</td>
</tr>
<tr>
<td>2</td>
<td>$\text{SeMet} + M^{2+} + \text{preincubation} + DNA + H_2O_2$</td>
</tr>
</tbody>
</table>

For each reaction, 12.5 µL of a 1.0 mM metal solution was used to provide the metal ions. Consequently, the final concentration of the metal ions in each reaction mixture was 25 µM. The Fe(II) solution was prepared from $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ immediately prior to the addition of the metal ion in each reaction scheme. This was done to min-
imize the amount of air oxidation of Fe(II). A 1.0 mM solution of CuSO₄ was used for the Cu(II) studies, and a 0.5 mM solution of CrCl₃ was for provide the Cr(III) studies.

The control reactions allowed for the quantification of the total oxidative DNA damage by ROS species in the absence of Se-Met. For control reactions, a solution containing 0.02 M Tris (pH 7), 0.12 M NaCl, 0.5 mg/µL DNA, and 0.025 mM metal ion were allowed to interact during a 30 minute preincubation period at 34.5°C. Following this incubation period, 20 µL of 3% H₂O₂ was added, resulting in a total sample volume of 500 µL. The reaction mixtures were then incubated for 1 hour.

Condition 1 reactions were used to determine if Se-Met can lower oxidative damage after metal ions have interacted with DNA. Solutions containing the same concentrations as the control reactions were allowed to interact during a 30 minute preincubation period at 34.5°C. Following the preincubation period, 0.01 M Se-Met and 3% H₂O₂ were added. The reaction mixtures were then incubated at 34.5°C for 1 hour.

Condition 2 reactions were used to determine whether or not Se-Met is acting as a preventative force in the reaction mixture by coordinating free metal ions before they are able to interact (and bind) to DNA. Solutions containing the same concentrations as the control were allowed to interact during a 30 minute preincubation period at 34.5°C. Following the preincubation period, 1 mg/mL DNA and 3% H₂O₂ were added. The reaction mixtures were then incubated at 34.5°C for 1 hour.

Following the 1 hour incubation period, 1 µL of bovine catalase was added to each of the reaction mixtures that contained H₂O₂ in order to decompose the H₂O₂ and quench the reaction. 100 µL of 1 M NaOAc buffer (pH 5) was then added to each of the reaction mixtures to adjust the pH of the system so that digestion is optimized. The reaction mixtures were then inverted five times to ensure that they were appropriately mixed, and
placed in a block heater at 95°C for 5-10 minutes to denature the DNA. Upon completion of the 5-10 minutes, the reaction mixtures were immediately cooled in an ice bath for 10 minutes. 5 µL of P₁ nuclease was added to each of the reaction mixtures to cleave the DNA phosphodiester bonds. The reaction mixtures were inverted. The reaction mixtures were then incubated at 34.5°C overnight.

Upon the completion of the incubation period, the samples were removed. 40 µL of 1 M Tris HCl (pH 8, 2.4 g Tris in 20 mL) and 1 µL of alkaline phosphatase were then added to each of the reaction mixtures in order to hydrolyze the phosphate groups of each nucleotide. The samples were then inverted six times and incubated at 34.5°C overnight.

Upon the completion of the second overnight incubation period, the samples were transferred to GE Healthcare Life Sciences Whatman™ syringeless filter devices (Mini-UniPrep™, Nylon Filter Media, Polypropylene Housing, 0.2 µm pore size) for HPLC analysis.

All HPLC analyses were performed using a Shimadzu Prominence-i liquid chromatograph. A Beckman Coulter™ Ultrasphere octadecylsilane column was used for all HPLC studies (45 x 4.6 mm, 5 µm particle diameter), in conjunction with a guard cartridge (4.0 x 3.0 mm). Injection volumes were 50 µL. A 1.0 mL/min flowrate was employed. UV absorption detection was performed at wavelengths of 254 nm and 297 nm.

The mobile phase consisted of 100 % millipure water (resistivity of 18 MΩ cm at 25°C) for the first 0.50 minutes, and then changed to 97% 85 mM AmAc/3% acetonitrile (ACN) at 0.6 min. At 7.50 minutes, the mobile phase was then increased to 100% ACN over the period of time between 7.50 and 12.50 minutes. From 17.50 to 18.0 minutes, the ACN was decreased from 100 to 0% composition, and the millipure water was increased from 0 to 100%.
4.3 Description of Experimental Procedures for UV-Vis Spectroscopy Studies with Cu(II) and Cr(III)

UV-vis spectroscopy was used to probe whether or not the effect of SeMet on Cu(II) or Cr(III)-mediated oxidative DNA damage was due to metal coordination between the SeMet and metal ions. A Varian Cary 4000 UV-vis spectrophotometer with a 1 cm path length quartz micro-cuvette was employed in this study.

Solutions containing 0.02 M Cu(II) or 0.01 M Cr(III) in 40 mM Tris (pH 7.0) and 120 mM NaCl were prepared and placed in a micro-cuvette. A baseline spectrum of the resulting solution was then obtained. Incremented volumes of 0.01 M Se-Met were then added to the micro-cuvette in the spectrophotometer, and the resulting spectrum obtained. The concentration of Se-Met added ranged from 0 to 8 mM.

4.4 Description of Experimental Procedures for Isothermal Titration Calorimetry Studies with Cu(II), and Cr(III)

ITC was used to further examine the potential interactions between SeMet and the metal ions: Cu(II), and Cr(III). Millipore-grade ultrapure water was used for all of the experimental processes involved in this study. A TA Instruments Nano ITC Low Volume instrument was used in conjunction with the TA Instruments Degassing Station, TA Instruments ITCRun$^{TM}$ software [58] and the TA Instruments ITCNanoAnalyze$^{TM}$ software [59]. The stir rate was 350 rpm. Injection volumes were 2.49 $\mu$L. The syringe size was 50 $\mu$L. Therefore, 20 total injections were performed over the course of the titration. The Cu(II) samples were injected at 300 s intervals, while the Cr(III) samples were injected at 700 s intervals. The injection intervals allow the heat of the sample cell to come down to baseline between injections—enabling a more accurate measurement.

Before each trial was run, the ITC was thoroughly cleaned. The titration syringe was
removed from the ITC and allowed to soak in millipore water. 50 mL detergent (Fisher Scientific Contrad 70) was diluted to 1 L with millipure water and pumped through the cell. Following the detergent, 3 L millipure water was pumped through the cell.

The metal ion solutions were titrated into the Se-Met solution. The titration buret contained 3 mM Cu(II), or 8 mM Cr(III) in 40 mM Tris (pH 7.0) and 120 mM NaCl. Each metal ion study was performed at three different concentrations of Se-Met–0.5mM, 1mM, and 2mM–in 40 mM Tris (pH 7.0) and 120 mM NaCl.

All solutions were degassed in a TA Instruments Degassing Station Model No.6326 for 10 minutes. The ITC sample cell was then cleaned by injecting and retracting 300 µL of the SeMet solution three times. After cleaning, 300 µL of the SeMet solution was injected into the sample cell. To ensure that there were no air bubbles in the cell, the syringe tip was rotated inside the cell.

The ITC syringe was cleaned by pulling the metal ion solution through it three times. 50 µL of metal ion solution was pulled into the ITC syringe, and it was ensured that there was an air bubble right above the 50 µL mark on the syringe. The end of the ITC syringe was then dried on a ChemWipe to ensure that none of the syringe sample reacted with the cell sample before the first injection.

4.5 Description of Experimental Procedure for Computational Studies

To fully understand the potential mechanisms of the preventative actions of SeMet against oxidative DNA damage, it is advantageous to fully characterize SeMet using computational methods. All of the computational analyses were performed using Spartan '16 computational software from Wavefunction, Inc [57].

Prior to any computations, SeMet was built in the Spartan software and energy minimized. This molecule was then characterized with four different solvation models.
(water, nonpolar solvent, polar solvent, and gas) using HF/3-21G* calculations.

