Investigating Arsenic−Protein Interactions Using Mass Spectrometry: From Hemoglobin to p53

by

Aleksandra Popowich

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Department of Chemistry
University of Alberta

Abstract

Chronic exposure to arsenic from groundwater is one of the greatest public health concerns in the world, putting over 170 million people at risk of arsenic-related diseases, including cancer, diabetes, and cardiovascular disease. Although our understanding of the mechanisms by which arsenic induces these biological effects remains limited and unclear, it is known that the binding of arsenic to the thiol groups (R−SH) of cysteine residues in proteins is a contributing factor. The binding of arsenic to proteins may alter their conformation and stability and inhibit their function. This could impede the recruitment of and interaction with other proteins and DNA, resulting in unrepaired DNA and carcinogenesis. Studying the interactions between arsenic and proteins is indispensable for understanding how arsenic affects the body.

A method combining size-exclusion high performance liquid chromatography (SE-HPLC) with inductively coupled plasma mass spectrometry (ICPMS) that enables the separation and detection of protein-bound arsenic and free arsenic was developed. Simultaneous detection of arsenic and sulfur oxides (from cysteine and methionine residues present in proteins) provided supporting information for the identification of the arsenic−protein complex. The quantitative ICPMS detection allowed for the determination of apparent binding constants. Using this method, the interactions between trivalent arsenicals and four proteins, hemoglobin, peroxiredoxin-1, high mobility group box 1 protein (HMGB1), and p53, have been characterized.

Investigating the interaction between arsenic and hemoglobin from seven animal species (cow, guinea pig, human, mouse, pig, rabbit, and rat) aimed to address the interspecies

differences seen in arsenic distribution, retention in the blood, and toxicity. Results demonstrated that rat hemoglobin generally has the highest affinity for trivalent arsenic of all the species studies.

Studying the interactions between arsenic and p53 proteins aimed to determine how known amino acid mutations impact the ability of the protein to bind to transcription factor DNA. The developed HPLC-ICPMS method confirmed the binding of small amounts of PAO^{III} and MMA^{III} to wild type p53. The interaction between wild type or mutant p53 and its transcription factor DNA was investigated using an enzyme-linked immunosorbent assay (ELISA) to monitor the capabilities of the p53 proteins to bind DNA. The ability of various trivalent arsenicals to inhibit the interaction between p53 and DNA was studied by performing the assay in the presence or absence of arsenic, providing further insight into the possible mechanism of arsenic-induced carcinogenesis.

Preface

This thesis is original work by Aleksandra Popowich. No part of this research has been previously published. Components of Chapter 2 have been drafted as a manuscript describing the methodology of the developed technique. A manuscript has been prepared based on the work described in Chapter 3. A manuscript is in progress on the research discussed in Chapter 4.

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*Special thanks to those who had the privilege of living with me and act as my second alarm clock.

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A	Adenine
a.a.	Amino acid
AAS	Atomic absorption spectroscopy
APL	Acute promyelocytic leukemia
AsB	Arsenobetaine
AsO	Arsenic oxide
C	Cytosine
CE	Capillary electrophoresis
cHb	Cow hemoglobin
Cys	Cysteine
Da	Dalton
DMA^{III}	Dimethylarsinous acid
DMA^{V}	Dimethylarsinic acid
DNA	Deoxyribonucleic acid
DRC	Dynamic reaction cell
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ESIMS	Electrospray ionization mass spectrometry
$FlAsH\text{-}EDT_2$	Fluorescin arsenical hairpin binder-ethanedithiol
G	Guanine

gHb	Guinea pig hemoglobin
GSH	Glutathione
GST	Glutathione s-transferase
Hb	Hemoglobin
hHb	Human hemoglobin
His	Histidine
HMGB1	High mobility group box protein 1
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
iAs^{III}	Inorganic arsenite
iAs^{V}	Inorganic arsenate
IC_{50}	Half maximal inhibitory concentration
ICP-OES	Inductively coupled plasma optical emission spectroscopy
ICPMS	Inductively coupled plasma mass spectrometry
LD_{50}	Median lethal dose
mHb	Mouse hemoglobin
MMA^{III}	Monomethylarsonous acid
MMA^{V}	Monomethylarsonic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NanoESI	Nanoelectrospray ionization
$NADP^{+}$	Nicotinamide adenine dinucleotide phosphate

nd	Not detected
nK	Apparent binding constant
nm	Nanometers
OH	Hydroxyl group
PAGE	Polyacrylamide gel electrophoresis
PAO^{III}	Phenylarsine oxide
PAO^{V}	Phenylarsonic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pHb	Pig hemoglobin
PDB	Protein data bank
Prdx1	Peroxiredoxin-1
rbHb	Rabbit hemoglobin
rHb	Rat hemoglobin
R−SH	Thiol group
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
Ser	Serine
SH	Sulfhydryl group
T	Thymine

TMA	Trimethyl arsine
TMAO	Trimethylarsine oxide
TOF	Time of flight
TP53	Tumour protein p53 gene
Trp	Tryptophan
Trx	Thioredoxin
TrxR	Thioredoxin reductase

Chapter 1 Introduction

1.1 Introduction

All matter and living beings are made up of chemicals; they are a ubiquitous part of daily life. Many chemicals contribute to the improvement of the quality of life, while other chemicals are hazardous and can affect human health negatively. Environmental contaminants are a continuous concern because of their detrimental health effects and pervasive nature. Understanding the geographical distribution, sources of exposure, biological mechanisms, and health effects of environmental contaminants is essential to their management. My research focuses on one environmental contaminant, arsenic, and its interactions with proteins.

1.2 Arsenic in the Environment

1.2.1 Sources of Arsenic

Arsenic (As) is naturally-occurring in the environment in both organic and inorganic compounds and in varying oxidation states (-3, 0, +3, +5).[1] The natural abundance of arsenic in Earth's crust is around 3.4 ppm,[2] although it rarely exists in its elemental form. Arsenic frequently is found in high concentrations in sulfur-rich deposits, where it is present as sulfide minerals or sulfur-containing salts, such as arsenopyrite (FeAsS) and realgar (As_4S_4).[3] Arsenic is distributed widely in surface waters, such as oceans, lakes, and rivers, groundwater, and finished drinking water, in varying concentrations, usually in the range of 1–5 µg/L.[4–6] Arsenic is released into water through the weathering and leaching of arsenic-containing rocks and soils. The inorganic form of pentavalent arsenic, arsenate (iAs^V), is the predominant form of

arsenic in most natural surface waters.[1,7–12] Arsenic is present in both fish and crustaceans in the mg/kg level, although the major arsenic species is arsenobetaine (AsB), a non-toxic organoarsenical believed to be an osmolyte.[13] A variety of plants also contain arsenic as iAs^{V} and arsenite (iAs^{III}), which are both easily taken up from water and soil by the cells in roots.[14] There exist several anthropogenic activities that emit arsenic into the environment, including mining and smelting, arsenic-containing pesticides, and fossil-fuel combustion.[15] The extent to which human inputs contribute to environmental arsenic levels remains unclear. Table 1-1 lists the arsenic compounds relevant to this thesis.

Table 1-1. Relevant Arsenic Species

Arsenic Species	Abbreviation	Chemical Formula
Arsenite	iAs^{III}	$As(OH)_3$
Arsenate	iAs^{V}	$AsO(OH)_3$
Monomethylarsonous acid	MMA^{III}	$CH_3As(OH)_2$
Monomethylarsonic acid	MMA^{V}	$CH_3AsO(OH)_2$
Dimethylarsinous acid	DMA^{III}	$(CH_3)_2AsOH$
Dimethylarsinic acid	DMA^{V}	$(CH_3)_2AsO(OH)$
Phenylarsine oxide	PAO^{III}	C_6H_5AsO
Phenylarsonic acid	PAO^{V}	$C_6H_5AsO(OH)_2$
Trimethyl arsine	TMA	$(CH_3)_3As$
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$

1.2.2 Human Exposure to Arsenic

Exposure to arsenic occurs through air, water, soil, and food, with water being the predominant source.[16] Despite the fact that the World Health Organization, the United States Environmental Protection Agency, and Health Canada have set guidelines for arsenic in drinking water at 10 μg/L,[17–19] it is estimated that over 170 million people world-wide are exposed to arsenic at concentrations surpassing this recommendation.[20–22] Inorganic arsenic (iAs^{V} and iAs^{III}) contaminated groundwater poses a major public health risk in numerous countries, including Argentina, Bangladesh, Chile, China, India, Mexico, and the United States of America.[23] The use of contaminated water via drinking, irrigation, and food preparation all contribute to arsenic exposure. The maximum daily intake of arsenic from water, when consuming 2 L of drinking water containing arsenic levels below 10 μg/L, is under 20 μg. Conversely, individuals consuming drinking water contaminated with arsenic (50–300 μg/L) will have a significantly higher daily intake of arsenic (100–6000 μg). One country that is affected devastatingly by arsenic contaminated groundwater is Bangladesh. In 2005, it was estimated that over 35 million are exposed chronically to arsenic concentrations exceeding 50 μg/L through their drinking water.[20,24–26] Studies also have found that 6.8–20% of wells tested in Bangladesh have arsenic levels surpassing 300 μg/L.[20,26,27] Since the late 1990's, significant efforts have been made to remedy the arsenic problem in Bangladesh, as a result, the number of individuals exposed to arsenic levels above 50 μg/L has been reduced to 20 million.[28] In Canada, 30.3% of the population relies on groundwater as a drinking water source,[29] and although the majority of municipal water has arsenic concentrations below 10 μg/L, there are

several localized areas in British Columbia, Alberta, Saskatchewan, Manitoba, Quebec, New Brunswick, Newfoundland and Labrador, and Nova Scotia above this guideline.[8]

1.3 Arsenic in the Body

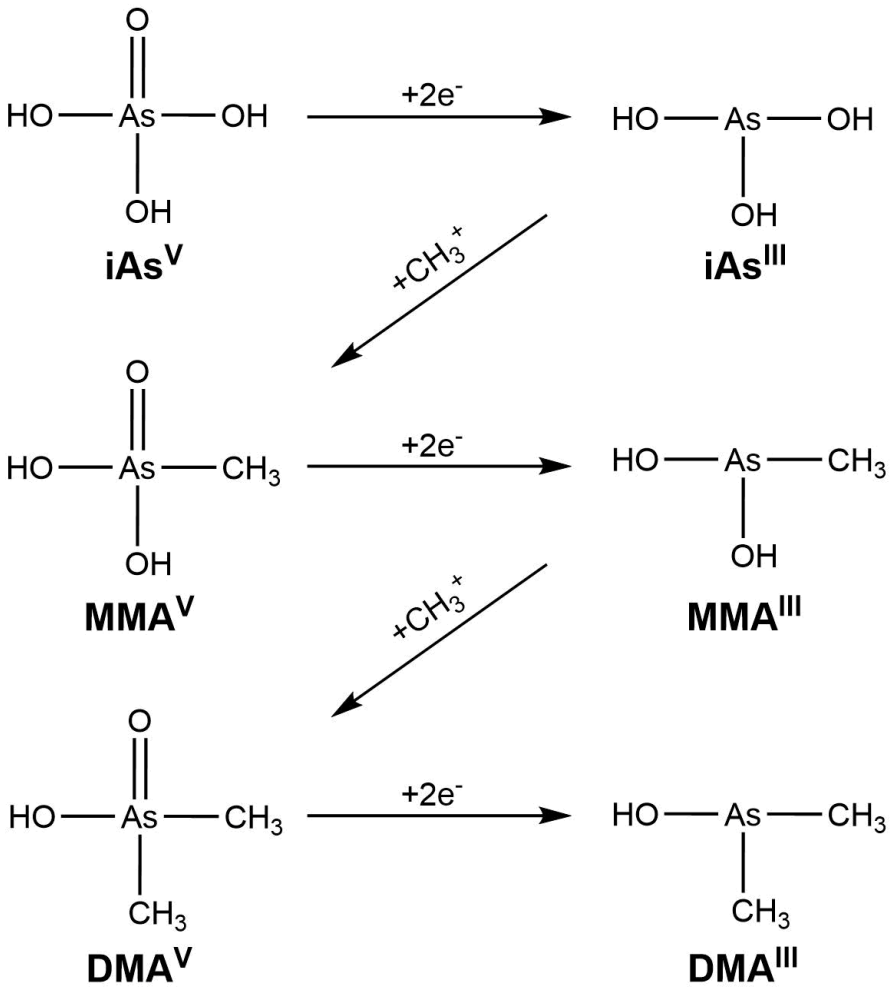
1.3.1 Health Effects of Arsenic

Chronic exposure to arsenic from groundwater puts people at risk of arsenic-related diseases,[21] including bladder, kidney, liver, lung, and skin cancers, skin lesions, hyperpigmentation and hyperkeratosis,[30] diabetes,[31] and cardiovascular diseases,[22,31–37] such as Blackfoot disease.[38,39] Epidemiological studies of arsenic-exposed populations in Argentina,[40,41] Bangladesh,[20,42,43] Chile,[33,44] India,[20,45] Inner Mongolia,[46,47] Taiwan[35,48,49], and the United States[50] reveal a strong correlation between exposure to high levels of arsenic in drinking water and the prevalence of a multitude of cancers.[51–53] Concentrations as low as 50 μg/L of arsenic in drinking water have been shown to be associated with increased cancer risk.[7] The International Agency for Research on Cancer (IARC) has classified arsenic as carcinogenic to humans.[54] Although it is known that many detrimental health effects arise from exposure to arsenic, our understanding of the mechanisms by which arsenic induces these biological effects remains limited and unclear.[7,22]

1.3.2 Arsenic Metabolism and Toxicity

Inorganic arsenic (iAs^{III} or iAs^{V}) is the predominant species of arsenic in nature. Following ingestion, inorganic arsenic is metabolized by the liver.[55,56] Scheme 1-1 shows the recognized pathway of arsenic metabolism, known as the Challenger pathway.[57] This pathway involves

sequential two-electron reductions and oxidative methylations of arsenic, where s-adenosyl methionine (SAM) is the methyl donor and the methylation is catalyzed by methyltransferases.[58–60] It is likely that glutathionine acts as the reducing agent. Arsenic metabolites produced along this pathway include: monomethylarsonic acid (MMA^V), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^V), dimethylarsinous acid (DMA^{III}), trimethylarsine oxide (TMAO), and trimethylarsine (TMA).[59–64] In humans and most mammals, evidence suggests that this pathway stops with the formation of the dimethylated arsenicals (DMA^V and DMA^{III}),[56] as shown in Scheme 1-1, although methylation varies greatly between mammals and some species, including mice and rats, which can further metabolize arsenic to the trimethylated forms.[65–68]



Scheme 1-1. Challenger pathway of arsenic biomethylation in humans.[57]

It has been thought for long that the metabolism of arsenic was a detoxification pathway because the major metabolites found in human urine, MMA^V and DMA^V, are less toxic than inorganic arsenic.[7] However, analytical advances allowed for the more recent detection of the less stable MMA^{III} and DMA^{III} metabolites,[69–72] which are more toxic than inorganic arsenic,[73–76] in human urine, negating the belief that the metabolism of arsenic is a detoxification pathway. The cytotoxicities of arsenic compounds relevant to this thesis are listed in Table 1-2. As these data illustrate, the toxicity of arsenic is strongly dependent on the

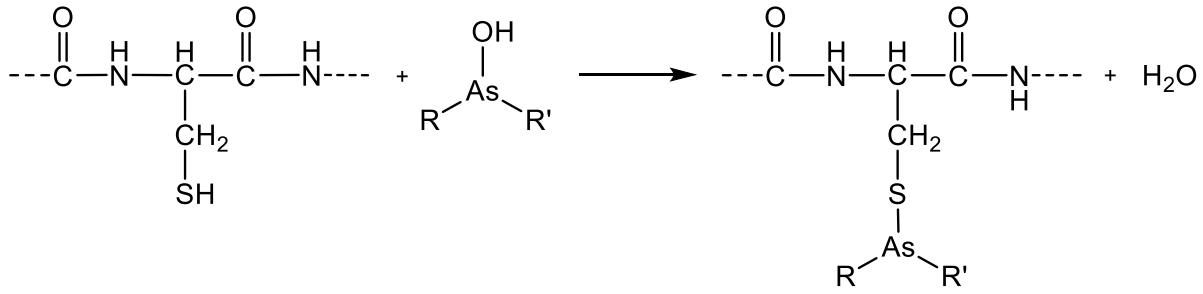
chemical speciation, therefore, it is important to study and understand the effects of each individual arsenic metabolite in the body.

Table 1-2. Cytotoxicity of Arsenic Compounds[73]

Arsenic Compound	Abbreviation	24 Hour IC_{50} (μM)	
		A549 human lung cells	T24 human bladder cells
Phenylarsine oxide	PAO^{III}	0.7	0.08
Dimethylarsinous acid	DMA^{III}	14	1.9
Monomethylarsonous acid	MMA^{III}	14	5
Arsenite	iAs^{III}	74	6.9
Arsenate	iAs^{V}	1400	85
Dimethylarsinic acid	DMA^{V}	6030	1500
Monomethylarsonic acid	MMA^{V}	28000	2600

1.3.3 Mechanisms of Action

The biological effects of arsenic are numerous, and the mechanisms of arsenic toxicity and carcinogenesis are complex and not fully resolved. It is believed that the toxicity of trivalent arsenicals is due to the binding of trivalent arsenic to sulfhydryl (SH) groups in proteins, of which the amino acid cysteine is the only source.[1,77,78] Scheme 1-2 shows the binding of a trivalent arsenic compound to a cysteine (Cys) residue; the arsenical can bind as many sulfhydryl groups as it has hydroxyl (OH) groups. The binding of arsenic to proteins may alter their conformation and stability and inhibit their function. This could impede the recruitment of and interaction with other proteins and DNA, resulting in unrepaired DNA and carcinogenesis.[79] Studying the interactions between arsenic and proteins is indispensable for understanding the mechanisms of arsenic toxicity and carcinogenicity. Furthermore, studying arsenic binding to proteins can assist in the discovery of treatments and methods for preventing arsenic-induced cancers and in the development of arsenic-based therapeutics.



Scheme 1-2. Reaction of a trivalent arsenic compound with a cysteine residue. If R is an OH group, the arsenic compound can bind to a maximum of two cysteines. If R and R' are OH groups, the arsenic compound can bind to a maximum of three cysteines.

1.4 Arsenic Binding to Proteins

1.4.1 Hemoglobin

1.4.1.1 Structure and Function

Hemoglobin (Hb) is the most abundant protein in red blood cells (RBCs), comprising approximately 97% of the dry content of mammalian RBCs;[80] it has two alpha and two beta globin chains, as shown in Figure 1-1. Each protein chain is covalently bound to a heme cofactor. The heme molecule is made of a central iron atom in a heterocyclic ring, known as porphyrin; the iron atom is capable of binding one oxygen molecule (O_2). Hemoglobin functions as an oxygen-transporter, transporting inhaled O_2 from the lungs to the other parts of the body and carbon dioxide (CO_2) back to the lungs, where it is exhaled. A unique feature of hemoglobin is the cooperativity observed during oxygenation; after an O_2 molecule binds to one hemoglobin subunit, the conformation of the tetramer changes, facilitating the binding of oxygen to the other three subunits.[81] The amino acid sequence of the globin chains varies between animal species. Table 1-3 summarizes the locations of cysteine residues in hemoglobin from the animal species relevant to this thesis.