Two different association complexes for each metal ion were then built in the Spartan software. One association complex positioned the metal ion near the N and Se atoms of SeMet. The second association complex positioned the metal ion near the carboxylate group of SeMet. These complexes were analyzed using Semi-Empirical/PM3 and DFT ωB97X-D/6-31G* for the Cu(II) system, HF/3-21G and DFT ωB97X-D/6-31G* for the Fe(II) system, and Semi-Empirical/PM3 and PM6 for the Cr(III) system.
5. RESULTS

5.1 Results of the Cu(II)-Mediated DNA Damage System

The oxidative DNA damage was quantified by examining the amounts of damaged DNA (represented by the 8-OH-dG peak) relative to the undamaged DNA (dG peak) for the control and Conditions 1 and 2 reactions. Peak area values of the 8-OH-dG (DNA damage marker) and dG (undamaged DNA marker) were obtained from the chromatograms generated by the HPLC studies. The mobile phase composition and detector wavelengths provided a good separation of the products of the HPLC reactions. Figure 5.1 presents representative chromatograms for each of the reactions for the 1 mM SeMet concentration study. This data was taken at 254 nm. The 8-OH-dG peak appears absent due to the higher absorbance of the DNA bases at 254 nm. The intensity of dA N-1-Oxide, the oxidized form of the adenine base (dA), provides an indication of the effects of excess $H_2O_2$ on non-radical oxidation of adenosine (dA).
Figure 5.1: Representative chromatograms for the three reaction conditions at 1 mM SeMet for the Cu(II) HPLC system. The chromatograms for the three conditions are displayed as a y-offset plot, indicating the change in the peak heights as the addition order of the reagents is changed. The dG and 8-OH-dG peaks are the peaks of interest. dA and dA N-1-Oxide denote the adenine and oxidized adenine peaks. dC and dT represent the cytosine and thymine peaks, respectively.

The peak area values from the chromatographic studies are significant because they are directly related to the quantities contained in the DNA digests. Using calibration curves constructed for 8-OH-dG and dG standards, the peak areas were converted to nmol. To normalize the amount of oxidative DNA damage, the ratio of nmol 8-OH-dG/nmol dG was calculated, Figure 5.2. Due to the nature of the reactions performed, the HPLC results cannot be compared across the different concentrations. Instead, relative percent DNA damage was computed for each of the concentration studies and presented in Figure 5.3.
Figure 5.2: (a) HPLC chromatograms were used to calculate the mole ratios of 8-OH-dG to dG for each of the reaction conditions. These mole ratios are products of the reactions outlined in the Experimental section. The effects of three concentrations of SeMet (0.5 mM, 1 mM, and 2 mM) were observed. The error shown represents the standard deviation in the average of 5 trials for each reaction condition. (b) To observe the concentration trends more easily, the peak area ratios were replotted.

Figure 5.2 reveals that the oxidative damage in the control group (which contains no SeMet) far exceeds that of either of the Condition 1 and 2 reactions. This indicates that
SeMet exerts an overall antioxidant effect on the Cu(II)-mediated system, independent of the concentrations that were examined. Comparison across Conditions 1 and 2 reveals a further antioxidant effect under Condition 2. This suggests that SeMet may be acting as a metal chelator when allowed to interact with Cu(II) prior to the introduction of the DNA. However, the significant decrease in oxidative damage across both Conditions 1 and 2 indicate that SeMet may act primarily as a radical scavenger in this system.

For each of the concentration studies, the control group represents the maximum amount of DNA damage. For some of the metal systems, there is large variance in this value. This may be a product of the method used to perform this analysis. The data for each of the concentration studies was obtained in a single batch. Digestion of the DNA by the enzymes across batches may vary. To account for the variances in oxidative DNA damage of the control groups, the percent difference between the control and each of the conditions was calculated and subsequently plotted, Figure 5.3. This approach allows for only the relative effects of SeMet on metal-mediated DNA oxidation to be examined, thereby allowing for comparison of the effects across concentrations.
The relative percent damage between the control group and the experimental conditions at each concentration of SeMet was calculated. This allows for a more accurate comparison between the concentrations, due to the variability in the observed oxidative damage of the control groups. It is evident that the 2 mM concentration of added SeMet resulted in the greatest decrease in oxidative damage. Additionally, there appears to be a direct relationship between concentration of SeMet and the amount of oxidative damage under Condition 2.

The Condition 1 reactions reveal the effects of increased concentrations of SeMet on the Cu(II)-mediated system. As the concentration of SeMet is increased from 1 mM to 2 mM, there is a statistically significant decrease in the amount of oxidative damage. This suggests that increased concentrations of SeMet results in increased antioxidant activity. The results of Condition 2 reactions further support the results of Condition 1. As the SeMet concentration is increased, the amount of oxidative damage is decreased. Further, the results of Condition 1 reactions suggest that SeMet may work to reduce oxidative damage by radical scavenging, ultimately working better at higher concentrations. Comparing the results between Conditions 1 and 2 also indicate that SeMet may act as a metal chelator as well. This is determined by the statistically significant decrease in oxidative damage between the conditions, as well as between the concentrations. As the SeMet concentration is increased, a greater decrease in the amount of oxidative damage
is observed in Condition 2. This reveals that SeMet may also work by metal chelation when allowed to interact with Cu(II) ions prior to the introduction of DNA. In comparing the effects of varying the concentration of SeMet, it is clear that 2 mM of added SeMet has the greatest antioxidant effect on DNA oxidation across both conditions.

Table 5.1 presents the mole ratio values and subsequent uncertainties for the Cu(II) HPLC studies. These values enumerate the oxidative effect of the addition of SeMet to the reaction mixtures, in addition to presenting the percentage difference between the conditions and the control group at each added SeMet concentration.

**Table 5.1:** The data obtained from the HPLC studies with Cu(II) was used to calculate the ratio of damaged DNA to undamaged DNA. The percentage difference between the controls and Conditions 1 and 2 was then calculated, and is presented beneath each trial. The rates are x1000 to illustrate a clearer depiction of the difference between the control and each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.5 mM</th>
<th>1 mM</th>
<th>2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M^{n+} + DNA$</td>
<td>11.3 ± 0.4</td>
<td>11.1 ± 0.2</td>
<td>12 ± 0.2</td>
</tr>
<tr>
<td>$DNA + M^{n+} + SeMet$</td>
<td>7.8 ± 0.3</td>
<td>6 ± 1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(75 ± 2%)</td>
<td>(75 ± 9%)</td>
<td>(40 ± 0.8%)</td>
</tr>
<tr>
<td>$SeMet + M^{n+} + DNA$</td>
<td>7.4 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(70 ± 1%)</td>
<td>(63 ± 1%)</td>
<td>(33 ± 1%)</td>
</tr>
</tbody>
</table>

Ultimately, the results of the HPLC studies indicate that metal chelation may play a role in the observed antioxidant effect of SeMet on the Cu(II)-mediated system. To further examine whether SeMet coordinates Cu(II) ions, UV-vis studies were performed. UV-vis spectra of samples containing Cu(II) ions with increasing amounts of SeMet are presented in Figure 5.4. The absorbance spectra were corrected for dilution, due to the added SeMet to the system. Dilution correction resulted in an upward shift in the overall absorbances of the system, this was remedied by correcting the spectra so that they showed 0 absorbance at a wavelength of 450 nm.
Figure 5.4: UV-vis spectra were obtained for Cu(II) ions with increasing amounts of SeMet. The plots represent the data after correcting for dilution. This plot presents an overlay of the spectra obtained over increased concentrations of SeMet (0 mM - 8 mM). The lack of change in the absorbance of the sample indicates that metal chelation does not occur for the mole ratios that were examined.

Figure 5.4 reveals that addition of SeMet to the Cu(II) solution results in a lack of coordination between Cu(II) ions and SeMet, as determined by the lack of change in the spectra once corrected for dilution and baseline absorbance. There are two potential explanations for the lack of metal chelation in this study. First, SeMet and Cu(II) may not directly coordinate. Second, the mole ratios that were studied do not reach a 1:1 (SeMet:Cu(II)) ratio.

To further determine whether or not metal chelation occurs at higher mole ratios of SeMet:Cu(II) ions, ITC studies were performed. These studies quantified the thermodynamics of the interactions between SeMet and Cu(II) ions in Tris and HEPES buffers, independently, at varying mole ratios. The mole ratios (SeMet:Cu(II)) that were studied included 1:1, 2:1, and 3:1. Figure 5.5 presents the results of the Cu(II)-SeMet ITC studies.
Figure 5.5: ITC binding curve for the Cu(II)-SeMet binding studies. 3.2 mM Cu(II) metal ions in 100 mM Tris Buffer was titrated into varying concentrations of SeMet in 100 mM Tris Buffer. The lack of a distinct equivalence point in the data suggests weak coordination between SeMet and Cu(II) ions.