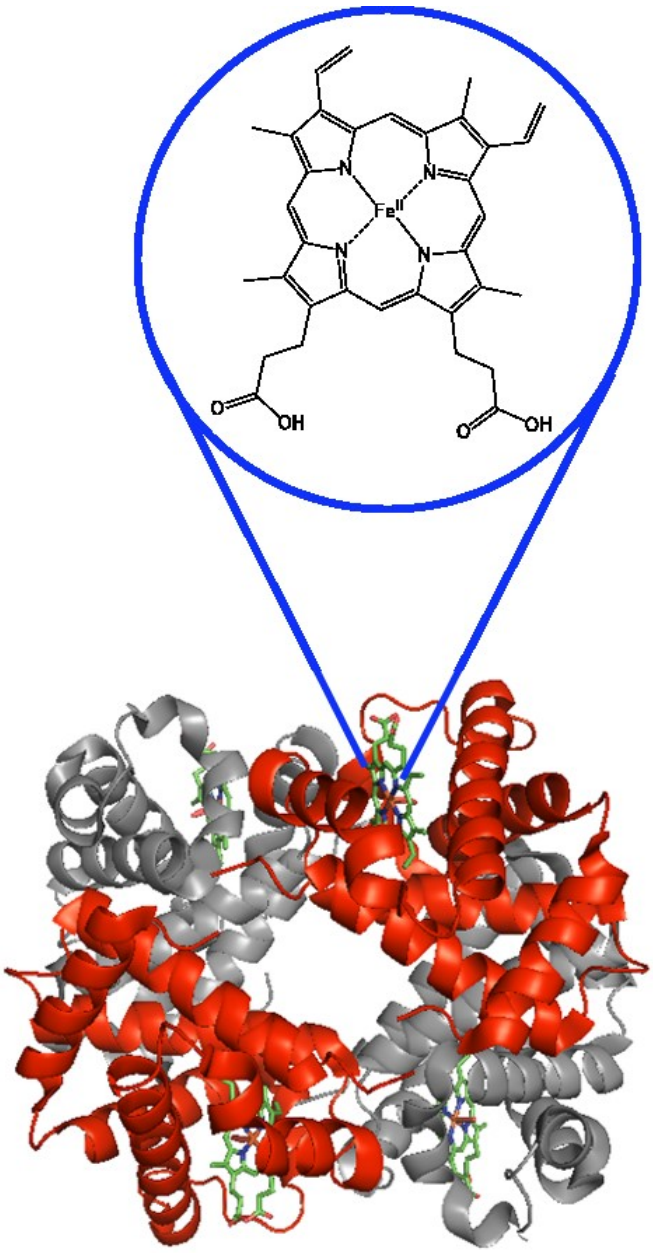


Figure 1-1. The three-dimensional structure of human hemoglobin showing the structure of the heme cofactor. The three dimensional structure of hemoglobin was displayed using PyMol 2.0 (Schrödinger, LLC, New York, NY) and the crystal structure from the Protein Data Bank (PDB) 1GZX.[82]

Table 1-3. Locations of Cysteines in Hemoglobin from Various Animal Species

Species	Cysteines Position in the Alpha Chain	Cysteines Position in the Beta Chain
Cow		91
Guinea Pig	104	93 125
Human	104	93 112
Mouse	104	13 93
Pig	141	93
Rabbit	103	93
Rat	13 104 111	93 125

1.4.1.2 Interactions with Arsenic

Due to its abundance in red blood cells, hemoglobin is known to form complexes with xenobiotics in the blood stream. This interaction can affect both the distribution and toxicity of the chemical.[83] Hemoglobin is predicted to be responsible for the retention and accumulation of arsenic in red blood cells as well as for the transportation of arsenic throughout the body.[84] The retention of arsenic in the blood of rats (around 90 days),[55,85–89] is significantly longer than that in humans (about 1 hour).[90,91] The binding of arsenic to hemoglobin has been deemed responsible for the retention of arsenic in rat blood.[84] Additionally, the binding of arsenic to hemoglobin may reduce the amount of free arsenic in the body, which is consistent with rats having a higher resistance to acute arsenic toxicity than humans. The lower binding of trivalent

arsenic to hemoglobin may lead to more free trivalent arsenic for distribution to other organs and tissues, resulting in higher incidences of arsenic toxicity and arsenic induced cancers.[92]

1.4.2 p53

1.4.2.1 Structure and Function

The p53 protein is a tumour suppressor protein that plays an important role in preventing cancer. Despite its name, p53 has a molecular weight of only 43.5 kDa. The name p53 arises from the mobility of the protein in SDS-PAGE analysis, where p53 migrates slower than normal because it is proline rich, giving it an apparent mass of 53 kDa. Full-length p53 is 393 amino acids in length. The DNA binding domain of p53 is residues 102–292 and contains a tetrahedrally coordinated zinc atom, which is bound to three cysteines and one histidine residue. The three-dimensional structure of the DNA binding domain of p53 complexed with DNA is shown in Figure 1-2.

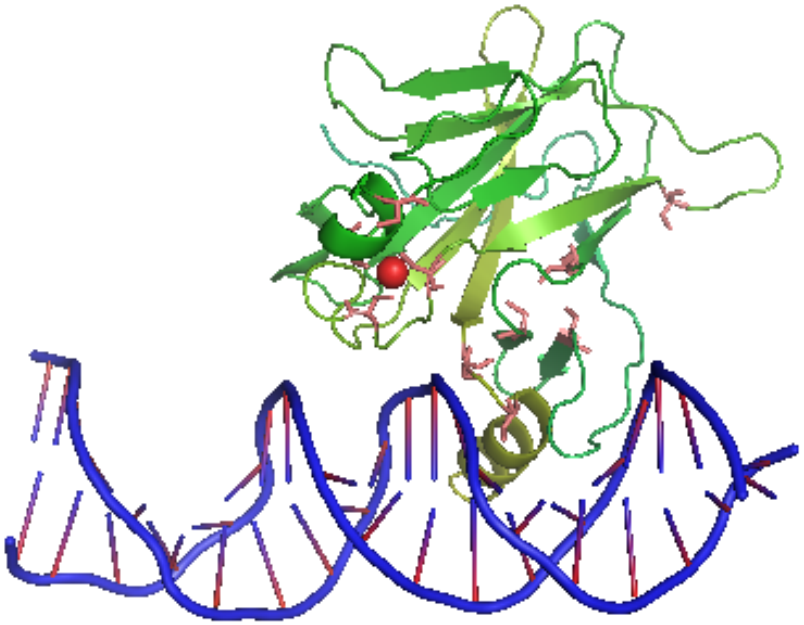


Figure 1-2. The three-dimensional structure of the DNA binding domain of p53 complexed with DNA. Displayed using PyMol 2.0 (Schrödinger, LLC, New York, NY) and the crystal structure from PDB 1TUP.[93]

The p53 protein regulates cell cycles and plays many important roles in tumour suppression.[94–97] Cellular stress, such as oxidative stress or DNA damage, triggers the activation of the p53 protein, which has many anticancer mechanisms, including growth arrest, DNA repair, and apoptosis.[98] Growth arrest stops the progression of the cell cycle, preventing the replication of damaged DNA. This provides additional time for DNA repair proteins to be activated and to fix the abnormal DNA in the cell, prior to the cell cycle continuing. The p53 protein also functions as a transcription factor and can activate expression of DNA repair proteins. Finally, if DNA repair fails, p53 can trigger apoptosis, or programmed cell death, to prevent proliferation of the cells containing damaged DNA. The p53 protein is essential for

tumour suppression and has been dubbed “the guardian of the genome” because of the significant role it has in preventing genomic mutations.[99]

1.4.2.2 Mutations

Inactivation of the tumour suppressor protein p53 frequently occurs during carcinogenesis,[100] to the extent that the p53 gene (*TP53*) is mutated in over 50% of all human cancers. Furthermore, *TP53* mutations have been associated with nearly all types of cancers, with rates varying from 10% in hematopoietic malignancies, for example,[101] to nearly 100% in high-grade ovarian cancers.[102] The most common mutations to the *TP53* gene are single nucleotide substitutions, as opposed to deletions or truncating mutations, which are typical of most tumour suppressor genes.[103] As a result, the cells continue to express full-length p53, but with a single amino acid substitution. There are numerous sites on the *TP53* gene that are susceptible to nucleotide substitution during carcinogenesis, but the mutations generally result in reduced thermal stability and inactivation of p53, and the loss of the protein’s ability to bind to DNA and function as a transcription factor.[104] Research has shown that mutation and inactivation of the p53 protein can provide cells with additional survival strategies, including chemoresistance, the ability to evade apoptosis, and increased proliferation.[105,106]

Trivalent arsenic has been shown to bind to wild type p53.[107] It is possible that this interaction may be a mechanism for arsenic-induced carcinogenesis as the binding of arsenic to p53 could result in reduction of the protein’s stability and inactivation. Moreover, mutant p53 proteins containing cysteine substitutions may have stronger interactions with trivalent arsenic because of the addition of sulfhydryl (SH) groups.

1.5 Hypothesis and Objectives

1.5.1 Hypothesis

Trivalent arsenic can bind to cysteine residues in proteins due to its affinity for sulfhydryl groups. The binding of arsenic to specific proteins may alter their conformation and inhibit their function, affecting the recruitment of and interaction with other proteins and DNA. My thesis focuses on the binding of trivalent arsenic species to two biologically relevant proteins, hemoglobin and p53. I hypothesize that:

1. Trivalent arsenic species bind to hemoglobin of various animal species. The differences in arsenic-hemoglobin interactions are possibly related to the difference in the retention and toxicity of the trivalent arsenic compound for different animal species.
2. Reduction in p53 stability and activity because of arsenic binding is a possible mode of action for arsenic-induced carcinogenesis.

1.5.2 Objectives

The main objective is to study the interactions between arsenic and proteins. The first phase is to develop an analytical method for the identification and characterization of arsenic protein binding. Once this is achieved, the interaction between trivalent arsenic metabolites and four biologically relevant proteins, hemoglobin, peroxiredoxin-1, HMGB1, and p53, will be studied. Investigating the interaction between arsenic and hemoglobin from various animal species aims to address the interspecies differences seen in arsenic distribution, retention in the blood, and toxicity. Studying the interactions between arsenic and p53 proteins aims to

determine how known amino acid mutations affect the strength of the binding and the ability of p53 to bind DNA. Overall, I intended to demonstrate the biological impact of the interaction of arsenic with two important proteins.

1.6 Study methods

1.6.1 Size Exclusion Chromatography-Inductively Coupled Plasma Mass Spectrometry

There are numerous analytical approaches that enable the study of the interaction between arsenic and proteins, many of which are summarized in review papers.[108,109] The methods are generally two-fold, often involving the on-line coupling of complementary analytical techniques. The complex composition of biological samples makes high-resolution protein separation essential, and the trace levels of arsenic require highly sensitive elemental detection. Common protein separation techniques include electrophoresis, particularly sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[110] capillary electrophoresis (CE),[111] and size-exclusion chromatography (SEC).[112,113] Arsenic can be detected using various analytical techniques, including atomic absorption spectroscopy (AAS),[114] inductively couples plasma optical emission spectrometry (ICP-OES),[115] and inductively coupled plasma mass spectrometry (ICPMS),[103] with the ICPMS technique having the highest sensitivity. The facile online coupling of SEC to ICPMS makes it an invaluable technique for studying arsenic–protein interactions. SEC allows for separation of protein-bound arsenic from free arsenic, and ICPMS allows for sensitive and quantitative detection of arsenic. This technique is invaluable for identifying whether an arsenic species binds to a protein and for quantifying the strength

of the binding by determining apparent binding constants. This thesis will describe SEC-ICPMS method that enables studies of arsenic–protein interactions.

Size exclusion chromatography (SEC)[117] separates molecules in solution based on their size. A column contains a stationary phase, which consists of small porous polymer beads that have pores and channels of varying sizes. As the solution flows through the column, smaller molecules can enter the pores easily, spending more time in the stationary phase, whereas larger molecules cannot enter as many of the pores and pass through the column in a shorter time. The result is quicker elution of larger molecules and longer retention of smaller molecules on the column. Figure 1-3 depicts the separation of different sized molecules using SEC.

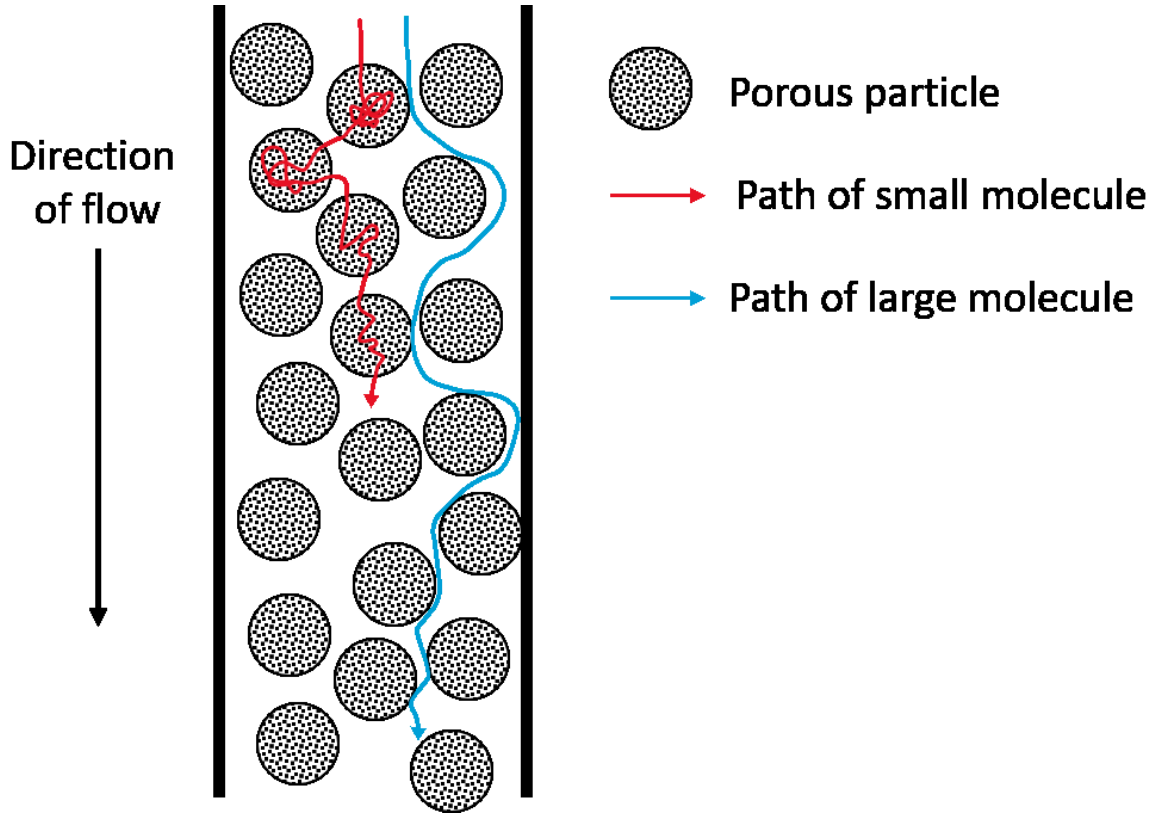


Figure 1-3. Diagram showing the separation of different sized molecules using size exclusion chromatography.

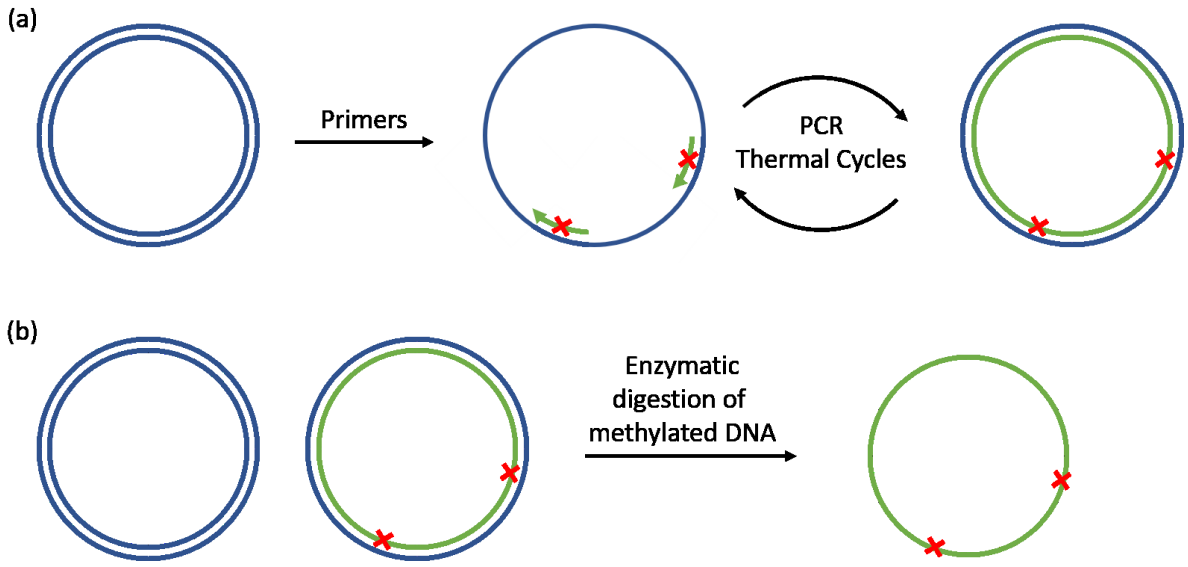
Inductively coupled plasma mass spectrometry (ICPMS)[118] is an analytical technique capable of measuring ultra-low concentrations of elements. A sample is introduced into a high temperature argon gas plasma (6,000–10,000 K) where elements are atomized and ionized. The ions are then separated based on their mass-to-charge ratios and detected using a mass spectrometer. Generally, a quadrupole mass spectrometer is used in ICPMS, which simultaneously can detect multiple elements quantitatively, with high sensitivity and selectivity.

1.6.2 Molecular Cloning and Protein Expression

Molecular cloning[119] allows for the replication of DNA within a living organism. Since the chemical structure of DNA is constant across all living organisms, a segment of foreign DNA can be inserted into the DNA of a host organism, where it will be replicated along with the host's own DNA. Bacteria cells are used commonly as the host organism, with the foreign DNA being inserted into plasmids. A plasmid is a small molecule of double stranded circular DNA that is replicated independently of chromosomal DNA. If the foreign DNA encodes for a protein of interest, the protein can be expressed by the bacteria cells and the plasmid will also encode for a molecular tag to be added to one terminus of the expressed protein. Common tags include His-tag (6 histidine residues) and GST-tag (glutathione S-transferase). These tags allow for easy purification of the recombinant protein using affinity chromatography. The purified protein can be used for a variety of applications.

1.6.3 Site-Directed Mutagenesis

Site-directed mutagenesis[120] is an *in vitro* technique for creating specific mutations in double stranded plasmid DNA. Possible mutations include insertion, deletion, or substitution of nucleotides. Scheme 1-3 shows the basic steps of polymerase chain reaction (PCR) site directed mutagenesis. (a) Oligonucleotide primers containing a single nucleotide mutation from the plasmid DNA containing the gene of interest are annealed to the plasmid. Then, the primers are extended and the mutant plasmids are amplified by PCR. (b) A mixture of original plasmid and PCR product remains. DNA produced by specific strains of *E. coli* is methylated, whereas DNA produced by PCR is not. The methylated non-mutated DNA is digested enzymatically, and only the mutant plasmid remains. Next, the produced mutant plasmid DNA can be transformed into bacteria to be cloned and for use in expression of mutant recombinant proteins.



Scheme 1-3. Schematic showing polymerase chain reaction (PCR) site directed mutagenesis. (a) Primers for producing mutants annealed to plasmid DNA and are extended. PCR thermal cycles amplify plasmid DNA. (b) Methylated DNA is digested enzymatically leaving only the mutant plasmid.

Site-directed mutagenesis was used to create *TP53* plasmids coding for mutant p53 proteins. Specific mutant p53 proteins containing cysteines substituted for other residues were designed. The substitution of each cysteine involved the mutation of only one nucleotide in the plasmid DNA. Additionally, all sites of cysteine substitution have been identified previously as mutations associated with human cancers. The mutant *TP53* plasmids produced using site directed mutagenesis were used for bacterial expression of the mutant p53 proteins.

1.6.4 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA)[121] is a technique used to detect and quantify the presence of a substance or antigen, such as peptides or proteins. ELISA generally is performed in a 96-well plate, where an antigen is immobilized in the well. Next, the antigen is complexed with a specific antibody that is linked to an enzyme. For detection, the enzyme catalyzes a specific reaction of a substrate, producing a measurable readout, such as a colour change. Horseradish peroxidase (HRP) is used frequently as the detection enzyme as it yields a distinct colour change when it catalyzes the oxidation of a substrate by hydrogen peroxide.

There are several different types of ELISA, including direct, indirect, and sandwich assays, as shown in Figure 1-4. For direct ELISA, the antigen is immobilized and directly detected using a primary antibody–enzyme conjugate. In indirect ELISA, the antigen is immobilized and then complexed with a primary antibody, then the detection is enabled by addition of an enzyme-labelled secondary antibody. Using a secondary antibody is more cost effective and creates flexibility in the assay as different primary antibodies can be used with the same secondary antibody. Therefore, fewer labelled antibodies are required. In sandwich ELISA, a capture antibody is first immobilized on the well, then the sample is added, and any antigen present binds to the capture antibody. Detection generally is accomplished in the same way as indirect ELISA, with the addition of a primary antibody followed by an enzyme-labelled secondary antibody.

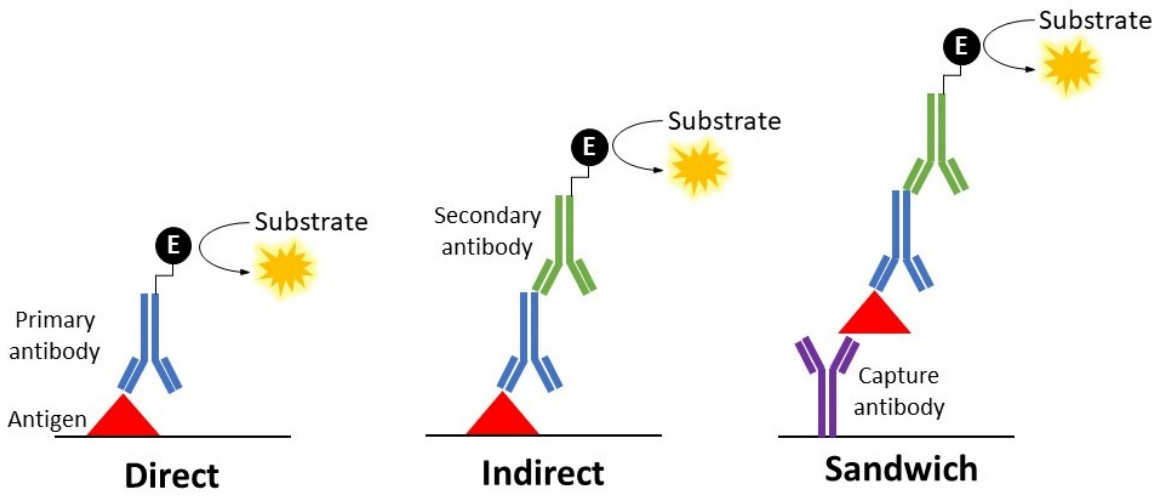


Figure 1-4. Diagram depicting the different types of ELISA using enzyme labelled antibodies for detection.

In Chapter 4, a modified sandwich ELISA is used to investigate the effects of arsenic on the ability of wild type and mutant p53 proteins to bind to transcription factor DNA. Rather than an antibody, a specific double-stranded DNA (dsDNA) sequence that binds active p53 is immobilized in the wells of a 96-well plate, as shown in Figure 1-5. Detection of DNA-bound p53 is enabled by the addition of a primary antibody binding to p53, followed by the addition of a HRP conjugated secondary antibody providing a sensitive colourimetric readout at 450 nm. The changes in DNA binding of p53 and specifically designed mutant p53 proteins in the absence or presence of toxic trivalent arsenicals were studied using this modified p53 transcription factor assay.

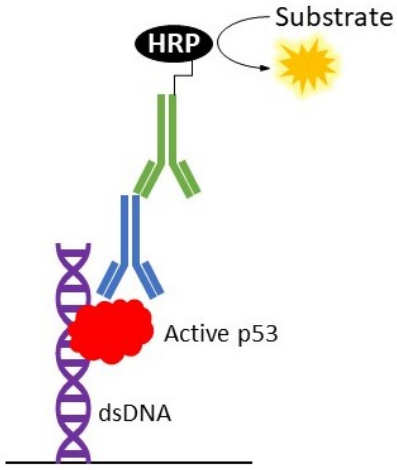


Figure 1-5. Schematic of the p53 transcription factor binding assay.

1.7 Design of Project and Overview of the Contents of the Thesis

The design of the project was threefold:

- First, an HPLC-ICPMS method was developed and validated by studying the interaction between arsenic compounds and hemoglobin from rats as a model system. The interactions between arsenic compounds and two additional proteins, the antioxidant enzyme peroxiredoxin-1 and the chromatin protein high mobility group box protein HMGB1, were also studied to demonstrate the capabilities of the method (Chapter 2).
- Second, the analytical method was applied to study the binding of arsenic species to the hemoglobin from six additional animal species in order to evaluate the interspecies differences of arsenic reactivity (Chapter 3).
- Third, the HPLC-ICPMS method was applied to investigate the binding of arsenic compounds to the tumour suppressor protein p53. Molecular cloning techniques were

employed to express and purify mutant p53 proteins containing amino acid mutations associated with cancer. An enzyme-linked immunosorbent assay (ELISA) was used to characterize the ability of arsenic to deactivate the DNA binding site of p53 (Chapter 4).

Finally, the pertinent conclusions from this thesis project are discussed and potential future studies are proposed (Chapter 5).

Chapter 2 Mass Spectrometry Studies of Arsenic Binding to Proteins

2.1 Introduction

Given the complexity of protein–metal interactions, a technique capable of separating high molecular weight proteins from small molecules is essential. Size-exclusion high-performance liquid chromatography (HPLC) is used widely for the fractionation of proteins because of its biocompatibility, speed, and reproducibility.[113] Due to its ability to detect elements sensitively, selectively, and quantitatively, inductively coupled plasma mass spectrometry (ICPMS) is a superb analytical tool for the analysis of trace elements, such as arsenic.[116] Hyphenating size-exclusion chromatography (SEC) with ICPMS capitalizes on the advantages of each technique, providing a powerful analytical tool for the analysis of metal–protein interactions. SEC separation with ICPMS detection is useful particularly for determining whether or not an arsenic species binds to a protein. When applying this on-line technique to the study of arsenic–protein interactions, SEC enables the separation of protein-bound arsenic from free arsenic. SEC alone is incapable of differentiating unbound protein from the arsenic–protein complex as they cannot be separated due to the minute differences in their sizes, but the arsenic-bound protein can be discriminated by using ICPMS for the selective detection of ^{75}As. Additionally, the quantitative capabilities of ICPMS allow for the determination of the binding constants between arsenic and proteins.

This chapter didactically describes an approach using the aforementioned techniques of SEC-ICPMS to systematically study arsenic−protein interactions. By studying the binding

of arsenic compounds to rat hemoglobin, as a model system, it is demonstrated how mass spectrometry and chromatography techniques are critical when addressing how strongly specific arsenic species bind to particular proteins and what the biological significance of the arsenic binding to a specific protein is. The interactions between arsenic compounds and two additional proteins, the antioxidant enzyme peroxiredoxin-1 and the chromatin protein high mobility group box 1 protein (HMGB1), also were studied to demonstrate the capabilities of the method.

2.2 Materials and Methods

Caution: *The arsenic species included in this study are toxic and are established or potential human carcinogens. Caution and care should be exercised when handling these materials.*

2.2.1 Materials

Sodium arsenite (iAs^{III}) (purity 96.7%) ($NaAsO_2$), sodium arsenate (iAs^{V}) (purity 99.4%) ($Na_2HAsO_4 \bullet 7H_2O$), and cacodylic acid (dimethylarsinic acid, DMA^{V}) (purity 98%) were obtained from Sigma (St. Louis, MO). Monomethylarsonic acid (MMA^{V}) (purity 99.0%) was purchased from Chem Service (West Chester, PA). Methylarsine oxide (CH_3AsO) and iododimethylarsine, $(CH_3)_2AsI$, were prepared following literature procedures and were both stored at -20°C.[122,123] Dilute solutions of these precursors were prepared fresh daily in deionized water to form monomethylarsonous acid (MMA^{III}), $CH_3As(OH)_2$, and dimethylarsinous acid (DMA^{III}), $(CH_3)_2AsOH$, respectively.

Standard rat hemoglobin (lyophilized, purity 98%) was purchased from Sigma and used without further purification. Stock solutions of hemoglobin (40 μM or 2.58 mg/mL) were

prepared fresh weekly in 10 mM ammonium bicarbonate (pH 7.4) and were stored at 4˚C. Lyophilized peroxiredoxin-1 (purity >85%) and HMG-1 (purity >90%) were purchased from Sigma (St. Louis, MO). Following dilution in deionized water, both protein solutions underwent buffer exchange with PBS, using 10kDa Amicon Ultra-4 cellulose centrifugal filters from Merck Millipore Ltd. (Cork, Ireland). The concentrations of protein solutions were determined using the Bio-Rad Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA).

All other reagents used in the experiments were HPLC grade or analytical grade.

2.2.2 Methods

2.2.2.1 Size Exclusion Chromatography Separation with Inductively Coupled Plasma Mass Spectrometry Detection

A PerkinElmer 200 series HPLC system (PerkinElmer Instruments, Shelton, CT) fitted with an autosampler was used to separate the unbound from protein-bound arsenic. A Superdex 200 Increase 10/300 GL column (10 mm internal diameter, 300 mm length) with an exclusion limit of 1.3×10^6 Da (General Electric Healthcare Life Sciences) was used to separate the protein-bound arsenic from the unbound arsenic species. An ammonium bicarbonate buffer (10 mM, pH 7.4) was used as the mobile phase, with a flow rate of 0.75 mL/min. The sample injection volume was 30 µL. The effluent from the size exclusion column was coupled directly to the spray chamber of an Elan 6100 DRC plus ICP mass spectrometer (PerkinElmer Sciex, Concord, ON, Canada). The operating parameters of ICPMS were optimized to an RF power (1350 W), a plasma gas flow (15 L/min), an auxiliary gas flow (1.5 L/min), a nebulizer gas flow (0.89 L/min), and cell gas (0.7 mL/min). ICPMS performance was optimized and checked

daily by testing the PerkinElmer Pure Plus ELAN 6100 DRC Setup/Sta/Masscal Solution (PerkinElmer, Shelton, CT, USA) standard mixture containing 1 ng/mL In, Co, Mg, Ba, Pb, and As in 1% HNO_3.

2.2.2.2 Determination of Arsenic–Hemoglobin Binding

To study the interaction of standard hemoglobin with arsenic species, iAs^{III}, MMA^{III}, and DMA^{III} (1 μM) were incubated with rat hemoglobin (20 μM) in 10 mM ammonium bicarbonate (pH 7.4) at 37˚C for 24 h. The reaction mixtures were subjected to SEC-ICPMS analysis. Sulfur oxide, iron oxide, and arsenic oxide were detected simultaneously using the dynamic reaction cell (DRC) mode (O_2 cell gas 0.7 mL/min) of the ICPMS at m/z of 48, 72, 91, respectively, to confirm the binding of arsenic to hemoglobin; SO (m/z 48) allows for detection of the protein chain, ^{56}FeO (m/z 72) allows for detection of the iron atom contained in the heme group of hemoglobin, and AsO (m/z 91) allows for detection of the arsenic species. Arsenic concentrations were verified by total analysis using ICPMS. The resulting chromatograms were processed using TurboChrom Workstation (PerkinElmer).

2.3 Results and Discussion

2.3.1 Dynamic Reaction Cell Oxygen Mass-Shift

ICPMS is capable of detecting arsenic as As^+ (m/z 75), which unfortunately can be subjected to isobaric interference from $ArCl^+$ (m/z 75), a product of the reaction between the argon plasma gas and Cl^- that is present in biological samples. With the use of the dynamic reaction cell (DRC) function available with certain ICPMS instruments, this interference can be

eliminated. O_2 gas can be introduced into the DRC to react with As^+, transforming As^+ (m/z 75) to AsO^+ (m/z 91). By detecting AsO^+ rather than As^+, the isobaric interference from $ArCl^+$ is avoided. Figure 2-1 shows the ICP-DRC-MS O_2 mass-shift method for monitoring ^{75}As. Additionally, the production of oxides using the DRC mode permits for the detection of proteins by ICPMS by monitoring SO^+ (m/z 48). Arsenic binding proteins have cysteine and methionine residues, each of which contain a sulfur atom. The production of SO^+ is especially beneficial because the other elements found in proteins (hydrogen, carbon, nitrogen, and oxygen) exist in air and cannot be monitored for the detection of proteins because of the atmospheric pressure of the ICP interface. Furthermore, detection of proteins using S^+ (m/z 32) suffers from isobaric interference from O_2^+ (m/z 32). The production of oxides in the DRC converts S^+ to SO^+ and m/z 48 can be monitored, eliminating the interference from O_2^+.[124,125] Table 2-1 lists the isobaric interference for elements relevant to this thesis.

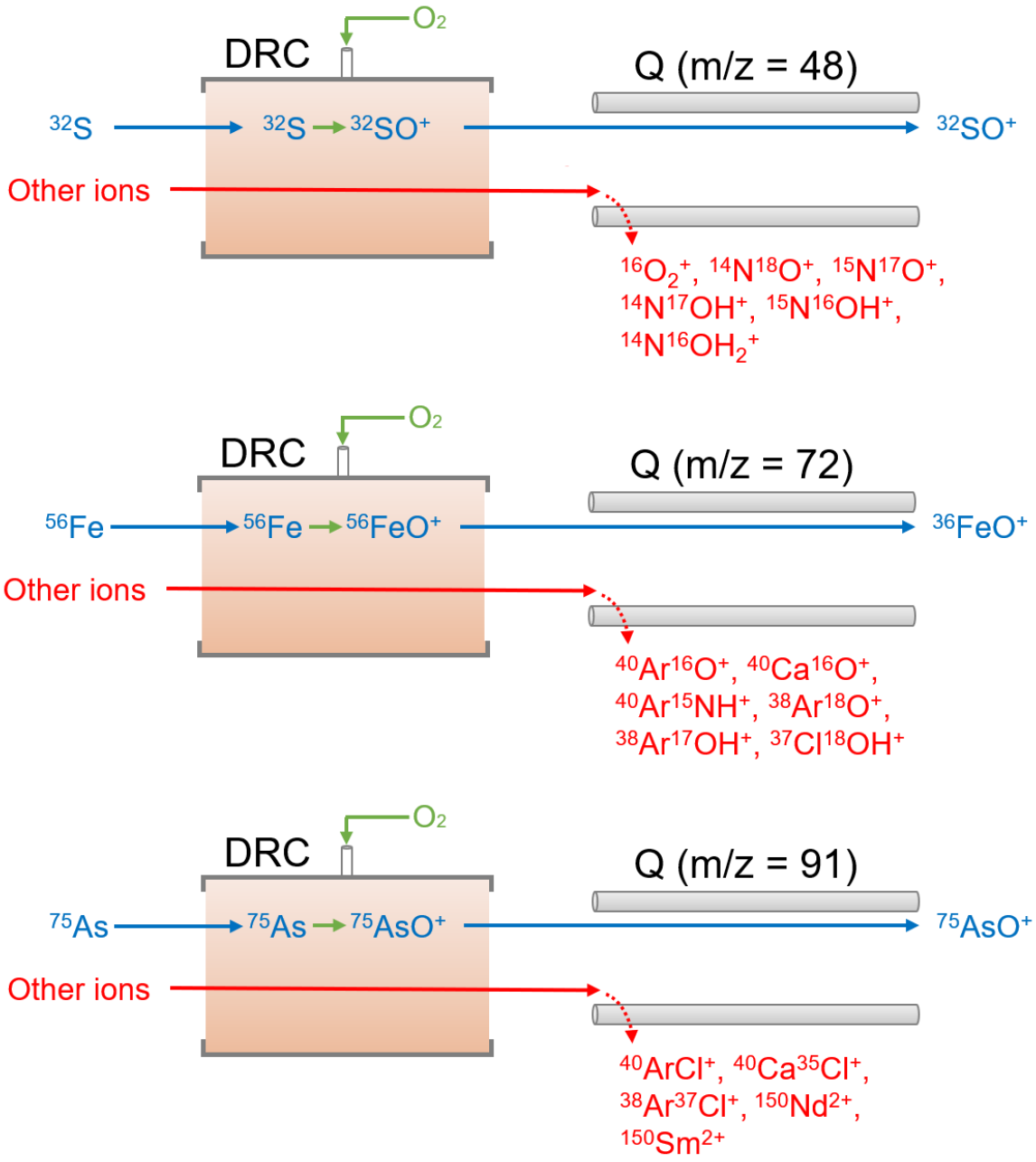


Figure 2-1. ICP-DRC-MS O_2 mass-shift method for measuring ^{32}S, ^{56}Fe, and ^{75}As.

Table 2-1. Isobaric Interference Observed in ICPMS for Relevant Elements[126]

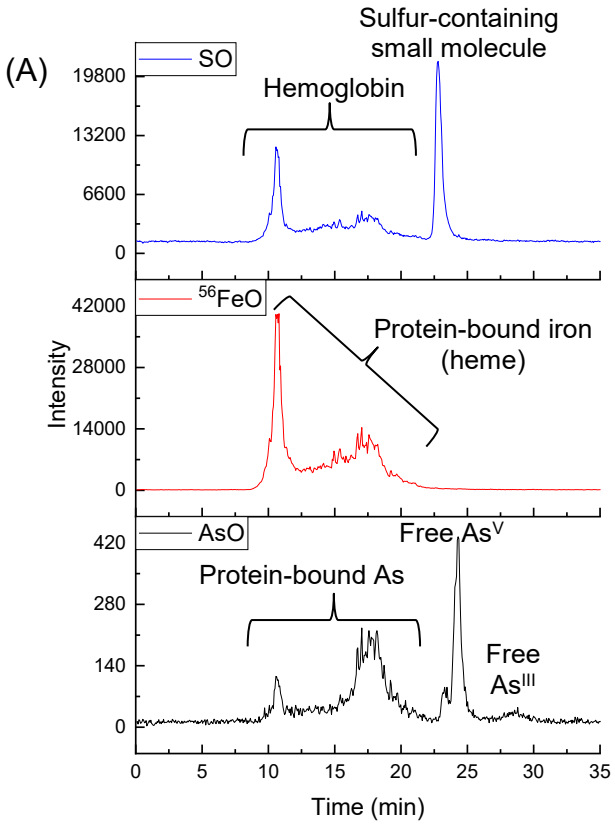
Element	Percent Abundance (%)	Isobaric Interfering Ions
^{32}S	95.02	$^{16}O_2^+$
^{54}Fe	5.8	$^{54}Cr^+$ (2.3565% abundance)
^{56}Fe	91.72	$^{40}Ar^{16}O^+$, $^{40}Ca^{16}O^+$
^{57}Fe	2.2	$^{40}Ca^{16}O^1H^+$, $^{40}Ar^{16}O^1H^+$
^{58}Fe	0.28	$^{58}Ni^+$ (68.077% abundance)
^{75}As	100	$^{40}Ar^{35}Cl^+$

With regards to hemoglobin, it is of interest to monitor iron to determine whether the central iron atom of the heme cofactor has been dissociated or displaced by arsenic. The most abundant isotope of iron, ^{56}Fe, is affected by interference from $^{40}Ar^{16}O^+$ and $^{40}Ca^{16}O^+$ (both m/z 56); the former is produced from the reaction of the plasma gas (Ar) and oxygen, the latter is produced from the reaction of calcium, which is present in biological samples, and oxygen. The second most abundant isotope of iron, ^{54}Fe, suffers from isobaric interference from $^{54}Cr^+$. Conventionally, $^{57}Fe^+$ is monitored because it has minimal isobaric interference, despite minor formation of $^{40}Ar^{16}O^1H^+$ and $^{40}Ca^{16}O^1H^+$. Unfortunately, monitoring $^{57}Fe^+$ has low sensitivity as ^{57}Fe has a natural abundance of only 2.2%. Finally, ^{58}Fe, which has an abundance of a mere 0.28%, is subjected to interference from ^{58}Ni, which is 68.077% abundant, making this an infeasible method for detecting iron as even small quantities of nickel would produce significant interference. I have developed a method that allows for the most abundant isotope of iron to be monitored by employing the DRC function to produce $^{56}FeO^+$ (m/z 72), which is

detected by ICPMS without any isobaric interference and with high sensitivity. Table 2-2 lists the oxides produced in the DRC and monitored by ICPMS for the detection of arsenic−hemoglobin interactions. Figure 2-2 demonstrates the use of SEC-ICP-DRC-MS to produce and monitor SO^+, $^{56}FeO^+$, and AsO^+ for a sample of purified rat hemoglobin incubated with DMA^{III}. From the chromatograms, two protein-bound arsenic peaks can be identified due to the SO^+ and AsO^+ peaks with identical retention times (11 and 18 min). Iron peaks at the same retention times signify that the iron atom is still bound to the heme moiety and has not been displaced by arsenic. This supports the idea that arsenic binds to cysteine residues within hemoglobin as opposed to binding to the heme moiety. A small sulfur-containing molecule is eluted at 23 min. Free arsenic has been confirmed using DMA^V and DMA^{III} standards, which have 25 and 30 min retention times, respectively. Chromatograms showing the separation of other trivalent and pentavalent arsenic species can be found in Figures 3-1C (iAs), 4-6B (PAO), and 4-7B (MMA).