As depicted in Figure 5.5, there is no discernible equivalence point. However, each of these systems reported a stoichiometric ratio greater than one. 1:1 SeMet:Cu(II) ratio resulted in a mole ratio of 2.526. 2:1 SeMet:Cu(II) ratio resulted in a mole ratio of 6.705. 3:1 SeMet:Cu(II) resulted in a mole ratio of 9.78. The relationship between the varying mole ratios examined is presented in Figure 5.6.
Figure 5.6: ITC binding curves for the varying mole ratios of Cu(II)-SeMet system.

Figure 5.6 further indicates either an absence of metal ion coordination or, at best, a weak coordination that occurs between SeMet and Cu(II). In prior literature, Cu(II) has been shown to bind Tris buffer. To examine whether the weak coordination suggested by the ITC studies presented in Figure 5.5 is due to a competition between Tris and SeMet for C(II), the reaction between SeMet and Cu(II) ions was examined in HEPES buffer, Figures 5.7a and 5.7b.

Figure 5.7: ITC binding curves for the titrations of 3.2 mM Cu(II) 25 mM HEPES buffer into SeMet in 25 mM HEPES buffer.

Cu(II) and SeMet show little evidence of metal coordination in the HEPES buffer system. The 0.23 mM SeMet-Cu(II)-HEPES buffer system resulted in a stoichiometric
ratio of 0.134, while the 0.46 mM SeMet-Cu(II)-HEPES buffer system exhibited a stoichiometric ratio of 0.100. The low stoichiometric ratios coupled with the lack of an equivalence point in each of the plots suggests that metal coordination does not occur between Cu(II) ions and SeMet in HEPES buffer. Furthermore, the discrepancy between the stoichiometric ratios reported by each of the buffer systems (Tris and HEPES) suggests that the coordination may be affected by metal-buffer coordination.

5.2 Results of the Fe(II)-Mediated DNA Damage System

Similar to the Cu(II) HPLC results analysis, the oxidative DNA damage was quantified by examining the amounts of damaged DNA (represented by the 8-OH-dG peak) relative to the undamaged DNA (dG peak) for each of the conditions. Figure 5.8 presents representative chromatograms for each of the conditions for the 1 mM SeMet concentration study. These chromatograms are similar to the chromatograms presented for the Cu(II)-mediated system.
Figure 5.8: Representative chromatograms for the three conditions at 1 mM SeMet for the Fe(II) HPLC system. The chromatograms for the three conditions are displayed as a y-offset plot, indicating the change in the peak heights as the addition order of the reagents is changed. The dG and 8-OH-dG peaks are the peaks of interest. dA and dA N-1-Oxide denote the adenine and oxidized adenine peaks. dC and dT represent the cytosine and thymine peaks, respectively.

To normalize the amount of oxidative DNA damage, the ratio of nmol 8-OH-dG/nmol dG were calculated and presented for each concentration study, Figure 5.9.
Figure 5.9: (a) HPLC chromatograms were used to calculate the mole ratios of 8-OH-dG to dG that were products of the reactions outlined in the Experimental section. The effects of three concentrations of SeMet (0.5 mM, 1 mM, and 2 mM) under a control and two distinct conditions were examined. (b) To observe the concentration trends more easily, the mole ratios were replotted as a line graph. This plot indicates that the 0.5 mM concentration study resulted in a pro oxidant effect under Conditions 1 and 2. Secondly, the HPLC study reveals that there is a greater antioxidant effect under Condition 2 – where the DNA is added after the SeMet and Fe(II) ions are allowed to interact.
The Fe(II) HPLC results reveal the concentration-dependence of the overall oxidant activity of SeMet. At 0.5 mM SeMet, a pro oxidant effect is observed. This is evidenced by the greater increased oxidative DNA damage of Condition 1 and 2 over the control. There is a statistically insignificant difference between Conditions 1 and 2 at 0.5 mM SeMet. Potential mechanisms for the pro oxidant activity of SeMet are presented in the Discussion section. At 1 mM SeMet, there is a statistically insignificant difference between Condition 1 and the control group, while an antioxidant effect is observed under Condition 2. This suggests that SeMet may act as a metal chelator in the Fe(II)-mediated system at 1 mM concentration, due to the decrease in oxidative damage when SeMet and Fe(II) are allowed to interact prior to the addition of DNA. Lastly, at 2 mM SeMet, an overall antioxidant effect is observed – indicating that increased concentration of SeMet results in decreased oxidative damage.

Similar to the Cu(II)-mediated system, there is variance in the amounts of oxidative DNA damage across the control groups. To account for these differences in oxidative DNA damage reported by the control groups, the percent difference between the control and each of the conditions were calculated and plotted, Figure 5.10. Theoretically, the control samples should yield the same amount of oxidative DNA damage across all concentration studies – calculating the percentage difference accounts for the fact that they do not. The percent difference was calculated assuming that the Control trial \((DNA + Fe(II))\) represented 100% DNA oxidation for that system. Furthermore, the reported percentages that are greater than 100% represent the pro oxidant activity of SeMet – where SeMet increased the amount of DNA oxidation from the control trial.
Figure 5.10: The difference between the control group and the experimental conditions at each concentration of added SeMet was calculated to account for the difference in oxidative damage of the control groups. This allowed for a more accurate comparison between the concentrations. It is evident that all of the concentrations offer statistically significant differences between Conditions 1 and 2.

The results of Condition 1 reveal that increased concentrations of SeMet results in an increased antioxidant effect — represented by the decrease in the percent relative DNA damage depicted in Figure 5.10. This result is mirrored in Condition 2, where increased amounts of SeMet results in decreased oxidative DNA damage. Furthermore, the statistically significant decrease in oxidative damage between Conditions 1 and 2 for the 1 mM and 2 mM concentration studies, indicates potential metal coordination between Fe(II) and SeMet. The mole ratios and uncertainties for each of the conditions for the Fe(II) system are presented in Table 5.2.
Table 5.2: The data obtained from the HPLC studies with Fe(II) was used to calculate the ratio of damaged DNA to undamaged DNA. The percentage difference between the controls and Conditions 1 and 2 was then calculated, and is presented beneath each trial. The rates are x1000 to illustrate a clearer depiction of the difference between the control and each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.5 mM</th>
<th>1 mM</th>
<th>2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M^{n+} + DNA$</td>
<td>19 ± 3</td>
<td>9 ± 1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>$DNA + M^{n+} + SeMet$</td>
<td>30 ± 6</td>
<td>8.6 ± 0.6</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>(123 ± 5%)</td>
<td>(99 ± 3%)</td>
<td>(66 ± 3%)</td>
</tr>
<tr>
<td>$SeMet + M^{n+} + DNA$</td>
<td>25 ± 4</td>
<td>6.4 ± 0.7</td>
<td>5 ± 3</td>
</tr>
<tr>
<td></td>
<td>(112 ± 3%)</td>
<td>(78 ± 3%)</td>
<td>(43 ± 5%)</td>
</tr>
</tbody>
</table>

As discussed in the Description of Model Section, UV-vis and ITC studies were not performed with the Fe(II)-mediated system due to the high reactivity of Fe(II) in air.

5.3 Results of the Cr(III)-Mediated DNA Damage System

Similar to the Cu(II) and Fe(II) HPLC results analysis, the oxidative DNA damage was quantified by examining the amounts of damaged DNA (represented by the 8-OH-dG peak) relative to the undamaged DNA (dG peak) for each of the conditions. Figure 5.11 presents representative chromatograms for each of the conditions for the 1 mM SeMet concentration study. These chromatograms were collected at 254 nm. The 8-OH-dG peak appears absent due to the higher absorbance of the DNA bases at 254 nm.
Figure 5.11: Representative chromatograms for the three conditions at 1 mM SeMet for the Cr(III) HPLC system. The chromatograms for the three conditions are displayed as a y-offset plot, indicating the change in the peak heights as the addition order of the reagents is changed. The dG and 8-OH-dG peaks are the peaks of interest. dA and dA N-1-Oxide denote the adenine and oxidized adenine peaks. dC and dT represent the cytosine and thymine peaks, respectively.

To normalize the amount of oxidative DNA damage, the ratio of nmol 8-OH-dG/nmol dG were calculated and presented for each concentration study, Figure 5.12. Relative percent DNA damage was computed for each of the concentration studies and presented in Figure 5.13.
Figure 5.12: (a) HPLC chromatograms were used to calculate the mole ratio of 8-OH-dG to dG. These mole ratios are products of the reactions outlined in the Experimental section. The effects of two concentrations of SeMet (1 mM, and 2 mM) under three distinct conditions were observed. (b) To observe the concentration trends more easily, the mole ratios were replotted.