Table 2-2. Oxides Produced by the Dynamic Reaction Cell and Detected by ICPMS

Oxide	Mass (amu)	Used to Detect
SO	48.064	proteins (cysteine and methionine)
^{56}FeO	71.934	iron atom in heme
AsO	90.9206	arsenic


(A)
SO
Sulfur-containing
small molecule
Hemoglobin
19800
13200
6600
0
^{56}FeO
Protein-bound iron
(heme)
42000
28000
14000
0
Intensity
AsO
Free As^{V}
Protein-bound As
Free
As^{III}
420
280
140
0
0
5
10
15
20
25
30
35
Time (min)

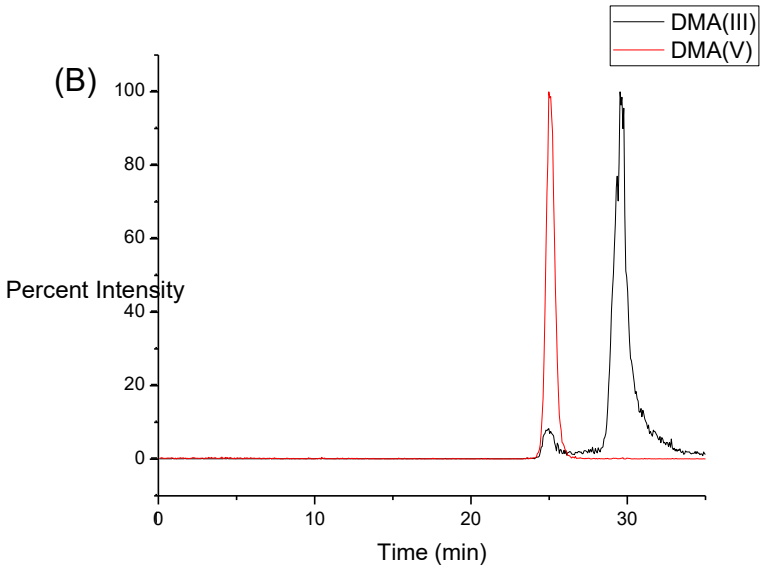

DMA(III)
DMA(V)
(B)
100
80
60
40
20
0
Percent Intensity
0
10
20
30
Time (min)

Figure 2-2. Chromatograms showing DMA^{III} that is free or bound to rat Hb after being subjected to SEC separation coupled with ICP-DRC-MS to selectively detect three oxides, SO^+ at 48 m/z, $^{56}FeO^+$ at 73 m/z, and AsO^+ at 91 m/z. Rat Hb (20 μM) was incubated with DMA^{III} (1 μM) at 37˚C for 24 h in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis. (A) Rat hemoglobin incubated with 1 μM DMA^{III}, (B) DMA^{III} and DMA^{V} standards, only AsO^+ detection shown.

2.3.2 Purified Proteins

2.3.3.1 Rat Hemoglobin

Using the hyphenated SEC-ICPMS technique facilitates the analysis of *in vitro* reaction mixtures containing arsenic and purified proteins, such as commercially available standard hemoglobin. The retention times of high molecular weight proteins are much shorter than those of the low molecular weight trivalent arsenic compounds. Hence, SEC separates the protein-bound arsenic from the free arsenic species, and ICPMS selectively and quantitatively detects arsenic oxide (m/z 91). Figure 2-3 portrays the chromatograms from SEC-ICPMS analysis of mixtures containing 20 μM rat hemoglobin incubated with 1 μM iAs^{III}, MMA^{III}, or DMA^{III}; the earlier eluting peak corresponds to hemoglobin-bound arsenic and the later eluting peak corresponds to free arsenic. A small amount of bound arsenic and free pentavalent arsenic are always detected in the purified rat hemoglobin.

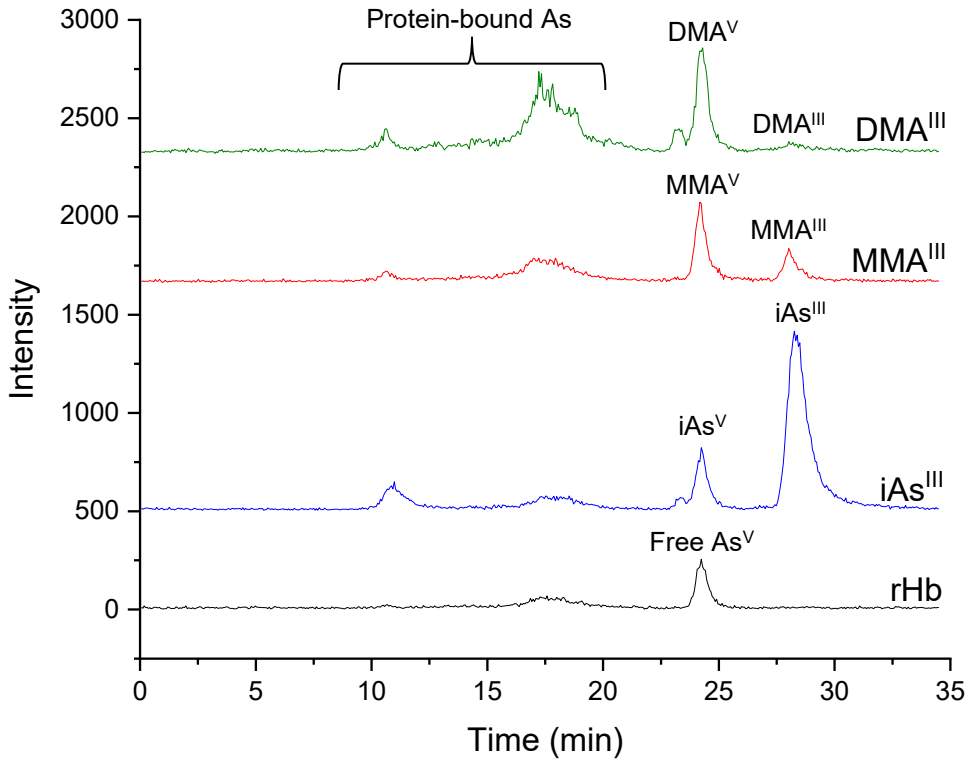


Figure 2-3. Chromatograms showing three trivalent arsenic species that are free or bound to rat hemoglobin (rHb) after being subjected to SEC separation coupled with ICPMS to selectively detect arsenic oxide at m/z 95. Hemoglobin (20 μM) was incubated with iAs^{III}, MMA^{III}, or DMA^{III} (1 μM) at 37ºC for 24 h in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis. Some As is detected in standard rat Hb and has been reported previously.[84]

The relative intensities of the protein bound arsenic complexes differ between the arsenic species. For example, DMA^{III} has substantially larger protein-bound peaks than the other arsenic compounds, suggesting that rat hemoglobin has varying affinities of the different arsenicals. The quantification of the fraction of hemoglobin-bound arsenic and the fraction of unbound arsenic species, using peak areas, permits the determination of the percentage of

arsenic bound to hemoglobin, shown in Figure 2-4.

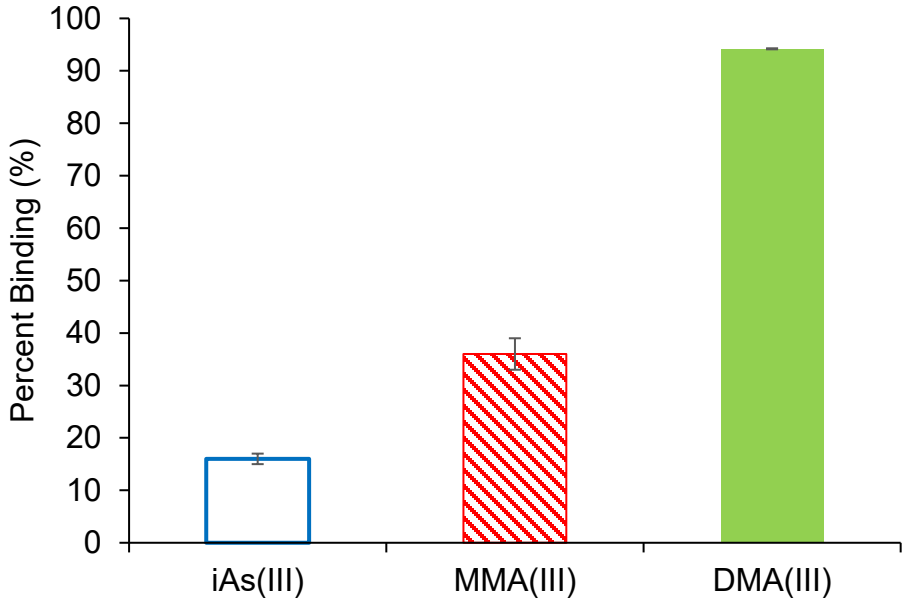


Figure 2-4. The comparison of the fraction of three trivalent arsenic species (iAs^{III}, MMA^{III}, or DMA^{III}) that are bound to rat hemoglobin. Rat hemoglobin (rHb) (20 μM) was incubated with iAs^{III}, MMA^{III}, or DMA^{III} (1 μM) at 37ºC for 24 h in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to SEC-ICP-DRC-MS analysis with the selective detection of ^{75}As) (m/z 91).

The apparent binding constants (nK) for the three trivalent arsenicals binding to rat hemoglobin, shown in Table 2-3, have been calculated from binding studies using a range of arsenic and hemoglobin concentrations, using Equation 1. Arsenic concentrations can be quantified using total elemental analysis by ICPMS, and protein concentrations can be determined using protein quantification techniques, such as the Bradford Assay.[127] The apparent binding constants allow for a direct comparison of the binding strength of different

trivalent arsenic compounds to hemoglobin from one animal species by examining their relative affinities. Trends of binding constants increase from iAs^{III} to MMA^{III} to DMA^{III}. Additionally, examining the ratio of the apparent binding constants allows for the comparison of the binding strength of the same arsenical to hemoglobin from different animal species; this is discussed in Chapter 3. Shen *et al*.[79] made a comparison of the binding affinity of trivalent and pentavalent arsenic for proteins, peptides, and thiols by summarizing the dissociation constants (K_d). The apparent binding constants determined for the various proteins in this thesis are within the range of other association constants (K_a) reported in literature.

Table 2-3. Percent Bound and Apparent Binding Constants (nK) for 1 μM Trivalent Arsenicals Binding to 20 μM Rat Hemoglobin

	Percent Bound	**nK (× 10^3) (M^{-1})**
iAs^{III}	16 ± 1	9.8 ± 0.6
MMA^{III}	36 ± 3	26 ± 3
DMA^{III}	94.2 ± 0.1	810 ± 40

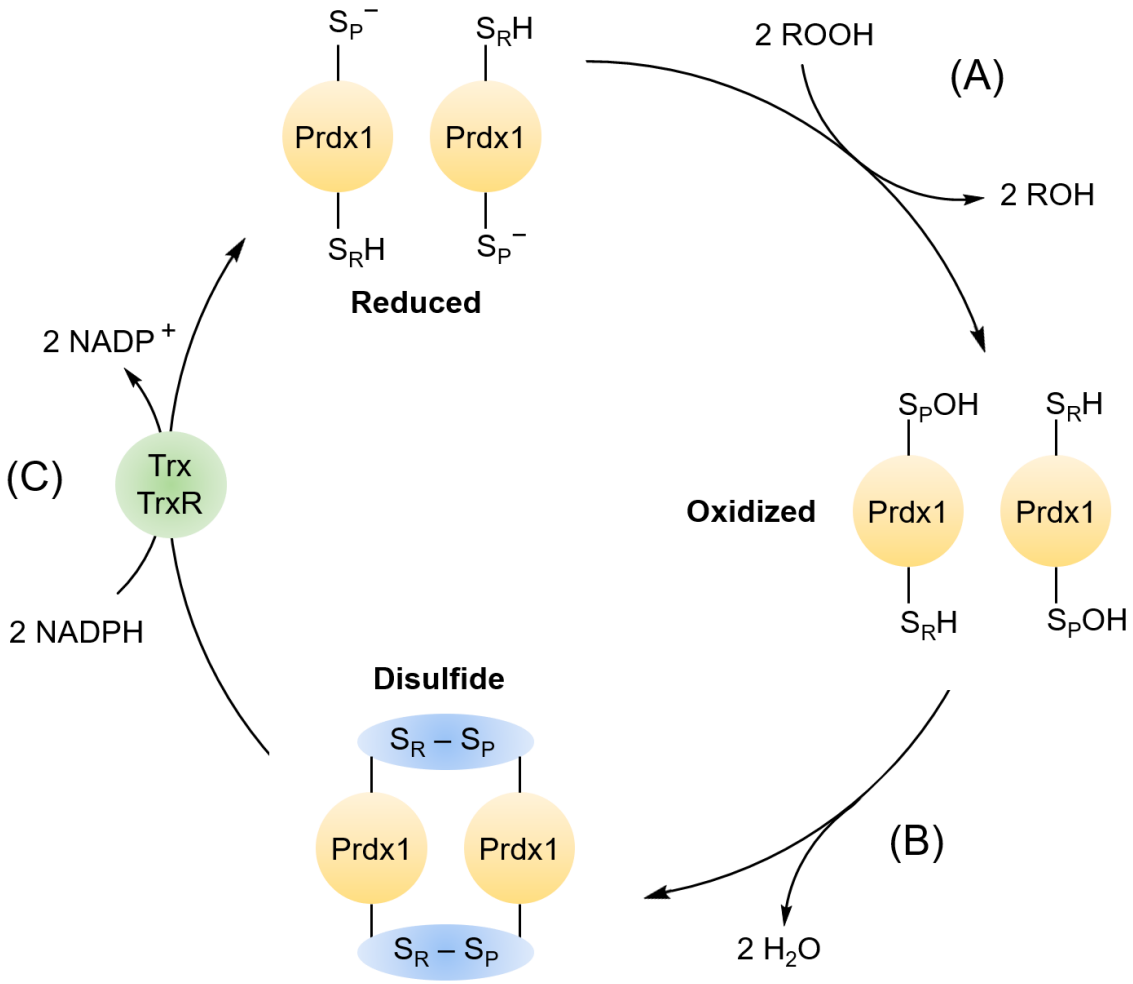
$$nK = \frac{[\text{bound As}]}{\left([\text{protein}] - \frac{[\text{bound As}]}{n}\right) \cdot [\text{free As}]} \cong \frac{[\text{bound As}]}{[\text{protein}] \cdot [\text{free As}]} \left(\text{when } [\text{protein}] \gg \frac{[\text{bound As}]}{n}\right) \quad \textbf{(1)}$$

where [bound As] is the molar concentration of protein-bound arsenic, [protein] is the total molar concentration of protein, [free As] is the molar concentration of free trivalent arsenic

species, and *n* is the number of available binding sites in each hemoglobin molecule. Total arsenic concentration was determined using total analysis ICPMS and [protein] determined using the Bradford assay.

2.3.3.2 Peroxiredoxin-1

Peroxiredoxins are a family of small (22−27 kDa) enzymes that combat cellular oxidative stress, playing an important role in human health and disease prevention.[128,129] They function as protective antioxidants in cells by reducing and detoxifying peroxides to alcohols. By regulating the peroxide levels, peroxiredoxins mediate cell signaling.[130] The catalytic cycle of peroxiredoxins involves an active site containing a redox-active cysteine residue, dubbed the peroxidatic cysteine, and is shown in Scheme 2-1: (A) a redox reaction oxidizes cysteine (R−SH) to sulfenic acid (R−SOH) and reduces peroxide (R′OOH) to an alcohol (R′OH), (B) a disulfide bond is formed from the reaction of sulfenic acid with a resolving cysteine on a second peroxiredoxin molecule, (C) the disulfide is reduced by an electron donor and is recycled, completing the catalytic cycle. For peroxiredoxin-1, thioredoxin acts as the electron donor.



Scheme 2-1. The catalytic cycle of peroxiredoxin-1 (Prdx1) involving the reduction of a peroxide by a redox-active cysteine with thioredoxin (Trx) as the electron donor. (A) a redox reaction oxidizes the peroxidatic cysteine ($-S_P$) to sulfenic acid and reduces peroxide to an alcohol, (B) a disulfide bond is formed for the reaction of sulfenic acid with a resolving cysteine ($-S_R$) on a second peroxiredoxin molecule, (C) the disulfide is reduced by electrons donated from the oxidation of thioredoxin, which is catalyzed by thioredoxin reductase (TrxR) and requires reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Peroxiredoxins may have a proliferative effect involved in cancer development and progression. Elevated levels of peroxiredoxin-1 are associated with pancreatic cancer.[131] Furthermore, peroxiredoxin-1 acts as a tumour suppressor in certain types of cancer and has been found to be overexpressed in some human cancers.[132] Studies have shown that peroxiredoxin-1 may be involved in lung cancer malignancy.[133,134]

Peroxiredoxin-1 has previously been identified by the Le group as a cellular arsenic-binding protein from A549 human lung carcinoma cells, using a p-azidophenylarsenoxide (PAzPAO) arsenic bait.[135] The developed SE-HPLC-ICPMS method was used to investigate the arsenic−peroxiredoxin-1 interaction using purified protein for the *in vitro* study. Chromatograms obtained with ICPMS detection of AsO^+ and SO^+ showed that peroxiredoxin-1 was able to bind to all four of the arsenicals tested (iAs^{III}, MMA^{III}, DMA^{III}, and PAO^{III}) using AsO and SO detection. Both the percentage of bound arsenic and the apparent binding constants were calculated using Equation 1, as described, and are listed in Table 2-4. Peroxiredoxin-1 was found to have the highest affinity for PAO^{III}, which was the molecular basis for the arsenic bait that previously identified the protein's ability to bind arsenic. The protein contains 4 cysteine residues (Cys 52, 71, 83, and 173), with Cys 52 being responsible for the peroxidase activity, and Cys 173 being involved in the disulfide bond during the catalytic cycle.[136] Based on the 3D model of the protein using the crystal structure from PBD 1QQ2, it is possible that Cys 52 also serves as the site for arsenic binding, which could result in inactivation of the enzyme, however, further experiments are needed to characterize the arsenic binding site on peroxiredoxin-1 fully as the other 3 cysteine residues are also exposed on the surface of the protein.

Table 2-4. Percent Bound and Apparent Binding Constants (nK) for 5 μM Trivalent Arsenicals Binding to 20 μM Peroxiredoxin-1. Samples run in triplicate.

	Percent Bound	nK ($\times 10^3$) (M^{-1})
iAs^{III}	$6._2 \pm 1._6$	3.3 ± 0.9
MMA^{III}	5 ± 1	2.4 ± 0.7
DMA^{III}	1.9 ± 0.4	1.0 ± 0.2
PAO^{III}	9 ± 1	4.9 ± 0.8

2.3.3.3 High Mobility Group Box 1 Protein

High mobility group box 1 protein (HMGB1), also known as HMG1 or amphoterin, is a chromatin protein. It is a single chain protein composed of two positively charged globular DNA-binding domains, HMG boxes A and B, and a negatively charged C-terminal region. When in the nucleus, HMGB1 binds DNA and functions as a transcription factor, regulating gene expression. When excreted from the nucleus and in its extracellular form, HMGB1 functions as a cytokine that mediates cellular responses to infection, inflammation, and injury.[137–140] The activity of HMGB1 depends strongly on the redox state of its cysteine residues. When HMGB1 contains an intramolecular disulfide bond between cysteine residues 23 and 45 and Cys 106 as a free thiol, it can induce the secretion of the important inflammatory cytokine tumour necrosis factor alpha (TNF alpha).[140,141] Alternatively, when the cysteines in HMGB1 are reduced, the protein acts as a chemoattractant for neutrophils and monocytes to

help fight infection.[142] The nuclear activity of HMGB1 also is attributed to the fully reduced form of the protein.

HMGB1 binds DNA in a non-sequence specific manner and bends DNA, facilitating the binding of other proteins and transcription factors to DNA.[143,144] For example, HMGB1 acts as a structural cofactor, bending DNA and enhancing the ability of p53 to bind DNA.[145,146] Also, it may enhance nucleotide excision repair (NER)[147] and contributes to base excision repair (BER) and mismatch repair (MMR) pathways,[148,149] by which HMGB1 is involved in DNA repair. HMGB1 is up-regulated in nearly all cancers, and it has been proposed as a possible target for cancer therapy.[150] Due to its role in the pathogenesis of a broad range of diseases, including chronic inflammatory diseases, such as sepsis, rheumatoid arthritis, and atherosclerosis, and autoimmune diseases, diabetes, Alzheimer's, and cancer,[139,151–154] it is important to study HMGB1 and how it interacts with other proteins, DNA, and xenobiotics.

The developed SE-HPLC-ICPMS method was used to investigate the interactions between HMGB1 and arsenicals. Results from the detection of AsO^+ and SO^+ showed that the protein was able to bind to all four of the arsenicals tested (iAs^{III}, MMA^{III}, DMA^{III}, and PAO^{III}) in small amounts, using AsO^+ and SO^+ detection. Both the percentage of bound arsenic and the apparent binding constants were calculated using Equation 1 and are listed in Table 2-5. Due to the cost of the protein, HPLC-ICPMS experiments were run only in duplicate, therefore, no error is given on the percentage of bound arsenic or the apparent binding constants. HMGB1 contains three cysteine residues (Cys 23, 45, and 106). Based on the 3D model of the protein using the crystal structure from PBD 4QR9, it is possible that any of the cysteines can serve as the site for arsenic binding. As the cysteines play a key factor in the activity of the protein,

binding of cysteine to arsenic could result in inactivation of the enzyme, however, further experiments are needed to characterize the arsenic binding site to HMGB1 fully.

Table 2-5. Percent Bound and Apparent Binding Constants (nK) for 5 μM Trivalent Arsenicals Binding to 10 μM HMGB1. Samples run in duplicate.

	Percent Bound	nK ($\times 10^3$) (M^{-1})
iAs^{III}	4.9	5.1
MMA^{III}	1.5	1.5
DMA^{III}	7.2	7.8
PAO^{III}	4.9	5.2

2.4 Conclusion

Arsenic is an infamous toxicant known for causing diverse cancerous and noncancerous health effects.[7,37] Arsenic trioxide has also been proven to be an effective treatment for acute promyelocytic leukemia (APL), with high remission rates.[155–157] Notwithstanding, numerous adverse health effects are associated with this treatment, including hepatic dysfunction, leukocytosis, peripheral neuropathies, QT prolongation, and even fatality.[156,158–160] Substantial research has been conducted on the biological mechanisms of arsenic, aiming to better our understanding of the therapeutic and detrimental health effects of arsenic, although minimal focus has been put on arsenic's interactions with cellular proteins.

HPLC-ICPMS can be used to characterize the interactions between proteins and arsenic species; the binding of trivalent arsenicals to rat hemoglobin was used to illustrate the validity

of this method. HPLC-ICPMS can separate and detect quantitatively the protein-bound and free arsenic, providing apparent binding constants that are a direct measure of the affinity of a protein for arsenic. Rat hemoglobin bound three arsenicals with varying affinities, with the affinity increasing from iAs^{III} to MMA^{III} to DMA^{III}. Using ESI-MS/MS, the Le group previously demonstrated that arsenic, in the form of DMA^{III}, in rat red blood cells is bound predominantly to the Cys 13 in the α-chain of rat hemoglobin.[161] DMA^{III} is accumulated effectively in rat blood, resulting in a reduced acute toxicity of arsenic and changes to the arsenic metabolism profile. The affinity of arsenic to the hemoglobin of different animal species is related to the capacity for arsenic accumulation in the RBCs.[162]

Four arsenicals (iAs^{III}, MMA^{III}, DMA^{III}, and PAO^{III}) were found to bind two additional proteins, peroxiredox-1 and HMGB1, using the developed method. The mechanisms through which arsenic causes biological effects, such as toxicity and carcinogenicity, remains unascertained, although the binding of arsenic to proteins is undoubtedly an inherent component of the process. Mass spectrometry and chromatography are essential techniques for the study of arsenic−protein binding.

Overall, the study of arsenic−protein interactions is of biological significance. The mass spectrometry methods highlighted in Chapter 2 provide a means for further investigations of these interactions, with the hope of expanding our understanding of the biological implications of arsenic−protein binding and guiding the discovery of treatments and methods for preventing arsenic-induced cancers.

Chapter 3 Mass Spectrometry Studies of Arsenic Binding to Hemoglobin from Different Animal Species

3.1 Introduction

Chapter 2 discusses the development of a method that employs size exclusion high-performance liquid chromatography (SE-HPLC) to separate protein-bound arsenic from free arsenic, paired with inductively coupled plasma mass spectrometry (ICPMS) to monitor oxides of arsenic and sulfur for the selective and quantitative detection of arsenic and proteins. The validity of this method was demonstrated using rat hemoglobin, which has a high affinity for trivalent arsenicals. The Le group previously identified hemoglobin (Hb) as the protein responsible for the retention and accumulation of arsenic in rat blood.[84] The retention of arsenic in rat blood is substantially longer than in humans,[55,85–88] where arsenic has a half-life of a mere 1 h.[90,91] Trivalent arsenic was found to bind strongly to the cysteine (Cys) in the 13th position of the alpha-chain of rat hemoglobin.[84]

Hemoglobin is the most abundant protein in red blood cells (RBC), making up approximately 97% of the dry content. Due to its abundance, in addition to being responsible for the retention and accumulation of arsenic in red blood cells, hemoglobin is predicted to be responsible for the circulation of arsenic in the body.[84] It is reasonable that lower binding of trivalent arsenic to hemoglobin may lead to more free (unbound) trivalent arsenic for transport and dispersal to other organs and tissues. The outcome would be more incidences of arsenic-induced toxicity and cancers.[92] The hypothesis is that trivalent arsenic binds to accessible cysteines in hemoglobin and that the differences in arsenic binding hemoglobin are related to

the variations in the toxicity of arsenic compounds between animal species. The goal of this study is to characterize the binding of hemoglobin from different animal species to trivalent arsenic in order to address the interspecies differences in the strength of the binding, the retention of arsenic in the blood, and arsenic-induced toxicity.

To test this hypothesis, I have studied the relative reactivity between hemoglobin and several arsenic compounds and compared the affinities of hemoglobin from seven animal species (cow, guinea pig, human, mouse, pig, rabbit, and rat) for arsenic compounds. The interaction of hemoglobin with three trivalent arsenic species, inorganic arsenite (iAs^{III}), monomethylarsonous acid (MMA^{III}), and dimethylarsinous acid (DMA^{III}), was tested because of their toxicological relevance.[74,75,163–165] Furthermore, these arsenic compounds are formed *in vivo* during the metabolism of arsenate (iAs^{V}), which is the most abundant form of arsenic in drinking water.[69,70,166,167] MMA^{III} and DMA^{III} have proven to be some of the most toxic arsenicals to both human cells and laboratory animals.[74,75,163–165,168] The binding of these trivalent arsenic compounds with hemoglobin from the seven animal species has been studied using pure hemoglobin to demonstrate their relative reactivities. These results provide evidence for a possible rationale for the interspecies differences in arsenic retention in the blood and arsenic-induced toxicity.

3.2 Materials and Methods

Caution: *The arsenic species included in this study are toxic and are established or potential human carcinogens. Caution and care should be exercised when handling these materials.*

3.2.1 Materials

Sodium arsenite (iAs^{III}) (purity 96.7%) ($NaAsO_2$), sodium arsenate (iAs^{V}) (purity 99.4%) ($Na_2HAsO_4 \bullet 7H_2O$), and cacodylic acid (dimethylarsinic acid, DMA^{V}) (purity 98%) were obtained from Sigma (St. Louis, MO). Monomethylarsonic acid (MMA^{V}) (purity 99.0%) was purchased from Chem Service (West Chester, PA). Methylarsine oxide (CH_3AsO) and iododimethylarsine, $(CH_3)_2AsI$, were prepared following literature procedures and were both stored at -20°C.[122,123] Dilute solutions of these precursors were prepared fresh daily in deionized water to form monomethylarsonous acid (MMA^{III}), $CH_3As(OH)_2$, and dimethylarsinous acid (DMA^{III}), $(CH_3)_2AsOH$, respectively.

Standard bovine (cow), human, porcine (pig), and rat Hb (lyophilized, purity 98%) were purchased from Sigma and used without further purification. Guinea pig, mouse, and rabbit Hb solutions (purity >95%) in phosphate buffered saline were purchased from Antibodies-Online (Atlanta, GA). The concentrations of hemoglobin solutions were determined using the Bio-Rad Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA). Stock solutions of hemoglobin (40 μM or 2.58 mg/mL) were prepared fresh weekly in 10 mM ammonium bicarbonate (pH 7.4) and were stored at 4˚C.

All other reagents used in the experiments were HPLC grade or analytical grade.

3.2.2 Methods

3.2.2.1 Size Exclusion Chromatography Separation with Inductively Coupled Plasma Mass Spectrometry Detection

A PerkinElmer 200 series HPLC system (PerkinElmer Instruments, Shelton, CT) fitted with an autosampler was used to separate the unbound arsenic from protein-bound arsenic. A Superdex 200 Increase 10/300 GL column (10 mm internal diameter, 300 mm length) with an exclusion limit of 1.3×10^6 Da (General Electric Healthcare Life Sciences) was used for the separation. An ammonium bicarbonate buffer (10 mM, pH 7.4) was used as the mobile phase with a flow rate of 0.75 mL/min. The sample injection volume was 30 μL. The effluent from the size exclusion column was coupled directly to the spray chamber of an Elan 6100 DRC plus ICP mass spectrometer (PerkinElmer Sciex, Concord, ON, Canada). The operating parameters of ICPMS were optimized to an RF power (1350 W), a plasma gas flow (15 L/min), an auxiliary gas flow (1.5 L/min), a nebulizer gas flow (0.89 L/min), and cell gas (0.7 mL/min). ICPMS performance was optimized and checked daily by testing the PerkinElmer Pure Plus ELAN 6100 DRC Setup/Sta/Masscal Solution (PerkinElmer, Shelton, CT, USA) standard mixture containing 1 ng/mL In, Co, Mg, Ba, Pb, and As in 1% HNO_3.

3.2.2.2 Determination of Arsenic−Hemoglobin Binding

To study the interaction of standard hemoglobin with the tested arsenic species, iAs^{III}, MMA^{III}, or DMA^{III}, (1, 5, or 50 μM) were incubated with hemoglobin (20 μM) in 10 mM ammonium bicarbonate (pH 7.4) at 37˚C for 24 h. Note DMA^{III} oxidizes overtime to DMA^{V} and reported

concentrations (1, 5, or 50 μM) are for initial levels of trivalent arsenic. MMA^{III} oxidized as well, but at a slower rate than its dimethylated counterpart. The reaction mixtures were subjected to SEC-ICPMS analysis. Sulfur oxide, iron oxide, and arsenic oxide were detected simultaneously using the dynamic reaction cell (DRC) mode (O_2 cell gas 0.7 mL/min) of the ICPMS at m/z of 48, 72, 91, respectively, to confirm the binding of arsenic to hemoglobin; SO allows for detection of the protein, ^{56}FeO allows for detection of the iron atom contained in the heme group of hemoglobin, and AsO allows for detection of the arsenic species. Hemoglobin concentrations were 10 μM, determined using the Bradford Assay. Arsenic concentrations were verified using ICPMS. The resulting chromatograms were processed using TurboChrom Workstation (PerkinElmer).

3.3 Results and Discussion

Hemoglobin from seven mammalian animal species were chosen to study the interaction between hemoglobin and trivalent arsenic. The arsenicals of interest are iAs^{III}, MMA^{III}, and DMA^{III} because of their known affinity for sulfhydryl groups in proteins, their high toxicities, and their formation during the biomethylation of arsenic. A unique mass spectrometry technique was employed to study the complex protein–arsenic interactions. Size-exclusion high-performance liquid chromatography (SE-HPLC) was used to separate the hemoglobin-bound from the unbound arsenic species. This was coupled on-line with inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) that enabled the formation of oxides in the DRC and the detection of sulfur oxide, iron-56 oxide, and arsenic oxide. By introducing $O_{2(g)}$ into the DRC of the ICPMS, oxides can be created and detected, and the isobaric interference that ^{75}As, ^{32}S, ^{56}Fe, ^{57}Fe, and ^{58}Fe are subjected to is avoided. This SEC-

ICP-DRC-MS technique provides information on whether an arsenic species binds to a protein and the relative strength (apparent binding constant) of this interaction; additionally, detection of FeO provides information on whether the central iron atom in the heme cofactor is dissociated or displaced by arsenic.

To study the binding of trivalent arsenicals to hemoglobin, SEC-ICP-DRC-MS was performed first on a series of mixtures containing purified hemoglobin from cow, guinea pig, human, mouse, pig, rabbit, or rat and each of the specific arsenic species. SEC enabled the separation of the hemoglobin-bound arsenic from free arsenic species. Additionally, separation of As^{III} and As^{V} was provided by a secondary interaction with the stationary phase of the column. Free arsenic peaks were identified using arsenic standards. Figure 3-1 depicts the SO, FeO, and AsO detection chromatograms obtained from the SEC-ICPMS analyses of 20 μM cow Hb (Figure 3-1A), a mixture of 20 μM cow Hb and 1 μM iAs^{III} after incubation at 37˚C for 24 h (Figure 3-1B), and of the AsO detection of iAs^{III} and iAs^{V} standards (Figure 3-1C). Monitoring of SO indicates the elution of cow Hb at 11 and 18 min (Figure 3-1A) due to the presence of sulfur in cysteine and methionine residues. The co-elution of FeO peaks at the same times indicates the intact iron atom in the heme groups. iAs^{III} (28 min) and its oxidation product, iAs^{V} (24 min), both of which are smaller molecules, are retained longer on the size-exclusion column. When iAs^{III} is bound to cow Hb (Figure 3-1B), the complex of arsenite with cow Hb elutes at the same retention time (11 min) as cow Hb (Figure 3-1A). The column is incapable of separating the arsenic-bound protein from the free protein due to the negligible differences in their size. The ICPMS monitoring of AsO allows for the arsenic–protein complex to be distinguished from the unbound protein.

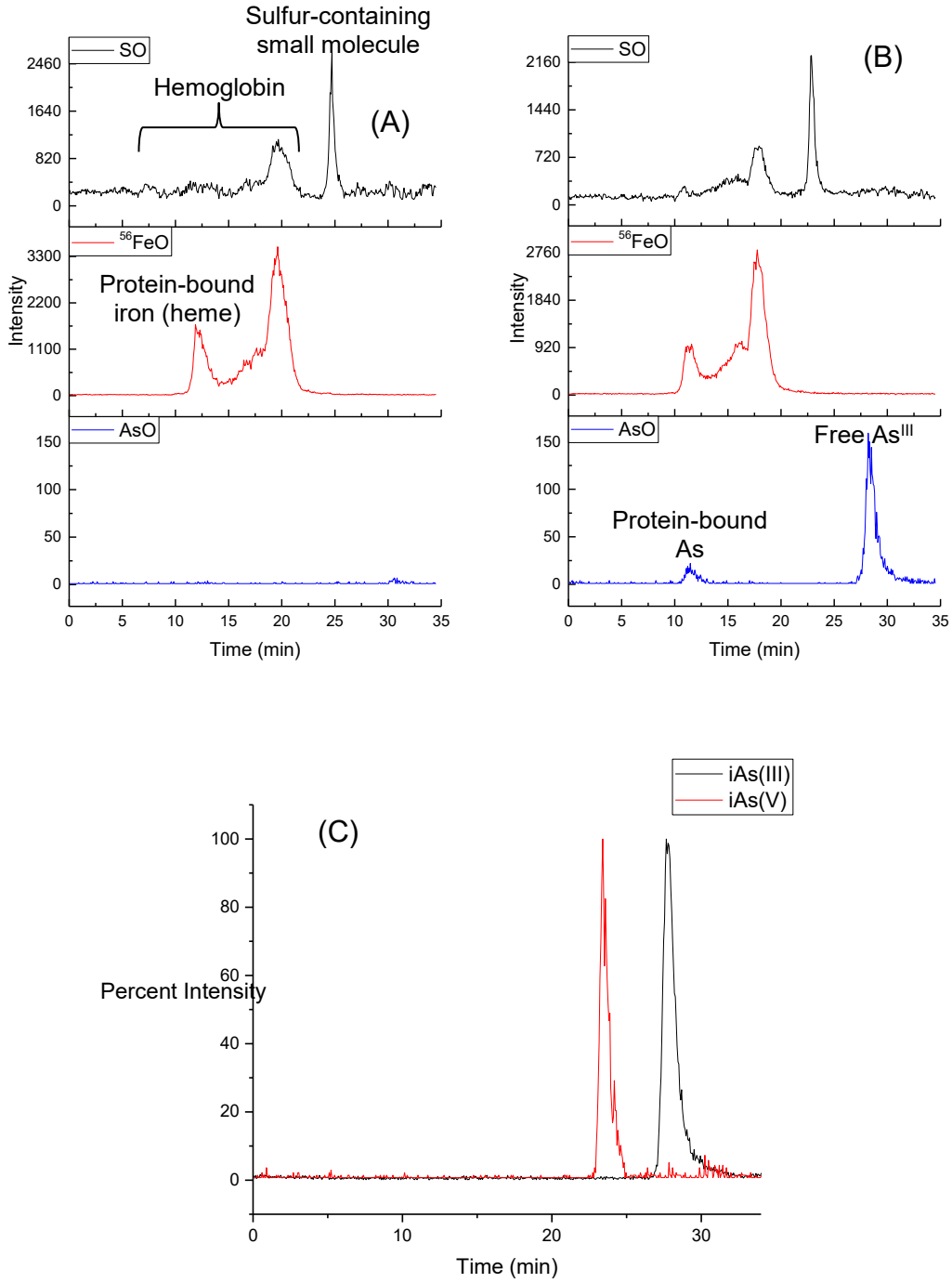


Figure 3-1. Chromatograms showing iAs^{III} that is free or bound to cow Hb after being subjected to SEC separation coupled with ICP-DRC-MS to selectively detect three oxides, SO^{+}

at 48 m/z, $^{56}FeO^{+}$ at 72 m/z, and AsO^{+} at 91 m/z. Cow Hb (20 μM) was incubated with iAs^{III} (1 μM) at 37˚C for 24 h in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis. (A) cow Hb only, (B) cow Hb incubated with 1 μM iAs^{III}, (C) iAs^{III} and iAs^{V} standards, only AsO^{+} detection shown.

Complexes of hemoglobin with MMA^{III} and DMA^{III} were resolved from the unbound arsenic species as well. Figure 2-3 shows the AsO detection of rat Hb incubated with each of the arsenic species. Unlike cow hemoglobin (Figure 3-2), rat hemoglobin can bind all three of the tested trivalent arsenicals. Equivalent SEC-ICPMS analyses of incubated solutions containing these arsenic compounds and the hemoglobin from the six other animal species were used to determine the percentage of arsenic bound to each hemoglobin when incubated with 1 or 5 μM arsenic, as seen in Figure 3-3. This demonstrates the differences in percent binding between the protein-bound complexes, suggesting interspecies differences in the affinity of hemoglobin for the arsenic species. Table 1-3 shows the different cysteine content between the studied animal species. Moreover, for the hemoglobin of one animal species, different affinities for the three arsenic species also are observed, suggesting differences in the affinity of one type of hemoglobin for each of the arsenic compounds.

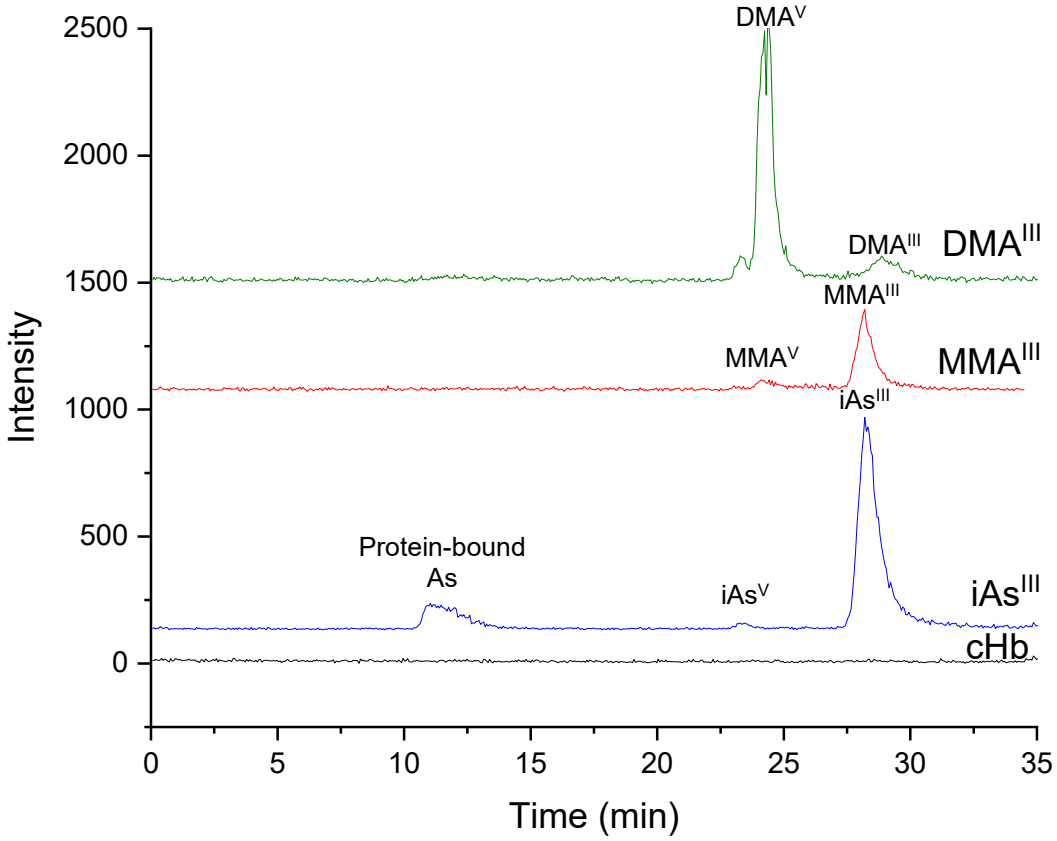


Figure 3-2. Chromatograms showing iAs^{III}, MMA^{III}, or DMA^{III} that is free or bound to cow Hb after being subjected to SEC separation coupled with ICP-DRC-MS. Detection of AsO^{+} (91 m/z) only shown. SO^{+} (48 m/z) was used to identify free from bound As. Cow Hb (20 µM) was incubated with trivalent arsenic (1 µM iAs^{III}, MMA^{III}, or DMA^{III} as labeled on right side of chromatograms) at 37˚C for 24 h in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis.