At a concentration of 1 mM, SeMet exerts a pro oxidant effect on the Cr(III)-mediated system. This is evidenced by the statistically significant increase in the oxidative DNA damage for both Conditions 1 and 2 relative to the control group. Contrarily, at a concentration of 2 mM, the activity of SeMet is condition dependent. Under Condition 1, SeMet exerts a pro oxidant effect. However, under Condition 2, SeMet exerts an antioxidant effect on the Cr(III)-mediated system. The antioxidant effect of SeMet is
only observed under Condition 2 at 2 mM SeMet, suggesting that metal coordination may play a role in the antioxidant activity of SeMet.

Similar to the Cu(II)- and Fe(II)-mediated systems, there is variance in the amounts of oxidative DNA damage between the two control groups. The percent difference between the control and each of the conditions was calculated and plotted, Figure 5.13. The percent difference was calculated assuming that the Control trial \((DNA + Cr(III))\) represented 100% DNA oxidation for that system. Furthermore, the reported percentages that are greater than 100% represent the pro oxidant activity of SeMet where SeMet increased the amount of DNA oxidation from the control trial.

![Figure 5.13](image)

**Figure 5.13:** The difference between the control group and the experimental conditions at each concentration of added SeMet was calculated to account for the difference in oxidative damage of the control groups. This allowed for a more accurate comparison between the concentrations.

As evidenced by Figure 5.13, the activity of SeMet in the Cr(III)-mediated system is both concentration- and addition-order-dependent. The results of Condition 1 reveal that increased SeMet concentration results in a statistically significant increase in oxidative DNA damage. Contrarily, the results of Condition 2 reveal that increased SeMet
concentration results in a statistically significant decrease in oxidative DNA damage. The potential mechanisms by which SeMet may be acting as a pro oxidant are proposed in the Discussion Section. Table 5.3 presents the mole ratios and percent difference values used to generate the Cr(III) plots.

**Table 5.3:** The data obtained from the HPLC studies with Cr(III) was used to calculate the mole ratio of damaged DNA to undamaged DNA, using the 8-OH-dG and dG markers, respectively. The percent difference between the controls and the independent conditions was then calculated, and presented beneath each trial. The presented data represents the data x1000 to more clearly illustrate the difference between the control and experimental conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 mM</th>
<th>2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M^{n+} + DNA)</td>
<td>61 ± 0.5</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>(DNA + M^{n+} + SeMet)</td>
<td>65 ± 0.5</td>
<td>76 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(108 ± 2%)</td>
<td>(125 ± 7%)</td>
</tr>
<tr>
<td>(SeMet + M^{n+} + DNA)</td>
<td>66 ± 0.6</td>
<td>93 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(107 ± 3%)</td>
<td>(81 ± 4%)</td>
</tr>
</tbody>
</table>

Ultimately, the results of the HPLC studies indicate that metal chelation may play a role in the observed antioxidant effect of SeMet at 2 mM under Condition 2 in the Cr(III)-mediated system. To further examine whether SeMet coordinates Cr(III) ions, UV-vis studies were performed. UV-vis spectra of samples containing Cr(III) ions with increasing amounts of SeMet are presented in Figure 5.14. The absorbance spectra were corrected for dilution, due to the added SeMet to the system. Dilution correction resulted in an upward shift in the overall absorbances of the system, this was remedied by correcting the spectra so that they showed 0 absorbance at a wavelength of 800 nm.
Figure 5.14: UV-vis spectra were obtained for Cr(III) metal ions with increasing amounts of SeMet. The plots represent the data after correcting for dilution. Increasing amounts of SeMet cause a decrease in the overall absorbance of Cr(III) in solution. The Cr(III) concentration of 12 mM in a buffer solution of 40 mM Tris solution (pH 7.0) and 20 mM NaCl.

Addition of SeMet to the Cr(III) solution resulted in an overall decrease in absorbance. The change in absorbance is indicative of Cr(III) ions being taken out of solution due to a potential metal coordination complex forming between Cr(III) and SeMet. Furthermore, the mole ratios that were studied fall below a 1:1 (SeMet:Cr(III)) ratio, suggesting there is strong coordination between SeMet and Cr(III) ions. To further elucidate the stoichiometry of the suggested metal coordination and quantify the thermodynamics of the reaction between SeMet and Cr(III) ions, ITC studies were performed for the Cr(III)-mediated system. Both 1:1 and 2:1 (SeMet:Cr(III)) mole ratios were examined for the Cr(III)-mediated system. There was no metal coordination indicated by the 1:1 mole ratio. The 2:1 mole ratio indicated metal coordination between Cr(III) and SeMet, Figure 5.15.
A multiple binding sites model was used to fit the Cr(III) ITC data. This model operates under the assumption that two distinct equilibrium and subsequent coordination structures are formed [Freyer]. This model was applied using the ITC NanoAnalyze Software package discussed in the Experimental Methods section. The multiple binding sites model is indicated through the parabolic nature of the curve, Figure 5.15. This is potentially indicative of two distinct mole ratio ranges that result in two different structures, which are generated by two different reactions. Table 5.4 presents the thermodynamic constants describing the 2:1 SeMet:Cr(III) system. The binding coefficients and the heat of formation do not directly describe the binding interactions between SeMet and Cr(III). However, the stoichiometry of the system described by the mole ratio is a direct indication of the behavior of the SeMet and Cr(III) ion interactions.
Table 5.4: The thermodynamic equilibrium constants describing the SeMet-Cr(III) system reveal the stoichiometry for each of the reactions in the 2:1 (SeMet:Cr(III)) system.

<table>
<thead>
<tr>
<th></th>
<th>Equilibrium 1</th>
<th>Equilibrium 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$ (M$^{-1}$)</td>
<td>$(1.89 \pm 2.2) \times 10^6$</td>
<td>$(4.16 \pm 4.3) \times 10^4$</td>
</tr>
<tr>
<td>Mole Ratio</td>
<td>$1.2 \pm 0.1$</td>
<td>$0.7 \pm 0.4$</td>
</tr>
<tr>
<td>$\Delta H$ (kJ/mol)</td>
<td>$-8.8 \pm 0.4$</td>
<td>$75 \pm 93$</td>
</tr>
<tr>
<td>$K_d$ (M)</td>
<td>$(2.33 \pm 3.10) \times 10^{-6}$</td>
<td>$(5.74 \pm 5.84) \times 10^{-5}$</td>
</tr>
<tr>
<td>$\Delta S$ (kJ/mol · K)</td>
<td>$84 \pm 14$</td>
<td>$335 \pm 301$</td>
</tr>
</tbody>
</table>

The thermodynamic information characterizing the Cr(III)-SeMet interactions reveals the mole ratio of each of the reactions. Equilibrium 1 results in a 1.2:1 ratio of SeMet:Cr(III), while Equilibrium 2 is characterized by a 0.7:1 ratio. This suggests that two SeMet molecules coordinate to one Cr(III) ion, as the concentration of Cr(III) ions is increased.

5.4 Results of the Computational Studies

In an effort to better understand the experimental results, computational methods were explored. These computational studies aim to elucidate potential coordination complexes that may form between SeMet and each of the metal ions. To do this, SeMet was characterized. Potential individually coordination points were identified using prior literature, which suggests that SeMet coordinates metals through the O, N, and Se atoms [28-29, 52], with the significant results of this study indicating that the carboxylate group is the primary site of coordination. Using this information, SeMet and the metal ions were brought closer together, to discern the shifts in energies using several computational methods, namely density functional theory (DFT) and Hartree-Fock (HF). The computational study results presented in this thesis are not conclusive, and require additional
experimental evidence to validate them.

5.4.1 Computational Characterization of SeMet

SeMet was built in Spartan '16 Suite from Wavefunction Inc. and subsequently geometry optimized. Figure 5.16 presents the skeletal structure of SeMet that was used as the model for part of the computational analysis.

![Figure 5.16](image)

Figure 5.16: The skeletal structure of SeMet used as a model for the computational study. More specifically, this structure represents L-SeMet, the conformer that is most biologically relevant.