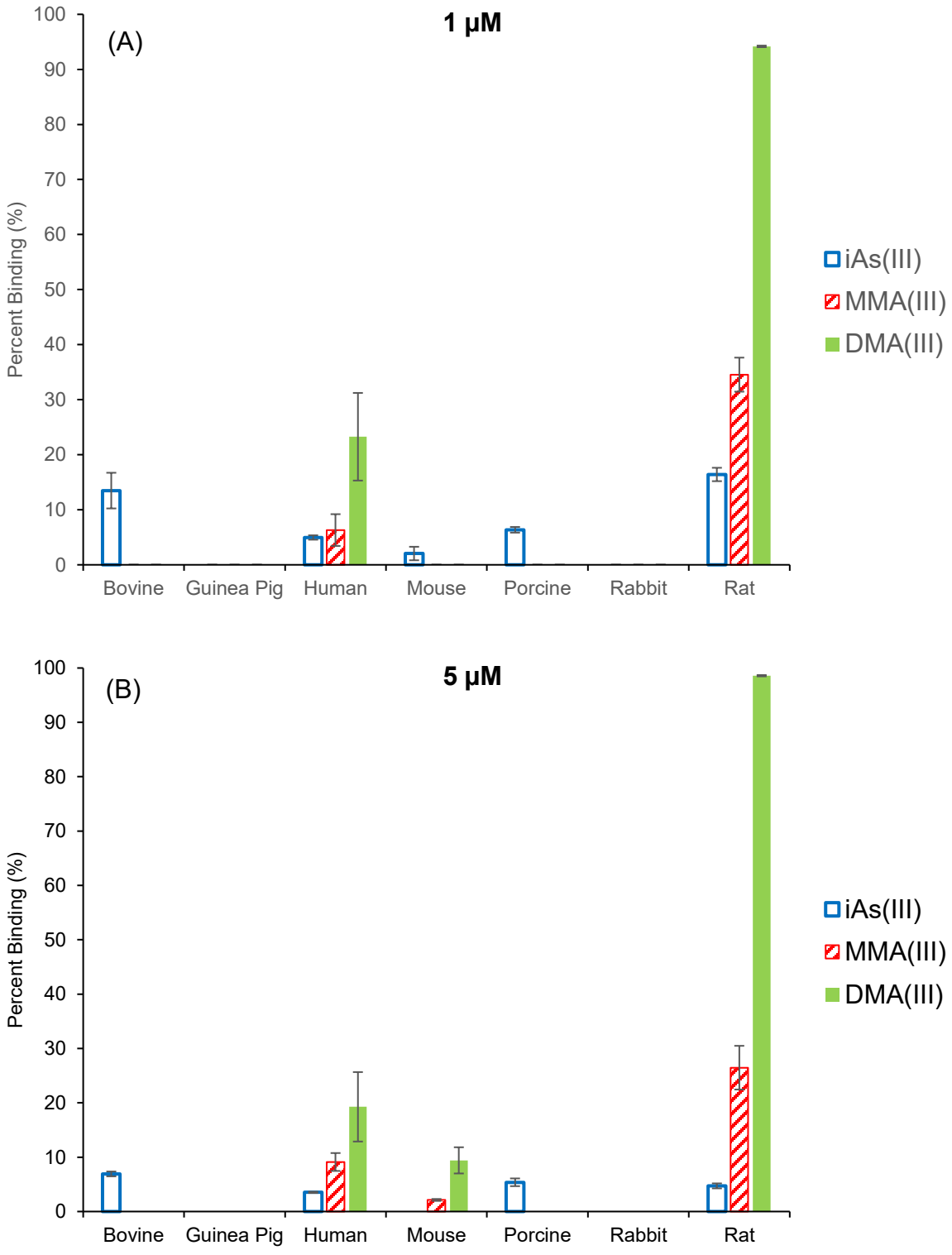


Figure 3-3. The comparison of the percent binding of three trivalent arsenic species (iAs^{III}, MMA^{III}, or DMA^{III}) that are bound to cow, guinea pig, human, mouse, pig, rabbit, and rat

hemoglobin. 20 μM hemoglobin was incubated with iAs^{III}, MMA^{III}, or DMA^{III} at 37˚C for 24 h in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to SEC-ICPMS analysis with the selective detection of SO^+ at 48 m/z, $^{56}FeO^+$ at 72 m/z, and AsO^+ at 91 m/z: (A) 1 μM As^{III}, (B) 5 μM As^{III}.

Additional experiments were carried out using a range of arsenics to hemoglobin molar ratios to determine the relative concentrations of each arsenic compound in the protein-bound and unbound forms, with each hemoglobin species. Solutions with arsenic to hemoglobin ratios of 1:20, 1:4, and 5:2, with the concentration of protein fixed at 20 μM, were incubated and analyzed by SEC-ICPMS allowing for the estimation of the apparent binding constants (nK) for the arsenic–hemoglobin complexes (Tables 3-1 and A-1), using Equation 1. Total elemental analysis by ICPMS was used to quantify arsenic concentrations, and the Bradford Assay was used to confirm the concentrations of hemoglobin solutions.

Examining the apparent binding constants allows for a comparison of the binding strength of different trivalent arsenic compounds to hemoglobin from one animal species. Further comparisons can be made by calculating the ratio of the apparent binding constants from one animal species to another, allowing for the determination of the relative affinity of the same arsenical to hemoglobin from different animal species. A comparison of the binding of the same arsenic compound to the hemoglobin from the various animal species shows that rat hemoglobin generally has higher apparent binding constants than all other hemoglobins. The Le group has previously identified Cys 13α as the target for As^{III} binding in rat hemoglobin using electrospray ionization tandem mass spectrometry (ESI-MS/MS).[162] A clear trend for stronger binding among the three arsenic species was not observed between the hemoglobin

from the different animal species studies.

Table 3-1. Apparent Binding Constants (nK) for 1 μM Trivalent Arsenicals Binding to 20 μM Hemoglobin*

	nK ($\times 10^3$) (M^{-1})						
	Cow	**Guinea Pig**	**Human**	**Mouse**	**Pig**	**Rabbit**	**Rat**
iAs^{III}	7.8 ± 0.8	nd	2.6 ± 0.3	1.0 ± 0.1	3.4 ± 0.3	nd	9.8 ± 0.6
MMA^{III}	nd	nd	3.3 ± 0.2	nd	nd	nd	26 ± 3
DMA^{III}	nd	nd	15 ± 2	nd	nd	nd	810 ± 40

*nd: complex not detected

The results indicated that hemoglobin from guinea pig (Cys 104α, 93β, and 125β) and rabbit (Cys 103α and 93β) did not bind to any of the trivalent arsenicals, despite the presence of available cysteine groups in their globin chains. Additionally, cow and pig hemoglobin each only bound to one of the three arsenic species tested. To confirm the low affinity of these proteins for the arsenicals, solutions were incubated with 50 μM As^{III} and 20 μM hemoglobin (Figure 3-4). Note that 50 μM is the initial concentration of As^{III}, as both MMA^{III} and DMA^{III} oxidize to MMA^{V} and DMA^{V}, respectively, over time. Even when exposed to high concentrations of arsenic, guinea pig hemoglobin did not bind to the arsenic species tested. This quantitative information can be used to study the trend in arsenic–hemoglobin binding as the concentration of arsenic increases. For example, for rat hemoglobin (Figure 3-5), there is

little difference between the concentration of DMA^{III} that binds to the protein, regardless of whether it is initially exposed to 5 or 50 µM DMA^{III}. For 20 µM of rat hemoglobin, it appears that its ability to bind DMA^{III} plateaus at approximately 4 µM of bound arsenic. It would be interesting to see if this is also the case for MMA^{III}, which was increasingly bound to rat hemoglobin at the highest concentration tested (50 µM).

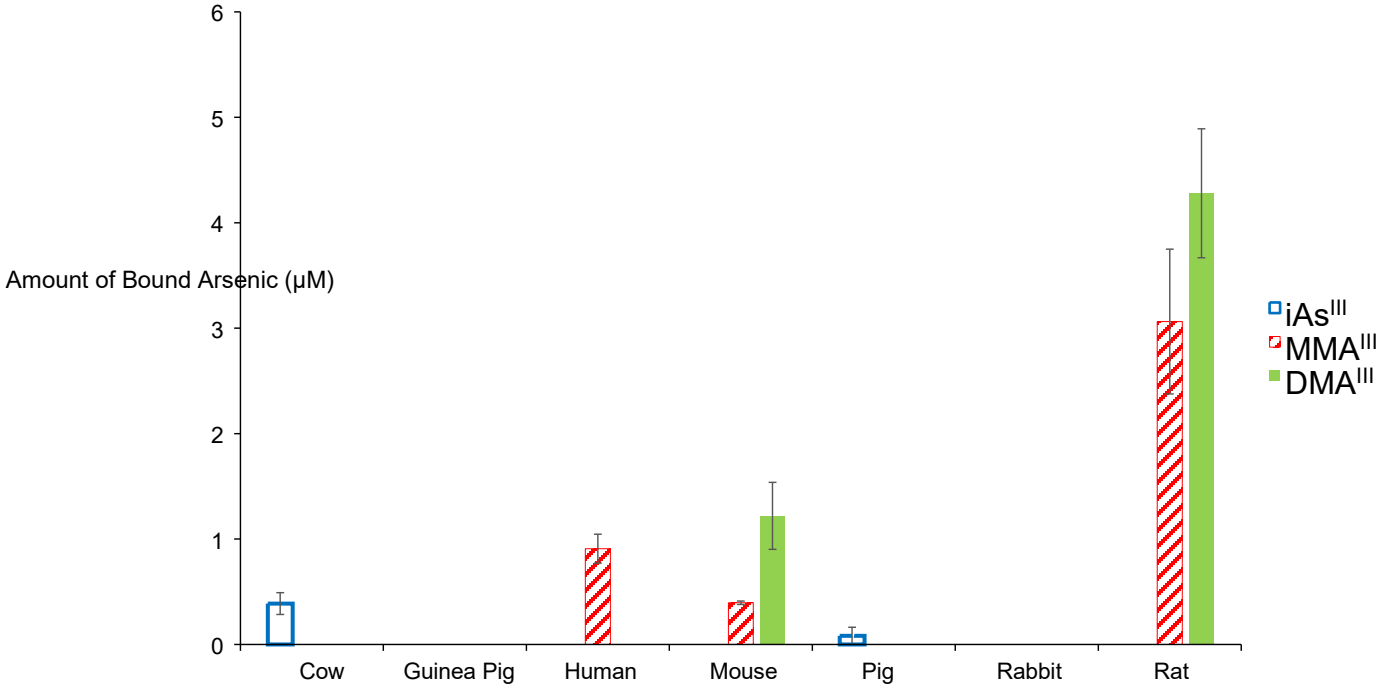
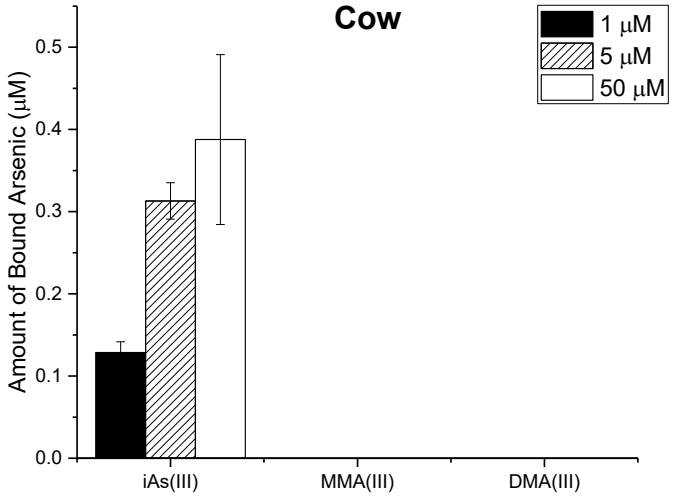
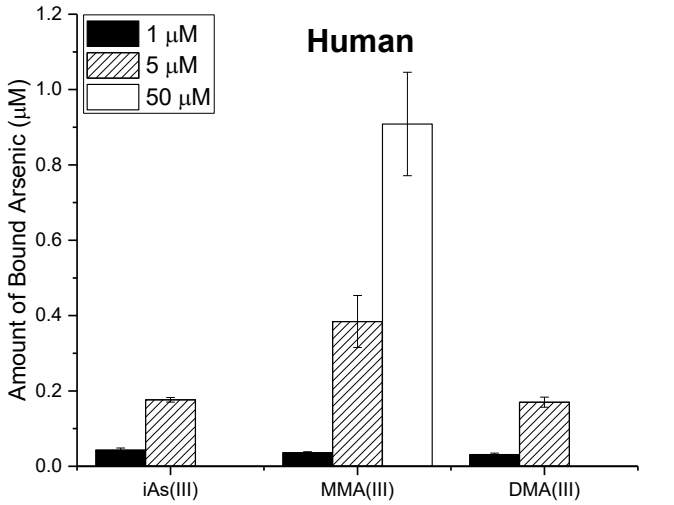
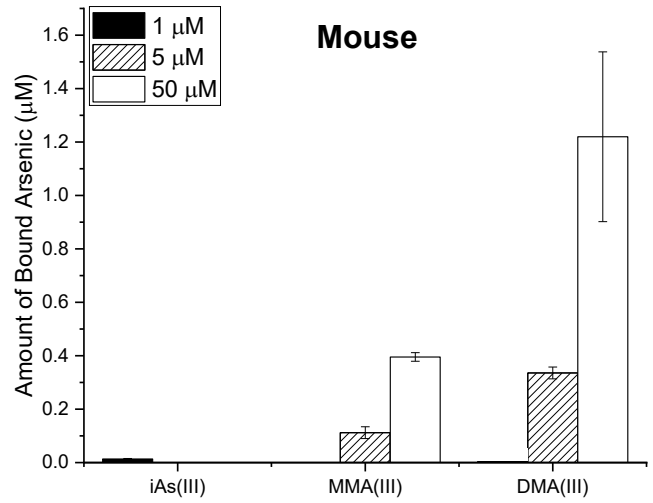
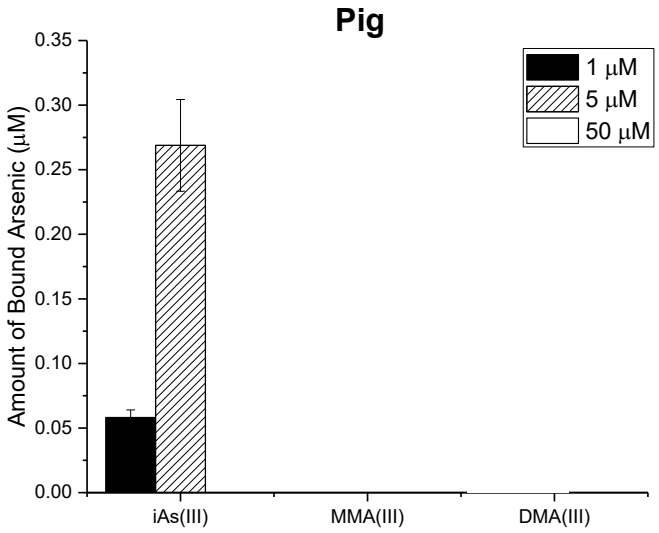


Figure 3-4. Amount of hemoglobin-bound As (µM) for cow, guinea pig, human, mouse, pig, rabbit, and rat Hb after incubation with 50 µM arsenic species. 20 µM hemoglobin was incubated separately with each of iAs^{III}, MMA^{III}, and DMA^{III} (50 µM), then was analyzed for protein-bound arsenic and free arsenic using SEC separation and ICPMS detection. No binding was detected for guinea pig or rabbit hemoglobin with 50 µM of any of the arsenic species tested.


Cow
1 μM
5 μM
50 μM
Amount of Bound Arsenic (μM)
0.0
0.1
0.2
0.3
0.4
0.5
iAs(III)
MMA(III)
DMA(III)


Human
1 μM
5 μM
50 μM
Amount of Bound Arsenic (μM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
iAs(III)
MMA(III)
DMA(III)


Mouse
1 μM
5 μM
50 μM
Amount of Bound Arsenic (μM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
iAs(III)
MMA(III)
DMA(III)


Pig
1 μM
5 μM
50 μM
Amount of Bound Arsenic (μM)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
iAs(III)
MMA(III)
DMA(III)

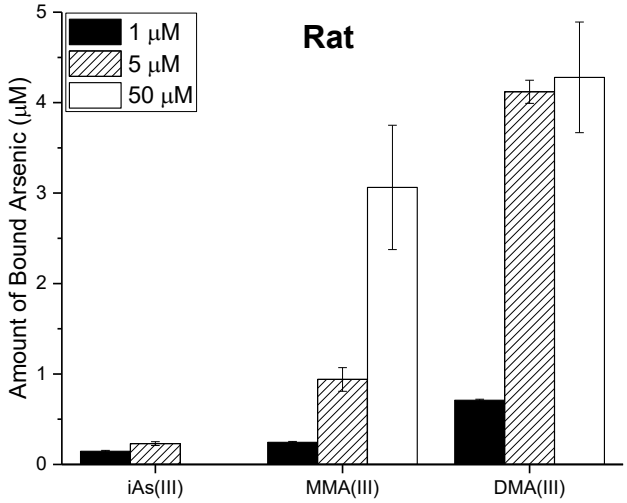


Figure 3-5. Concentration of hemoglobin-bound arsenic (μM) for cow, human, mouse, pig, and rat hemoglobin after incubation with arsenic species. Hemoglobin (20 μM) was incubated separately with each iAs^{III}, MMA^{III}, and DMA^{III} (1, 5, 50 μM), then was analyzed for protein-bound arsenic and free arsenic using SEC separation and ICPMS detection.

3.4 Conclusion

SE-HPLC-ICPMS was used to separate and detect protein-bound arsenic from free arsenic quantitatively, providing information on the interaction between hemoglobin and arsenic species. The binding between the trivalent arsenic species iAs^{III}, MMA^{III}, and DMA^{III} and cow, guinea pig, human, mouse, pig, rabbit, and rat hemoglobin were tested. Interspecies differences in the relative affinity of hemoglobin for the arsenic species *in vitro* were observed. This information could be related to the differences in the accumulation and retention of arsenic in the blood, as well as the distribution of arsenic throughout the body, excretion, and arsenic

toxicity. Based on these results and previous studies done in the Le lab, it is likely that the affinity of arsenic to the hemoglobin of different animal species is related to the capacity for arsenic accumulation in the red blood cells.[162]

To elucidate the mechanisms of arsenic toxicity and carcinogenesis *in vivo*, identifying an appropriate animal model is critical. The interspecies differences in arsenic accumulation, distribution, metabolism, and excretion may be related to the differences in toxicities of arsenic towards the different animal species. The interactions between arsenic and hemoglobin may play a key role in arsenic's varying mechanisms of action in different animal species. Unfortunately, at this time, only limited data is available regarding arsenic toxicity, metabolism, and retention in different animal species. Therefore, further research needs to be done to confirm whether a negative relationship exists between the differences in arsenic binding to hemoglobin and the differences in the toxicity of the arsenic compounds for different animal species. Elucidating the biochemical and chemical mechanisms of these differences could assist with human health risk assessment and the selection of appropriate animal models.

Chapter 4 Effects of Arsenic Compounds on the Binding Wild Type and Mutant p53 Proteins to Transcription Factor DNA

4.1 Introduction

The tumour suppressor protein, p53, functions as a transcription factor that regulates cellular responses to genotoxic stress.[94–97] The anticancer activity of p53 is mediated by several mechanisms, including growth arrest, DNA repair, and apoptosis.[98] Under normal cellular conditions, p53 is in its inactive form and the concentration is maintained at low levels through continuous degradation of the protein, which is regulated by the E3 ubiquitin-protein ligase Mdm2.[169] Mdm2 ubiquitinates p53, marking it for degradation. In stressed cells, p53 is activated by a variety of complex interactions with other cellular proteins. The ubiquitination of p53 is reversed by ubiquitin specific ligases, resulting in rapid accumulation of p53 in the cell. Additionally, conformational changes stabilize p53, resulting in its activation and enhanced DNA binding and transcriptional activity.

p53 is comprised of 393 amino acids and has seven domains: N-terminal transcription activation domain (TAD) or activation domain 1 (AD1) (residues 1–42) activates transcription factors;[170] activation domain 2 (AD2) (43–63), which is important for apoptotic activity; proline rich domain (64–92), which also is essential for apoptotic activity; central DNA-binding core domain (residues 102–292), which interacts with DNA in a sequence specific manner;[171] nuclear localization signaling domain (316–325); homo-oligomerization domain (307–355), which is critical for the tetramerization of p53 *in vivo*; and C-terminal domain

(356–393), which downregulates DNA binding to the central domain.[172] Each domain plays a significant role in the tumour suppressor activity of p53.

The p53 gene (*TP53*) is mutated in over 50% of human cancers, frequently resulting in inactivation of p53.[100] Single nucleotide substitutions are the most frequent mutations to the *TP53* gene; they cause continued expression of full-length p53 with a single amino acid substitution.[103] Mutations to p53 commonly result in loss of the protein's ability to bind to DNA and function as a transcription factor.[104] The HPLC-ICPMS method developed in Chapter 2 is used to demonstrate that wild type p53 was able to bind small amounts of certain trivalent arsenicals. The binding between p53 and arsenic could result in reduction of the protein's stability and inactivation. This interaction is a possible mechanism for arsenic-induced carcinogenesis. Additionally, mutations to p53 that result in an amino acid being substituted with cysteine, of which many natural variants associated with human cancer are known, may have stronger interactions with trivalent arsenic because of the addition of sulfhydryl (SH) groups. The interactions between trivalent arsenicals and p53 proteins are studied in order to address how known amino acid mutations affect the strength of the arsenic−protein binding and how arsenic-binding impacts the ability of p53 to bind to its transcription factor DNA.

4.2 Materials and Methods

Caution: *The arsenic species included in this study are toxic and are established or potential human carcinogens. Caution and care should be exercised when handling these materials.*

4.2.1 Materials

Sodium arsenite (iAs^{III}) (purity 96.7%) ($NaAsO_2$), sodium arsenate (iAs^{V}) (purity 99.4%) ($Na_2HAsO_4 \bullet 7H_2O$), and cacodylic acid (dimethylarsinic acid, DMA^{V}) (purity 98%) were obtained from Sigma (St. Louis, MO). Monomethylarsonic acid (MMA^{V}) (purity 99.0%) was purchased from Chem Service (West Chester, PA). Fluorescein arsenical hairpin binder-ethanedithiol (FlAsH-EDT_2) (purity >98%) was purchased from Cayman Chem Company (Ann Arbor, MI). Methylarsine oxide (CH_3AsO) and iododimethylarsine, $(CH_3)_2AsI$, were prepared following literature procedures and were both stored at -20°C.[122,123] Dilute solutions of these precursors were prepared fresh daily in deionized water to form monomethylarsonous acid (MMA^{III}), $CH_3As(OH)_2$, and dimethylarsinous acid (DMA^{III}), $(CH_3)_2AsOH$, respectively. FlAsH-EDT_2 was dissolved in ethanol using sonication and stored at -20°C.

Lysogeny broth was obtained from Fisher Scientific (Fair Lawn, NJ). SOB medium, lysozyme from chicken egg white, imidazole, piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), and magnesium chloride tetrahydrate ($MgCl_2 \bullet 4H_2O$) were purchased from Sigma (St. Louis, MO). Reduced glutathione and Halt protease and phosphatase inhibitor single use cocktail were obtained from Thermo Sci (Rockford, IL). Agar, ampicillin sodium salt, and zinc chloride ($ZnCl_2$) were purchased from Acros Organics (New Jersey). SOC medium, agarose, UltraPure isopropyl β-D-1-thiogalactopyranoside (IPTG), DNase I, and Ni-NTA agarose were purchased from Invitrogen (Carlsbad, CA). RNAse A and QIAprep Spin Miniprep Kit were obtained from QIAGEN (Hilden, Germany). Phosphate buffered saline (PBS) pH 7.4 was obtained from Gibco by Life Tech (Grand Island, NY). GST-tagged recombinant human p53

protein and pAb240 antibody was obtained from Abcam (Cambridge, UK). DNA primers were purchased from Integrated DNA Technologies (Coralville, IA).

GST-tagged recombinant human p53 protein from Abcam was used without further purification. GST-tag purified recombinant 82–292 fragment p53 proteins were purified using glutathione Sepharose 4B obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Following purification, the fragment p53 proteins were desalted by buffer exchange with PBS using 30kDa Amicon Ultra-4 cellulose centrifugal filters from Merck Millipore Ltd. (Cork, Ireland). His-tag purified recombinant wild type p53 underwent dialysis in Tris buffer using Spectra/Por molecular porous membrane tubing, MWCO 6–8 kDa standard RC tubing from Spectrum Laboratories Inc. (Rancho Dominguez, CA). The concentrations of desalted protein solutions were determined using the Bio-Rad Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA). Desalted proteins were stored at 4˚C for 1 month or in 50% glycerol at -80˚C for longer periods.

DH5α and BL21(DE3) *E. coli* strains, pEt15b plasmid containing the expression vector for His-tagged wild type p53, pGEX-2TK containing the expression vector for the GST-tagged 82–292 p53 fragment, and pAb246, and pAb1620 antibodies were kindly provided by Dr. Roger Leng.

All other reagents used in the experiments were HPLC grade or analytical grade.

4.2.2 Methods

4.2.2.1 Expression Vectors

pET15b served as the backbone bacterial expression vector for His-tagged wild type p53. pGEX-2TK served as the backbone expression vector for the GST-tagged 82–292 amino acid (a.a.) fragment of p53.

4.2.2.2 Site-Directed Mutagenesis

Mutants of the p53 fragment containing 82–292 a.a. were generated using the QuikChange Lightning (Agilent, Cedar Creek, TX) site-directed mutagenesis kit. Mutagenesis was performed using pGEX-2TK 82–282 $p53^{173}$ as the template of polymerase chain reaction (PCR) according to the manufacturer's instructions. Primers (Integrated DNA Technologies, Coralville, Iowa) for generating mutants were designed with natural variants associated with cancer that resulted in the substitution of an amino acid to cysteine. All mutants were confirmed by Sanger sequencing (The Amplified Genomics Core, Edmonton, AB) using the pGEX 3' sequencing primer.

Primers for mutant p53 were designed using the Agilent web-based QuikChange Primer Design Program (www.agilent.com/genomics/qcpd), except the primers for the DNA domain, which were modified to eliminate overlap of the primers along the template. The sequences of the primers used in site-directed mutagenesis are listed in Table 4-1.

Table 4-1. Primers Used for Generating p53 Mutants by Site-Directed Mutagenesis. Nucleotide mutations shown in bold.

Mutant Name	Nucleotide Mutation	Nucleotide Primer Sequence (5' to 3')	Resulting Amino Acid Mutation
DNA domain	a377g	G TCT GTG ACT TGC ACG T**G**C TCC CCT GCC C	Y126C
	t401g	C AAC AAG ATG T**G**T TGC CAA CTG GCC AAG ACC	F134C
FlAsH	c722g, g730t, and g733t	C ATG TGT AAC AGT T**G**C TGC ATG **T**GC **T**GC ATG AAC CGG AGG C	S241C, G244C, and G245C
Surface	g438t	C CCT GTG CAG CTG TG**T** GTT GAT TCC ACA CCC	W146C
	c680g	G CCG CCT GAG GTT GGC T**G**T GAC TGT ACC	S227C

4.2.2.3 Preparation and Purification of Recombinant Proteins

DNA sequences corresponding to wild type human p53 were subcloned into the bacterial expression vector pET15b (Novagen) to produce His-tagged p53 protein. The GST-tagged p53 fragment protein 82–292 a.a. and its mutants were expressed in bacteria using plasmids based on the pGEX-2TK backbone (GE Life Sciences). These vectors were expressed in *E. coli* BL21(DE3) (Novagen) and induced with 1 mM isopropyl-1-thio-α-D-galactopyranoside (IPTG) for 5 h at 28°C. Bacteria were lysed by sonication, and the recombinant GST- or His-tagged proteins were purified by affinity chromatography using glutathione-Sepharose beads (Amersham Pharmacia Biotech) or Ni-NTA agarose beads (Qiagen), respectively.

4.2.2.4 Size Exclusion Chromatography Separation with Inductively Coupled Plasma Mass Spectrometry Detection

A PerkinElmer 200 series HPLC system (PerkinElmer Instruments, Shelton, CT) fitted with an autosampler was used to separate the unbound arsenic from protein-bound arsenic. A Superdex 200 Increase 10/300 GL column (10 mm internal diameter, 300 mm length) with an exclusion limit of 1.3×10^6 Da (General Electric Healthcare Life Sciences) was used to separate the protein-bound arsenic from the unbound arsenic species. An ammonium bicarbonate buffer (10 mM, pH 7.4), with a flow rate of 0.75 mL/min, was used as the mobile phase. The sample injection volume was 30 µL. Wild type p53 concentration was 50 µM. Arsenic concentrations were kept constant at 5 µM. The effluent from the size exclusion column was coupled directly to the spray chamber of an Elan 6100 DRC plus ICP mass spectrometer (PerkinElmer Sciex,

Concord, ON, Canada). The operating parameters of ICPMS were optimized to an RF power (1350 W), a plasma gas flow (15 L/min), an auxiliary gas flow (1.5 L/min), a nebulizer gas flow (0.89 L/min), and cell gas (0.6 mL/min). ICPMS performance was optimized and checked daily by testing the PerkinElmer Pure Plus ELAN 6100 DRC Setup/Sta/Masscal Solution (PerkinElmer, Shelton, CT, USA) standard mixture containing 1 ng/mL In, Co, Mg, Ba, Pb, and As in 1% HNO_3.

4.2.2.5 Transcription Factor Assay

An enzyme-linked immunosorbent assay (ELISA) is used to investigate the effects of arsenic on the transcription factor DNA binding ability of wild type and mutant p53 proteins. As illustrated in Figure 1-5, a specific double-stranded DNA (dsDNA) sequence that can bind to active p53 is immobilized on the walls of a 96-well plate. Detection of DNA-bound p53 is enabled by the addition of a p53 binding primary antibody, followed by the addition of a horseradish peroxidase (HRP) conjugated secondary antibody that provides a sensitive colourimetric readout at 450 nm. The changes in the DNA binding ability of p53 and specifically designed mutant p53 proteins, in the absence or presence of toxic trivalent arsenicals, were studied using this modified p53 transcription factor assay.

The p53 transcription factor assay kit was purchased from Cayman Chem Company (Ann Arbor, MI) and used with several modifications to the manufacturer's instructions. In addition to the primary antibody provided by the supplier, which was monoclonal with an unmapped epitope, the p53 antibodies pAb1620, pAb246, and pAb240 also were used as they had mapped epitopes between residues 82 and 292 on p53. The additional primary antibodies were diluted 1:2000 in the antibody binding buffer provided with the kit. Purified proteins were

used in lieu of nuclear extracts in the assay. 10 µL of approximately 10 µM protein was used per well.

To test the effect of trivalent arsenic on the DNA binding ability of p53, 10 µM protein (His-tagged wild type, GST-tagged wild type, 82–292 fragment, and the four mutants) were incubated with 50 µM As^{III} (iAs^{III}, MMA^{III}, DMA^{III}, PAO^{III}, or FlAsH-EDT_2) overnight at 4ºC prior to performing the assay. Dithiothreitol (DTT) was not added to the Complete TFB Assay Buffer (CTFB) for wells containing arsenic. Tests using Nutlin-3-stimulated MCF-7 nuclear extract as a positive control showed minimal effect on the signal in the absence of DTT. An additional control of denatured full-length p53 was used to test for non-specific binding.

4.3 Results and Discussion

4.3.1 Mutant Design and Site Directed Mutagenesis of p53

Mutant p53 proteins were designed according to known amino acid mutations that resulted in a cysteine replacing another amino acid because of a single nucleotide substitution. The Uniprot database (www.uniprot.org) entry P04637 for the human cellular tumour antigen p53 lists the natural variants of p53 associated with various human cancers. Three mutant p53 proteins were designed based on the 82–292 fragment of p53 protein and were named DNA domain mutant, FlAsH mutant, and Surface mutant. Figure 4-1 gives the nucleotide and amino acid sequence for *TP53* and p53, respectively, and shows the locations of site-directed mutagenesis used to create the mutant p53 proteins.

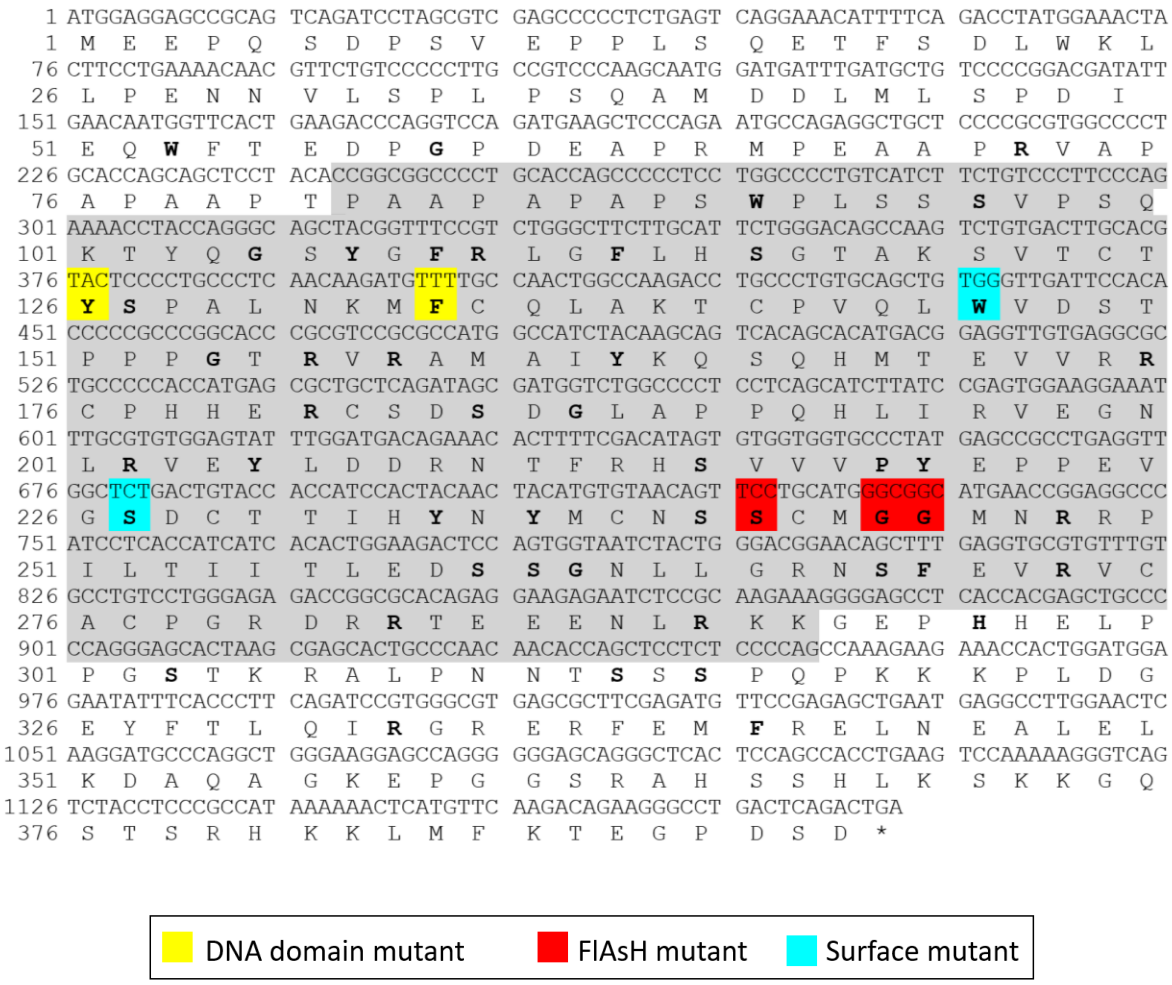


Figure 4-1. Nucleotide sequence of the p53 gene *TP53* and the amino acid sequence of the p53 protein. The grey region corresponds to the 82–292 fragment p53 protein. Mutation sites for p53 mutant proteins are shown as follows: yellow, DNA domain mutant, residues 126 and 134; red, FlAsH mutant, residues 241, 244, 245; and blue, Surface mutant, residues 146 and 227. Sites of known single amino acid cysteine substitutions associated with cancer shown in bold.

The central DNA binding core domain spans residues 102–292 and binds to DNA in a sequence specific manner. X-ray crystallography shows that this domain contains a zinc atom

that is tetrahedrally coordinated to three cysteine residues (Cys 176, 238, and 242) and one histidine (His 179).[93] Transition metals have been shown to be able to replace zinc. Amino acids 124–141 are known to interact directly with DNA, and studies using site-directed mutagenesis of cysteines 124, 135, and 141 in murine p53 demonstrated that these residues may cooperate to modulate the structure of the DNA binding domain.[174] The DNA domain mutant was designed to have two cysteine substitutions: tyrosine 126 coded by the TAC codon (nucleotides 376–378), mutated to cysteine with the single base substitution of adenine 377 to guanine, as TGC codes for cysteine; phenylalanine 134 coded by the TTT codon (nucleotides 400–402) mutated to cysteine with substitution of thymine 401 with guanine. Mutation Y to C 126 (VAR_044716126) is associated with familial cancer other than Li−Fraumeni syndrome and germline mutation, as well as a somatic mutation in sporadic cancers. Mutation F to C 134 (VAR_044749134) is a somatic mutation associated with sporadic cancers. Mutations and variants are summarized in Table 4-2.

Fluorescein arsenical hairpin binder-ethanedithiol (FlAsH-EDT_2) is an organoarsenic compound with a fluorescein core and two 1,3,2-dithiarsolane substituents. FlAsH is capable of binding proteins to the tetracysteine motif CCXXCC *in vitro* and *in vivo* and is used frequently for imaging proteins as it only fluoresces once bound. Based on the natural variants of p53, a CCXCC motif can exist at residues 241–245, and despite not being the preferred CCXXCC motif, FlAsH has been shown to bind this similar tetracysteine sequence with high efficiency.[175] Mutation 241 (VAR_045217241) involved substitution of a serine to a cysteine and a TCC codon (nucleotide 721–723) to a TGC codon; it is a somatic mutation associated with sporadic cancers. Mutation 244 (VAR_045231244) and 245 (VAR_005972245) both involved substitution of a glycine to a cysteine and GGC codons (nucleotides 730–732 and

733–735) becoming TGC codons. Both 244 and 245 variants are associated with sporadic cancers, while 245 is associated also with Li−Fraumeni syndrome and germline mutations. The CCXCC motif also involves cysteine 242, which is part of the zinc finger.