SeMet was first characterized in several environments to gain a better understanding of the solvent that would yield the most stable conformation. The results, Table 5.5, suggest that the computational solvent does not yield a significant difference in the stability of the molecule.

Table 5.5: Hartree-Fock (HF) HF/6-31G* Energy calculations were performed using Spartan '16. The thermodynamic information was calculated for SeMet in several computational environments supplied by Spartan: water, nonpolar solvent, and polar solvent.

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>Nonpolar Solvent</th>
<th>Polar Solvent</th>
<th>Gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy $\times 10^6$ (kJ/mol)</td>
<td>-7.35</td>
<td>-7.35</td>
<td>-7.35</td>
<td>-7.35</td>
</tr>
<tr>
<td>EHOMO (eV)</td>
<td>-8.72</td>
<td>-8.72</td>
<td>-8.72</td>
<td>-8.71</td>
</tr>
<tr>
<td>ELUMO (eV)</td>
<td>4.29</td>
<td>4.28</td>
<td>4.29</td>
<td>4.12</td>
</tr>
</tbody>
</table>

Table 5.5 indicates that there is no major difference in the results of the solvation models. Therefore, gas phase calculations are a suitable model for this system. This increases the efficiency of the overall calculations.

The HOMO and LUMO surfaces of SeMet were also generated using HF/3-21 com-
putations, Tables 5.7 and 5.6. The HOMO of SeMet lies over the Se atom, suggesting that this may be the most favorable binding site for potential metal coordination complexes. The next lower occupied orbitals (HOMO-1 and HOMO-2) are located about the N and O atoms. This is significant because prior literature suggests that SeMet most likely coordinates metal ions through these two functional groups.

**Table 5.6:** The HOMOs of SeMet were calculated using Hartree-Fock with a 3-21G basis set. The increased electron density at the Se atom of the HOMO indicates Se is directly involved in binding as a ligand. The shift towards the amine and carboxylate groups in the HOMO - 1 and HOMO - 2, respectively, elucidates the order in which SeMet would donate electrons in binding.

<table>
<thead>
<tr>
<th>HOMO</th>
<th>HOMO - 1</th>
<th>HOMO - 2</th>
</tr>
</thead>
</table>

The LUMO of SeMet is located at the carboxylate group, indicating the ability of SeMet to accept electrons into this orbital. The next highest unoccupied orbitals are located at the Se and N atoms of SeMet.

**Table 5.7:** The LUMOs for SeMet were calculated using Hartree-Fock with a 3-21G basis set. The increased electron density on the carboxylate group of the LUMO indicates that MLCT would occur at this location. The shift towards the Se and amine group in the LUMO + 1 and LUMO + 2, respectively, elucidates the order in which SeMet would accept electrons in binding.

<table>
<thead>
<tr>
<th>LUMO</th>
<th>LUMO + 1</th>
<th>LUMO + 2</th>
</tr>
</thead>
</table>
5.4.2 Computational Modeling of the Association Complexes

Due to the metal compounds used in the experimental studies, hydrated complexes of the metal ions were analyzed individually. These computations were performed using DFT B3LYP/6-31G* energy minimized structures, and subsequent DFT B3LYP/6-31G* and ωB97X-D/6-31G* energy calculations. These calculations were performed in the gas phase, as well as in water. This was done to gain a better understanding of the difference between the two calculations.

SeMet was modeled in the neutral form for the coordination geometry studies. This was done in light of prior coordination studies of Cu(I) with SeMet performed by Wang et al., which employed the neutral amine group to maintain the +1 charge of the system [28]. Furthermore, this approximation is supported by the calculations presented in the preceding section.

Table 5.8 presents the computational data for the association complexes that were formed between SeMet and Cu(II). Cu(II) was associated to the carboxylate group, and to the nitrogen and selenium atoms in SeMet. Only PM3 calculations were used to characterize the resulting association geometries due to the continual errors resulting from all other computational approaches. The association geometry generated when Cu(II) associates with the nitrogen and selenium atoms of SeMet is much more stable than association with the carboxylate group. Further, the total energies of SeMet associated with Cu(II) are more stable than those resulting from the association of SeMet and Fe(II). This discrepancy may also be due to the difference in the basis sets used to perform the calculations.
Table 5.8: Two potential association compounds between Cu(II) and SeMet were generated using Spartan ’16 semi-empirical/PM-3 and DFT ωB97X-D/6-31G* calculations. Several key characteristics are presented.

<table>
<thead>
<tr>
<th>Computation</th>
<th>Carboxylate Group</th>
<th>N and Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy*10^6 (kJ/mol)</td>
<td>PM3</td>
<td>DFT</td>
</tr>
<tr>
<td>Energy*10^6 (kJ/mol)</td>
<td>4.82</td>
<td>-11.66</td>
</tr>
<tr>
<td>EHOMO (eV)</td>
<td>-17.51</td>
<td>-15.33</td>
</tr>
<tr>
<td>ELUMO (eV)</td>
<td>-9.94</td>
<td>-8.78</td>
</tr>
</tbody>
</table>

Table 5.9 presents the computational data for the association complexes that were formed between SeMet and Fe(II). Fe(II) was associated to the carboxylate group, and to the nitrogen and selenium atoms in SeMet. Hartree-Fock 3-21G and DFT computations were used to determine the total energy of each of the association complexes that resulted. From the data, it is evident that Fe(II) associated to the carboxylate group yields a lower total energy, suggesting a more stable conformation of the association complex.
Table 5.9: Two potential association compounds between HS Fe(II) and SeMet were generated using Spartan '16 HF/3-21G and DFT ωB97X-D/6-31G* Equilibrium Geometry calculations. Several key characteristics are presented.

<table>
<thead>
<tr>
<th>Computation</th>
<th>Carboxylate Group</th>
<th>N and Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy*10^6 (kJ/mol)</td>
<td>HF</td>
<td>DFT</td>
</tr>
<tr>
<td>Energy*10^6 (kJ/mol)</td>
<td>HF</td>
<td>DFT</td>
</tr>
<tr>
<td>EHOMO (eV)</td>
<td>-20.49</td>
<td>-16.95</td>
</tr>
<tr>
<td>EHOMO (eV)</td>
<td>-17.8</td>
<td>-17.41</td>
</tr>
<tr>
<td>ELUMO (eV)</td>
<td>-5.68</td>
<td>-7.99</td>
</tr>
<tr>
<td>ELUMO (eV)</td>
<td>-9.67</td>
<td>-9.67</td>
</tr>
</tbody>
</table>

The HOMOs and LUMOs of each of the association complexes were generated, Tables 5.10 and 5.11.

Table 5.10: The HOMOs and LUMOs for the Fe(II) ion associated near the carboxylate group of the SeMet were generated.

![HOMO and LUMO for Fe(II) associated near carboxylate group]

Table 5.11: The HOMOs and LUMOs for the Fe(II) ion associated near the N and Se atoms of the SeMet were generated.

![HOMO and LUMO for Fe(II) associated near N and Se atoms]

Table 5.12 presents the computational data for the association complexes that were formed between SeMet and Cr(III). Cr(III) was associated to the carboxylate group, and
to the nitrogen and selenium atoms in SeMet. Semi-empirical PM3 and PM6 calculations were used because all others resulted in failures to converge—despite revisions to the starting geometries.

Table 5.12: Two potential association compounds between Cr(III) and SeMet were generated using Spartan ’16. Several key characteristics are presented.

<table>
<thead>
<tr>
<th>Carboxylate Group</th>
<th>N and Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Computation</td>
<td>PM3</td>
</tr>
<tr>
<td>Energy*10^6 (kJ/mol)</td>
<td>9.68</td>
</tr>
<tr>
<td>EHOMO (eV)</td>
<td>-23.00</td>
</tr>
<tr>
<td>ELUMO (eV)</td>
<td>-16.94</td>
</tr>
</tbody>
</table>

From the computational data alone, the best coordination geometry is inconclusive. This is because each computational basis set yields a different coordination geometry. The larger difference between the total energies ascertained using the PM6 data set is reason to suggest that Cr(III) coordinates with the N and Se atoms of SeMet. Additional computational trials, and experimental studies using X-ray diffractometry would be needed in order to come to a definitive conclusion.

The results of the Cr(III) ITC studies indicated the ratio Cr(III):SeMet was 1:2. Two separate systems were examined, Table 5.13. First, two SeMet molecules were modeled for equilibrium geometry using DFT B3LYP/6-311G*. A Cr(III) ion was then associated to the structure that resulted from the two SeMet molecules and the energies were calculated using DFT B3LYP/6-311G*. 
Table 5.13: Equilibrium Geometry DFT B3LYP/6-311G* calculations were performed for two SeMet molecules in the gas phase, and two SeMet molecules associated to a Cr(III) ion.

<table>
<thead>
<tr>
<th></th>
<th>E HOMO (eV)</th>
<th>E LUMO (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-5.92</td>
<td>-0.30</td>
</tr>
<tr>
<td></td>
<td>-13.75</td>
<td>-13.56</td>
</tr>
</tbody>
</table>

Association of the Cr(III) ion resulted in a significant decrease in the HOMO-LUMO band gap. The HOMOs and LUMOs for the Cr(III)-2SeMet complex reveal the HOMO to be the $dz^2$ orbital on the Cr(III) ion, Table 5.14.

Table 5.14: The HOMO and LUMO of the 1:2 (Cr(III):SeMet) coordination complex.

Due to the reported participation of the Se, N, and O atoms of SeMet in binding metal ions by prior studies [28-29, 52], SeMet may participate in bidentate or tridentate coordination with metal ions. Bidentate coordination is suggested by DFT studies performed with Cu(II) and SeMet by Wang et al. [28] and experimental potentiometric titration studies performed by Zainal et al. [52]. SeMet as a tridentate ligand is suggested by potentiometric titration and Raman spectroscopy studies performed by Zainal et al. [52]. Further computational and experimental studies are required to fully characterize the potential coordination geometries for SeMet and each of the metal ions in this study.
6. DISCUSSION

The HPLC studies worked to both quantify the oxidative effect of SeMet on each metal-mediated system, as well as illuminating potential mechanisms of action. Pro versus antioxidant activity of SeMet was assessed by examining the amount of oxidative damage on a DNA system with and without SeMet (Control compared to Conditions 1 and 2). Antioxidant activity is characterized by a decrease in the oxidative damage relative to the Control. Pro oxidant activity is characterized by an increased in oxidative damage relative to the Control. As stated in the Description of Model Section, the control for each batch is considered to be 100% oxidative DNA damage because SeMet is not introduced into that system.

Should antioxidant activity be observed, it is likely due to three potential mechanisms: (i) SeMet as a radical scavenger, (ii) SeMet as a metal chelator, or (iii) SeMet as a component of an antioxidant-metal ion-DNA adduct. These are visualized in Table 6.1.

Table 6.1: There are three potential mechanisms by which SeMet may act as an antioxidant: radical scavenging, metal chelation, or formation of a DNA-metal-SeMet adduct.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radical Scavenging</td>
<td>( \text{SeMet} + \cdot \text{OH} \rightarrow \text{SeMet} - \text{OH} )</td>
</tr>
<tr>
<td>Metal Chelating</td>
<td>( M^{n+} + m\text{SeMet} \rightarrow M^{n+} - \text{SeMet}_m )</td>
</tr>
<tr>
<td>Adduct Formation</td>
<td>( M^{n+} - \text{DNA} + \text{SeMet} \rightarrow \text{SeMet} - M^{n+} - \text{DNA} )</td>
</tr>
</tbody>
</table>

Decreases in oxidative damage under Condition 1 \((\text{DNA} + M^{n+} + \text{SeMet})\) suggest that SeMet acts as an antioxidant. This condition, alone, does not provide any indication of potential mechanisms of action. Decreases in Condition 2 \((\text{SeMet} + M^{n+} + \text{DNA})\)
that are greater than those observed in Condition 1, indicate the potential presence of metal chelation. Decreases in Condition 2 that are comparable to the decrease seen in Condition 1 may be explained two ways: either radical scavenging is the primary mechanism of action and metal chelation may not play a significant role in the system, or metal chelation is the primary mechanism of action independent of addition order. Increases in oxidative DNA damage indicate that SeMet exerts a pro oxidant effect on the system. This may be due to several different mechanisms, which will be discussed later in this section.

The results of the HPLC studies are summarized in Table 6.2. The results of the HPLC studies indicate the dependence of the oxidative activity of SeMet on metal ion identity. In this way, the chemistry of the oxidative effects of SeMet is dictated by the metal ion system. Therefore, it is viable to examine each metal ion system independently.

### Table 6.2: Summary of the Condition 1 \((DNA + M^{n+} + \text{SeMet})\) and Condition 2 \((\text{SeMet} + M^{n+} + DNA)\) results across all of the HPLC metal ion studies. The arrow directions represent the magnitude of the 8OHdG marker relative to the control. ↑ indicates an increase in 8-OH-dG marker, ↓ indicates a decrease, −− denotes a case where there is no statistically significant difference between that condition and the control, and X indicates no data. α on Condition 2 results indicates a statistically significant difference in oxidative activity relative to Condition 1.

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>DNA + M^{n+} + SeMet</th>
<th>SeMet + M^{n+} + DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mM 1 mM 2 mM</td>
<td>0.5 mM 1 mM 2 mM</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>↓  ↓  ↓</td>
<td>↓^α  ↓^α  ↓^α</td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td>↑  −−  ↓</td>
<td>↑^α  ↓^α  ↓^α</td>
</tr>
<tr>
<td>Cr^{3+}</td>
<td>X  ↑  ↑</td>
<td>X  ↑  ↓^α</td>
</tr>
</tbody>
</table>
SeMet is observed to exert an overall antioxidant effect on the Cu(II)-mediated system. This is observed as a decrease in oxidative damage relative to the control group for both Conditions 1 and 2. This suggests that SeMet is acting by one or more of the possible antioxidant mechanisms presented in Table 6.1. The statistically significant decrease in oxidative damage of Condition 2 relative to Condition 1 for all three concentration studies suggests the presence of metal chelation in the antioxidant activity of SeMet.

However, the presence of direct interaction between Cu(II) ions and SeMet in Tris buffer at pH 7.4 is not supported by the UV-vis studies of the Cu(II)-mediated system. Addition of SeMet samples of Cu(II) ions did not result in a change in absorbance or a shift in maximum absorbance. A decrease in overall absorbance would be expected should metal ions be taken out of solution due to binding with SeMet. There are two potential explanations for the lack of coordination. First, a 1:1 mole ratio (SeMet:Cu(II)) was never reached when adding SeMet to the system. Therefore, there may not have been enough SeMet in solution to fully coordinate the Cu(II) ions. Second, these results may also indicate that there is no direct interaction between SeMet and Cu(II) ions.

The lack of coordination indicated by the UV-vis studies is further supported by the weak coordination suggested by the ITC results. While there is a weak inflection point observed in the 3:1 (SeMet:Cu(II)) study, this may be due to interactions between Cu(II) and Tris buffer. This is further supported by the lack of equivalence point observed for the same Cu(II)-SeMet system in HEPES buffer. These results contradict prior literature, which cites the presence of metal coordination between Cu(II) and the N and O atoms of SeMet using Raman spectroscopy and pH potentiometric techniques [52]. The difference in experimental methods used to analyze the system may explain the discrepancy in the results. Zainal et al. used Raman spectroscopy to examine the solid
formed between Cu(II) and SeMet, which was reported to take the form $Cu(SeMet)_2$. Furthermore, these studies were performed in NaNO$_3$. Furthermore, Hordyjewska et al. cited that Cu(II) ions bind preferentially to nitrogen or oxygen donors, as opposed to the preference for thiol groups by Cu(I) ions [41].

While the experimental data of the present study does not indicate that SeMet directly coordinates Cu(II) ions, strong coordination between SeMet and Cu(I) ions has been cited by prior literature [15, 23, 28]. Wang et al. cited strong metal coordination between SeMet and Cu(I), which was indicated through mass spectrometry studies [28]. In light of these findings, an alternative explanation for the Cu(II) system may be proposed. Perron et al. reported that in the presence of hydrogen peroxide, Cu(II) undergoes redox cycling [5],

$$Cu^{2+} + H_2O_2 \rightarrow Cu^{1+} + OH + ^{\cdot}OH$$

Therefore, in the HPLC reaction system, it is possible that Cu(II) underwent the reaction to form Cu(I) ions, which then formed metal coordination complexes with SeMet. Another way of forming Cu(I) in the studied system is [8],

$$Cu^{2+} + DNA \rightarrow Cu^{2+} - DNA \rightarrow HOO - Cu^{+} - DNA$$

Kawanshi et al., proposes that Cu(II) binds preferentially to the phosphate group of guanine, and this complex is the reactive species of the Cu(II)-mediated system [8]. In this way, SeMet in the HPLC studies may participate in metal chelation by directly coordinates Cu(I) ions. Further studies into the exact binding interactions between Cu(II) and SeMet are needed to draw further conclusions. Potential future studies are presented in Section 7.
The HPLC results for the Fe(II)-mediated system reveal the importance of SeMet concentration in its observed effect on oxidative DNA damage. The SeMet concentration of the system appears to dictate the oxidative activity. At 0.5 mM SeMet, a prooxidant effect is observed. However, as the concentration of SeMet in the system is increased to 1 mM and 2 mM, it exerts an antioxidant effect.

Further, there is a statistically significant decrease in the observed oxidative damage between Conditions 1 and 2. This is indicative of metal chelation. While the present study did not perform ITC or UV-vis studies for the Fe(II)-mediated system due to the high reactivity of Fe(II) in air, prior literature indicates that Fe(II) binds with SeMet. Zimmerman et al. reported that Fe(II) is most likely to bind with the N and O atoms of SeMet. This is further supported by the computational studies presented in this study, which indicate the participation of the carboxylate group of SeMet. Further, association between an Fe(II) ion and SeMet resulted in a lower lying HOMO for Fe(II). Potential prooxidant mechanisms of action are discussed later in the section.

The Cr(III) system indicates that SeMet acts singularly as a prooxidant at 1 mM independent of addition order. However, at 2 mM SeMet, the oxidative activity is dictated by the addition order of the reagents. In this way, SeMet in the Cr(III) system is both concentration and addition order-dependent. One possible explanation for the oxidant activity differences between Conditions 1 and 2 for the 2 mM study, is the potential increased affinity of DNA for Cr(III) over SeMet. Strong interactions between Cr(III) and DNA have been suggest by prior literature [18-19, 43]. Moreover, increasing the concentration of SeMet from 1 mM to 2 mM under Condition 1 results in a statistically significant increase in oxidative DNA damage. This suggests that SeMet is accelerating the oxidative damage process in a concentration-dependent mechanism. Potential pro
oxidant mechanisms are discussed later in this section.

In light of the metal coordination between SeMet and Cr(III) suggested by the ITC studies, the antioxidant effect of SeMet at 2 mM under Condition 2 may be due to strong metal coordination between Cr(III) and SeMet. The ITC results indicate that two coordination reactions occur in the Cr(III)-SeMet system. Cr(III) coordinates SeMet in a 1.2:1 and a 0.7:1 (SeMet:Cr(III)) ratio. The ITC results suggest that Cr(III) coordinates two SeMet molecules. The computational results of the present study indicate that Cr(III) associates with the carboxylate groups of two SeMet molecules. However, the resulting complex is highly unstable relative to association between two SeMet molecules. This is concluded based on a decrease in the HOMO-LUMO Band Gap between the two systems.

The computational results are presented to supplement the experimental data. SeMet was first characterized in different solvent systems. These results indicated that the solvent system did not affect the chemistry of SeMet. The HOMO of SeMet is located over the carboxylate group across all solvent models. This indicates the overwhelming effect of the carboxylate group on the chemistry of SeMet. These results correlate with previous studies, which cite the involvement of N and O atoms in SeMet coordination with metal ions [28, 52].

The HOMO and LUMO of a molecule are a key feature of metal coordination. Therefore, it is important to note that the HOMO of SeMet is located at the Se atom. The HOMO-1 is located at the amine and carboxylate groups. The LUMO is located at the carboxylate groups, and the LUMO+1 is located at the Se atom. This is significant in illustrating the crucial role that the carboxylate group may play in binding. However, several sources cite the involvement of the amine and carboxylate groups in metal binding, suggesting that the extra electron pairs carried by the N and O atoms play a larger
To further elucidate potential metal coordination complexes, the metal ions were associated to two distinct binding sites on SeMet: (i) between the N and Se atoms, and (ii) near the carboxylate group. These association sites were determined in light of prior literature and the computational results of this study, which suggest that coordination between SeMet and metal ions occurs through the N, Se, and O atoms [28, 52]. The results are summarized in Figure 6.1.

**Figure 6.1:** The results of the semi-empirical PM3 computational trials were used to illustrate the relationship between the different coordination compounds. This data was used because it is the only common computational method across the three metal ions. -O refers to the compounds in which the metal ion is associated near the carboxylate group. -N refers to the compounds in which the metal ion is associated near the N and Se atoms.

The role of the carboxylate group in binding is further implicated in the summary of the association results. For both the Fe(II) and Cr(III) systems, the carboxylate asso-
cation complex (depicted as M(n)-O in Figure 6.1) yields a lower total energy than the corresponding amine-Se association complex (M(n)-N in Figure 6.1). This is interesting in light of prior literature, which cites that hexaaqua Cr(III) complexes, specifically, are known to bind easily with the N atoms of the nucleotides DNA, resulting in DNA-DNA cross-linking [51]. This may suggest that coordination between Cr(III) and SeMet may occur through the N atom. However, this is not supported by the computational results of the present study, which indicate that the carboxylate association complex is less reactive than the N and Se association complex. The Cu(II) results suggest that this metal ion may bind more preferentially with the amine group of SeMet. Further computational and experimental results are required to offer additional evidence for the hypotheses generated by these results, as well as validating the computational system.

Pro oxidant activity of SeMet is observed in both the Fe(II) and Cr(III) systems. This activity is dependent on the concentration and addition order, within each of the metal ion systems. The present study proposes two potential mechanisms of action for the observed pro oxidant effect. First, SeMet may generate oxidative DNA damage when it interacts with •OH to form a radical. Second, redox cycling of SeMet may promote redox cycling of the metal ion, which can then interact with addition hydrogen peroxide to produce additional •OH.

Mishra et al. report that SeMet interacts with •OH, like those generated by the Fenton and Fenton-like reactions, to produce a SeMet radical, Figure 6.2 [61]. This radical may interact with DNA, causing oxidative damage. In this way, SeMet would add to the metal-mediated oxidative damage, thereby resulting in an observed pro oxidant effect.
Secondly, SeMet may also engage in redox cycling which may work to reduce the oxidized metal ions in solution, thereby promoting •OH generation [50, 23, 60, 63]. This proposal mirrors the supported pro oxidant mechanism of vitamin C [60], Figure 6.3. In this mechanism, vitamin C aids in the redox cycling of Fe(III), reducing it to Fe(II). The Fe(II) ion can then interact with endogenous hydrogen peroxide to generate additional •OH.

Correspondingly, SeMet may act to reduce Fe(III) to Fe(II), thereby enabling it to react with hydrogen peroxide to generate additional hydroxyl radicals, Figure 6.4. This process is also a potential explanation for the pro oxidant activity of SeMet in the presence of Cr(III). In this case, SeMet may reduce Cr(IV) to Cr(III), which then generates hydroxyl radicals by reacting with hydrogen peroxide. Fe(III) and Cr(IV) may be present due to the redox cycling of each metal ion.
Figure 6.4: Similar to the vitamin C system, redox cycling of SeMet may promote generation of •OH by reducing metal ions.

To further support this claim, the redox potentials of each of the metal ions and SeMet were compared, Table 6.3. Redox potentials are important in understanding the system because compounds with lower redox potential can donate electrons to compounds with higher redox potentials.

Table 6.3: The relationship between the redox potentials of SeMet and the metal ions indicate whether or not SeMet may reduce the metal ion.

<table>
<thead>
<tr>
<th>Redox Reaction</th>
<th>Potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$HCrO_4 + 7H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O$</td>
<td>+1.20</td>
</tr>
<tr>
<td>$Fe^{3+} + e^- \rightarrow Fe^{2+}$</td>
<td>+0.77</td>
</tr>
<tr>
<td>SeMet + e^- → SeMet^-</td>
<td>+0.64 [15]</td>
</tr>
<tr>
<td>Cu^{2+} + e^- → Cu^+</td>
<td>+0.153 [15]</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>+0.058 [15]</td>
</tr>
</tbody>
</table>

The redox potentials presented in Table 6.3 indicate that SeMet has the ability to donate electrons to Cr(IV) and Fe(III). This further supports redox cycling of SeMet as a potential pro oxidant mechanism. Moreover, SeMet only exerts a pro oxidant effect in the Fe(II)- and Cr(III)-mediated systems. In the Cu(II)-mediated system, SeMet is only
observed to act as an antioxidant. This metal-specific activity is further supported by the inability of SeMet to donate electrons to Cu(II), due to the higher redox potential of SeMet relative to Cu(II). Thus, this may be an explanation for part of the metal-specific pro oxidant activity of SeMet. Moreover, this correlation further supports the proposed redox cycling mechanism as a potential pro oxidant mechanism for SeMet.

The pro oxidant effects observed in the Cr(III) HPLC studies may also be explained by another hypothesis. Redox cycling within the HPLC reaction mixtures may have increased the oxidation state of Cr(III), rendering it non-reactive with SeMet while still damaging DNA by generation of ROS. The observed increased oxidative damage may be explained by the generation of free radicals from the interaction of SeMet with hydrogen peroxide. This is supported by prior literature, which cites that redox cycling of Cr(III) results in the production of ROS [19]. Redox cycling being the main mode of DNA damage by chromium species is further supported by another study, which claims that interactions between Cr(V) or Cr(VI) and DNA generates the oxidative damage observed with Cr(II) in this study [18]. The oxidative damage may have further been compounded because interactions between antioxidants, like SeMet, and Cr ions of higher oxidation states readily form Cr(III) [18-19, 43, 51]. Further, the evidence of Cr(III)-DNA adducts formation in the presence of hydrogen peroxide [7-8] suggests that the observed pro oxidant activity of SeMet may be a result of a depression of undamaged DNA (dG). The level of dG may be depressed due to an adduct forming between DNA, Cr(III), and SeMet, thereby generating a new compound that may not appear in the dG peak used for the HPLC analysis.

Overall, the results of this study indicate that the oxidative activity of SeMet is first dependent on the identity of the metal ion. The indicated importance of the SeMet
carboxylate group in binding by the computational studies does not explain the metal ion dependency of SeMet activity. Therefore, a different mechanism may be at play. Alternatively, the computational results may reflect a local maxima, which is perturbing the system. Additional experimental and computational results would improve understanding of the direct interactions of SeMet in each of the metal-mediated systems. Potential future studies are presented in the Conclusions and Future Works Section.
7. CONCLUSION AND FUTURE WORKS

The results of this study indicate that the activity of SeMet is largely dependent on its chemical environment — namely the identity of the metal ion that is mediating the DNA oxidation. The varied behavior of SeMet in each of the metal-mediated damage environments, suggests that SeMet activity may be dependent on the oxidation state, and redox cycling.

In looking at Cu(II)-mediated DNA damage, the HPLC results revealed that SeMet decreased oxidative damage across all concentrations for both conditions, additionally indicating that the alleviation is, in part, due to metal coordination between Cu(II) and SeMet. The ITC and UV-vis studies suggested that direct metal coordination between SeMet and Cu(II) ions does not occur. As such, targeted radical scavenging may still be important in the observed antioxidant activity of SeMet. Additionally, redox cycling of Cu(II) to Cu(I) may be representative of the indicated role of metal chelation, due to proven coordination between Cu(I) and SeMet [28]. ITC studies aimed at characterizing the potential interaction between Cu(I) ions and SeMet, would help to verify this hypothesis.

In examining the Fe(II)-mediated system, the HPLC results revealed that activity of SeMet is largely concentration dependent. At 0.5 mM SeMet, SeMet exhibits a prooxidant effect on the system. Whereas, at 1 and 2 mM concentrations, SeMet exhibits an antioxidant effect. While no ITC studies were used to examine the Fe(II)-SeMet system, potential metal coordination was proposed to be weaker by prior literature [15]. While metal coordination may not be the primary mechanism of oxidative DNA damage, it may
be a part of the process. The dual nature of SeMet activity appears to be due to concentration. Further, SeMet may promote the redox cycling of Fe(III) to Fe(II) (which can generate additional hydroxyl radicals) due to the lower redox potential of SeMet relative to Fe(II).

Further analysis of the mechanisms of action for SeMet in the Fe(II)-mediated system is required. First, developing a scheme in which reliable and reproducible ITC data could be obtained for the interactions between SeMet and Fe(II) ions would be invaluable in determining the effect of metal coordination in the alleviation of Fe(II)-mediated DNA damage. Along these lines, conducting cyclic voltammetry studies to examine potential metal coordination in the Fe(II)-SeMet system would be beneficial due to the closed-system nature of this analytical technique. Shifts in voltages between a pure Fe(II) system and an Fe(II)-SeMet system would be indicative of metal coordination. If a shift does not occur, this would be indicative of SeMet decreasing oxidative DNA damage by a radical scavenging mechanism [15]. In light of prior literature presented by Battin et al., metal coordination demonstrated between Fe(II) and Se compounds is weak [15]. Furthermore, determining the concentration at which the shift in SeMet oxidative activity occurs (pro to antioxidant) would be helpful in further analyses as far as determining the mechanism of action for SeMet in both cases.

Cr(III)-mediated DNA damage was increased at 1 mM SeMet, as shown by the HPLC results. However, the addition order-dependence of SeMet is suggested by the pro and antioxidant activity of SeMet at 2 mM. Metal coordination was indicated by the ITC results. These results suggest that Cr(III) coordinates SeMet in multiple locations, and further suggests the presence of multiple reactions at different mole ratios of the two compounds. SeMet has been suggested to act as both a bi- and tridentate ligand depend-
ing on the metal ion system [28-29, 52]. Further experimental and computational work to determine the viability of SeMet as a bidentate ligand in the Cr(III)-system would be beneficial.

This study is not comprehensive in fully defining the behavior of SeMet in the presence of Cu(II)-, Fe(II), and Cr(III)-mediated DNA oxidation. Several additional studies would be useful in characterizing the activity of SeMet. From this study, it is apparent that SeMet coordinates with Cr(III) in a multiple binding site model. This could be further examined using additional analytical techniques that experimentally suggest geometries, which can then be correlated with computational studies. One such technique is NMR. While this study attempted to gain an NMR spectra for the SeMet-Cr(III) interaction, it was unsuccessful in minimizing the water signal of the spectra.

Using ITC studies to examine each of the metal-mediated systems in multiple buffers would also allow a curve to be produced in which the number of protons transferred in the reaction would be quantifiable. Further, well-defined reactions with EDTA could be used to determine the enthalpy of formation for each of the metal-SeMet interactions that were studied. These results would offer improved information on the metal coordination complexes that are formed.

Further, to examine whether SeMet is a HS or LS ligand in the metal coordination complexes that are proven, performing Electron Spin Resonance (ESR) spectroscopy would be illuminating. ESR is a common analytical tool used to examine metal coordination compounds [34-35].

The computational studies performed in this thesis suggests that SeMet binds through the carboxylate group. Additional computational studies are necessary to explore the stoichiometric ratios greater than one that are observed in the ITC studies.
for Cu(II) and Cr(III). Along these lines, modeling SeMet as a bi- and tridentate ligand would be illuminating, in light of prior literature. The experimental results indicated that the overall characteristics of the metal determine the activity of SeMet as a pro or antioxidant. Therefore, it would be beneficial to further explore what may cause each metal ion to interact differently with SeMet, and which coordination complexes may prove to be more detrimental to DNA. It should be noted that further experimental evidence is required to validate the results and provide a clearer direction in potential interactions between the different compounds and future computational studies.

Ultimately, this study supports previous literature as far as the overall effect of SeMet on metal-mediated oxidative DNA damage. SeMet was found to act as an antioxidant in Cu(II)-mediated DNA damage systems, but exhibited weak to no metal coordination as the mechanism of action. In the presence of Fe(II)-mediated DNA damage, the activity of SeMet was dependent on the concentration - acting as a prooxidant at low concentrations, and an antioxidant at higher concentrations. Lastly, SeMet acted as prooxidant in the presence of Cr(III)-mediated DNA damage. Further ITC studies revealed that this activity may be partly due to metal coordination between Cr(III) and SeMet. Cumulatively, the results of this study suggest that SeMet exerts its oxidative effect by different mechanisms. The overall activity and mechanism of action are determined by the identity of the metal ion. The computational results suggest that metal chelation as a potential mechanism of action may be dictate by the interactions of the metal ion and the carboxylate group of SeMet. In light of the reported benefits of SeMet, additional exploration of the effects of SeMet on a variety of metal-mediated systems would be beneficial—especially due to the importance of the identity of the metal ion.
LIST OF REFERENCES


368, 187-193.