The Surface mutant was designed to substitute two residues with cysteine that were exposed on the surface of the protein and, therefore, might be easily accessible to arsenic. Sites were selected based on the crystal structure data from PDB 1TUP,[93] which was displayed using the molecular visualization software PyMol 2.0 (Schrödinger, LLC, New York, NY), as shown in Figures 4-2 and 4-3. Both tryptophan 146 and serine 227 are conformationally close to cysteine 229 in folded wild type p53, as seen in Figure 4-2. Additionally, the functional group of all three residues are exposed on the surface of the protein, as shown in Figure 4-3, and are pointing in the same direction (the upper oxygen atom on the serine in Figure 4-3C is from the protein backbone, and the lower oxygen is from the functional group). Certain trivalent arsenicals (iAs^{III}, MMA^{III}, and PAO^{III}) can bind multiple cysteine residues. For the mutations that create multiple exposed cysteines, with similar orientation in proximity to one another, the strength of the interaction with trivalent arsenicals, that have more than one binding site, theoretically will be increased. Mutation 146 (VAR_044795146) involved substitution of a tryptophan with a cysteine, as a result of a TAC codon (nucleotides 436–438) becoming a TGC codon. Mutation 227 (VAR_045148227) involved substitution of a serine with a cysteine due to a TCT codon (nucleotides 679–681) becoming a TGT codon. Both are somatic mutations associated with sporadic cancers.

Table 4-2. Summary of Mutants and Natural Variants

Mutant	Nucleotide Mutation	Natural Variant	Amino Acid Mutation	Description
DNA domain mutant	a377g	VAR_044716126	Y126C	In a familial cancer not matching Li−Fraumeni syndrome; germline mutation and in sporadic cancers; somatic mutation
	t401g	VAR_044749134	F134C	In sporadic cancers; somatic mutation. Corresponds to variant dbSNP:rs780442292
FlAsH mutant	c722g	VAR_045217241	S241C	In sporadic cancers; somatic mutation. Corresponds to variant dbSNP:rs28934573
	g730t	VAR_045231244	G244C	In sporadic cancers; somatic mutation
	g733t	VAR_005972245	G245C	In Li−Fraumeni syndrome; germline mutation and in sporadic cancers; somatic mutation. Corresponds to variant dbSNP:rs28934575
Surface mutant	g438t	VAR_044795146	W146C	In a sporadic cancer; somatic mutation
	c680g	VAR_045148227	S227C	In sporadic cancers; somatic mutation

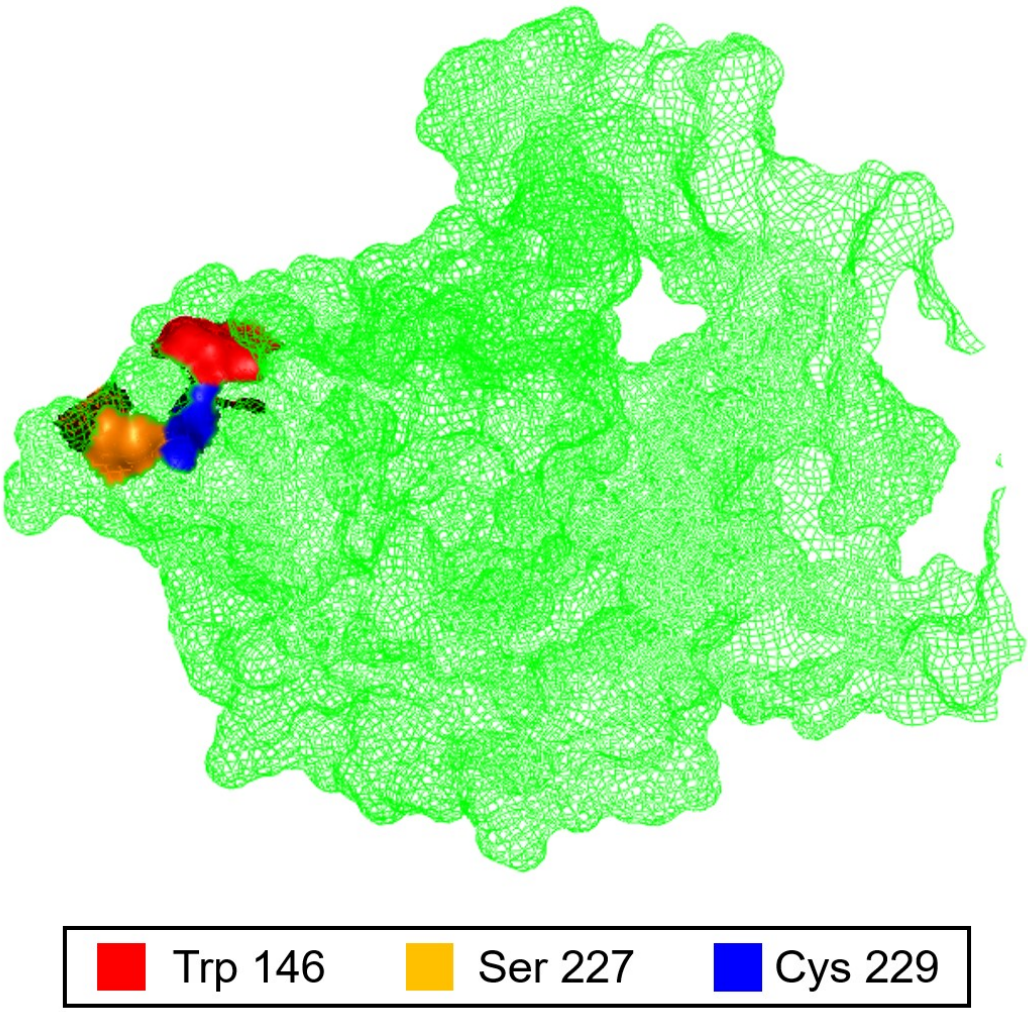


Figure 4-2. The three-dimensional structure of the DNA binding domain of p53 showing residues 146, 227, and 229 for Surface mutant design. Trp 146 shown in red, Ser 227 in orange, and Cys 229 in blue. Displayed using PyMol 2.0 and the crystal structure from PDB 1TUP.[93] (A) Mesh model of p53 with Trp 146 shown in red, Ser 227 in orange, and Cys 229 in blue.

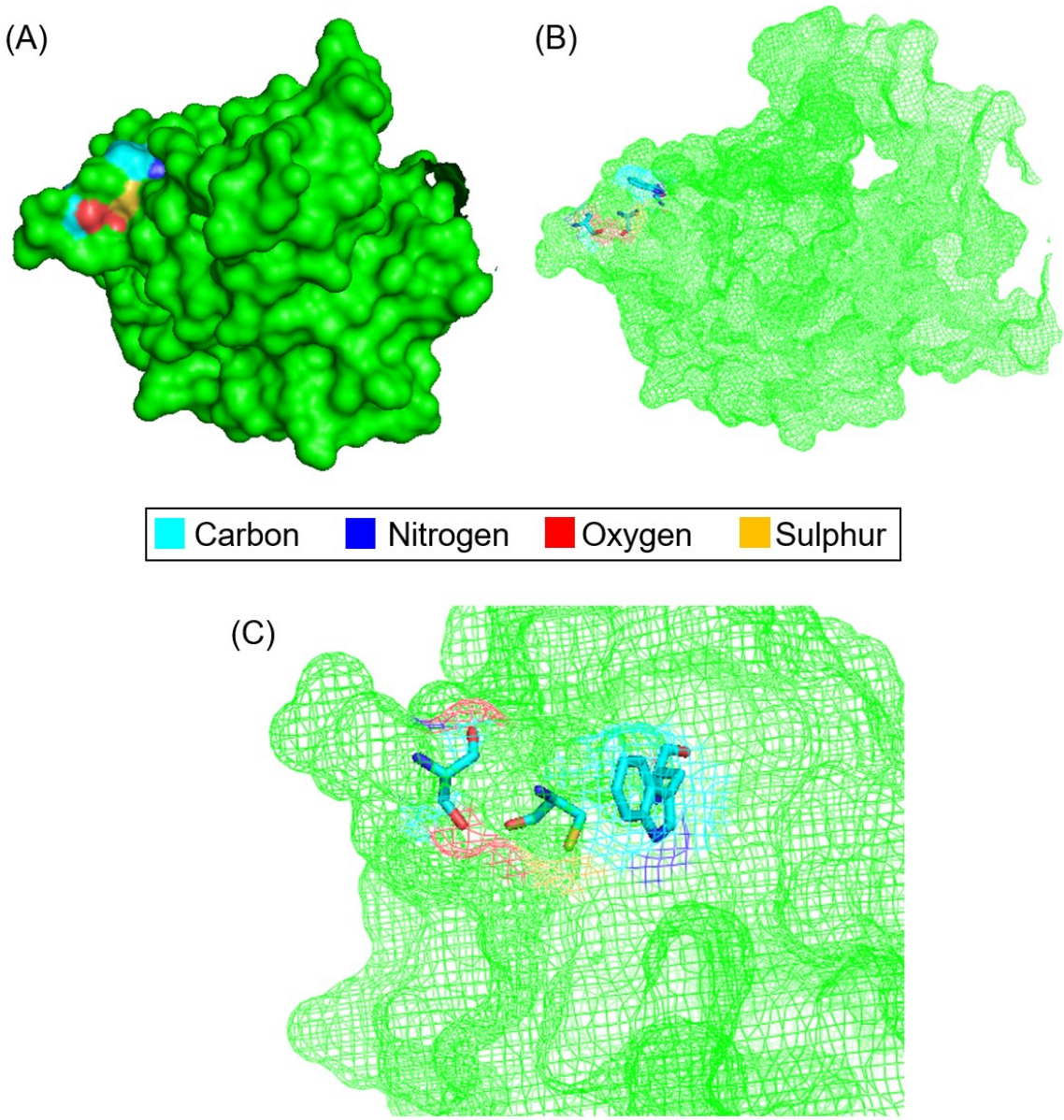


Figure 4-3. The three-dimensional structure of the DNA binding domain of p53 highlighting the orientation of the functional groups of residues 146, 227, and 229 for Surface mutant design. Displayed using PyMol 2.0 and the crystal structure from PDB 1TUP.[93] (A) Surface visualization of amino acids 94–292 of wild type p53, with Trp 146, Ser 227, and Cys 229 atoms shown in colour. (B) Mesh visualization of p53 showing mesh surface and stick model

of Trp 146, Ser 227, and Cys 229. (C) Different orientation, zoomed in mesh visualization of p53 with mesh surface and stick model of Trp 146, Ser 227, and Cys 229. Same orientation of protein shown for Figure 4-2 as Figure 4-3A–B.

Site-directed mutagenesis was confirmed by Sanger sequencing using the reverse primer pGEX 3'; an example of sequencing data is shown in Figure 4-4. The purity of plasmids was confirmed using a 1% agarose gel separation, an example shown in Figure 4-5.

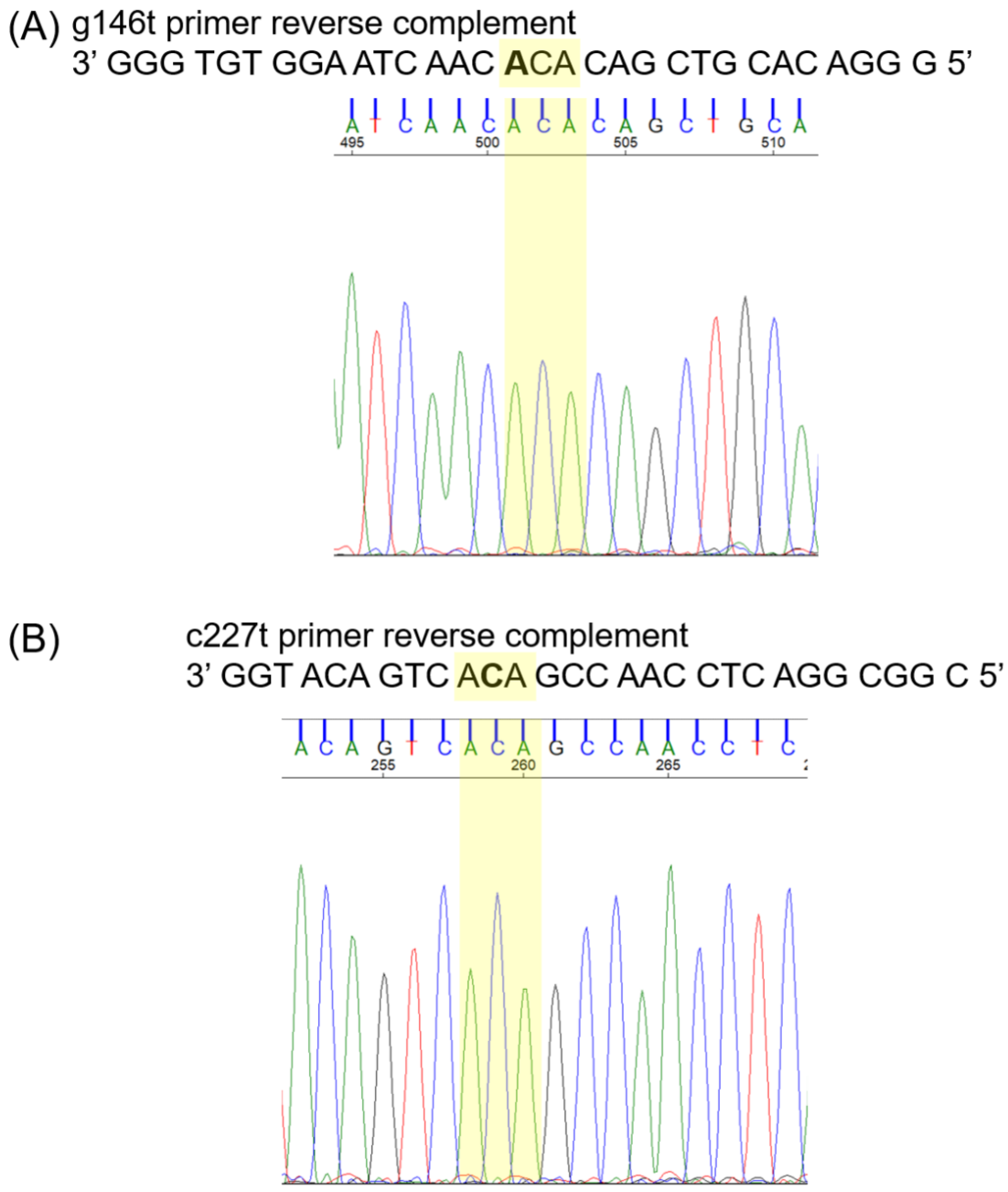


Figure 4-4. Example of Sanger sequencing data showing successful site-directed mutagenesis of Trp 146 to Cys (TGG to TGT) and Ser 227 to Cys (TCT to TGT) to produce the Surface mutant pGEX-2TK plasmid. Reverse primer pGEX 3' was used for sequencing. Primers used for generating the plasmid are listed in Table 4-1.

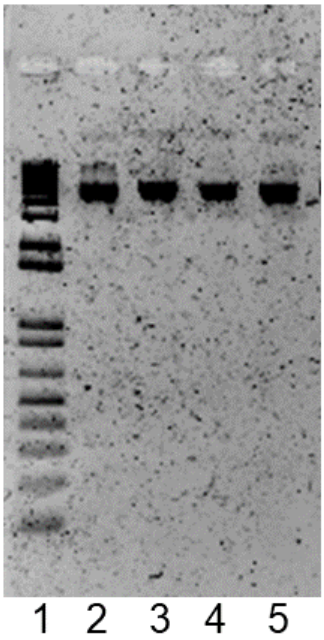


Figure 4-5. Agarose gel separation of purified p53 pGEX-2TK 82–292 p53 plasmids. Lane 1, 1 kilobase DNA marker; Lane 2, 82–292 fragment plasmid; Lane 3, DNA domain mutant plasmid; Lane 4, FlAsH mutant plasmid; Lane 5, Surface mutant plasmid. The gels were stained with ethidium bromide.

4.3.2 Purification of p53 Proteins

Wild type and mutant 82–292 fragment p53 proteins were extracted successfully using the described protocols. The purity of the final protein samples was established by SDS-PAGE, as shown in Figure 4-6. Free GST was removed using buffer exchange and 30kDa centrifugal filters and monitored using HPLC-ICPMS. His-tag purified recombinant wild type p53 underwent dialysis in Tris buffer using Spectra/Por molecular porous membrane tubing,

MWCO 6–8 kDa standard RC tubing from Spectrum Laboratories Inc. (Rancho Dominguez, CA).

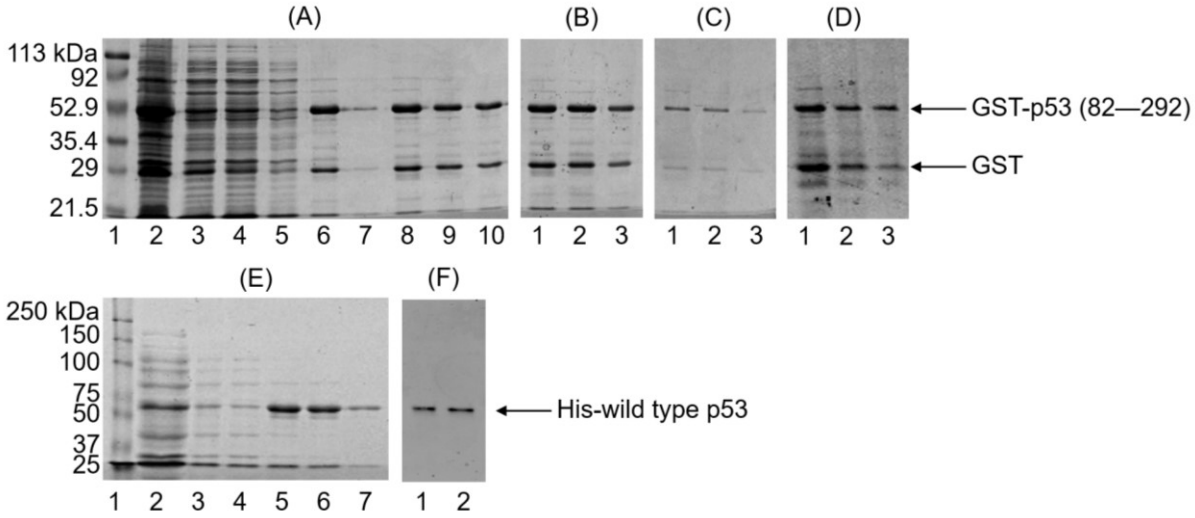


Figure 4-6. SDS-PAGE separation of 82–292 fragment, DNA domain mutant, FlAsH mutant, and Surface mutant GST-tagged p53 proteins, and of His-tagged wild type p53 before and after dialysis. The gels were stained with Coomassie blue. Features shown are: (A) GST-affinity column purification of GST-tagged wild type 82–292 fragment: Lane 1, molecular weight markers; 2, cell lysate; 3, soluble protein fraction; 4, flow through; 5, wash 1; 6, wash 2; 7, wash 3; 8, elution 1; 9, elution 2; 10, elution 3. (B) DNA domain mutant 82–292 p53: Lane 1, elution 1; 2, elution 2; 3, elution 3. (C) FlAsH mutant 82–292 p53: Lane 1, elution 1; 2, elution 2; 3, elution 3. (D) Surface mutant 82–292 p53: Lane 1, elution 1; 2, elution 2; 3, elution 3. (E) Wild type p53: Lane 1, molecular weight markers; 2, flow through; 3, wash 1; 4, wash 2; 5, elution 1; 6, elution 2; 7 elution 3. (F) Wild type p53 after dialysis: Lane 1, elution 1; 2, elution 2.

4.3.3 HPLC-ICPMS Analysis of Wild Type p53-Arsenic Interactions

The interaction between wild type p53 and five trivalent arsenicals (iAs^{III}, MMA^{III}, DMA^{III}, PAO^{III}, and FlAsH) was studied using the previously developed HPLC-ICPMS method. Size-exclusion high-performance liquid chromatography (SE-HPLC) was used to separate the p53-bound from the unbound arsenic species. This was coupled on-line with inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) that enabled the formation of oxides in the DRC and the detection of sulfur oxide and arsenic oxide. Zinc oxide ($^{68}ZnO^{+}$) detection using this method would allow for monitoring of the zinc finger and show whether or not arsenic was displacing the zinc atom. $^{68}ZnO^{+}$ (m/z 84) was monitored as opposed to the more abundant $^{64}ZnO^{+}$ (m/z 80) because of the interference that would be caused by $^{80}Se^{+}$. Although ICPMS was capable of detecting $^{68}ZnO^{+}$, the zinc was completely retained on the size-exclusion column and no zinc peak was observed from p53. Nonetheless, the SEC-ICP-DRC-MS technique provides information on whether an arsenic species binds to a protein and on the relative strength (apparent binding constant) of this interaction.

Figure 4-7 depicts the chromatograms obtained from the SEC-ICPMS analyses of (A) 50 μM p53, (B) a mixture of 50 μM p53 and 5 μM PAO^{III} after incubation at 4˚C for 30 min, and (C) AsO^{+} detection of PAO^{III} and PAO^{V} standards. Monitoring of SO indicates the elution of p53 at 11 and 15 min (Figure 4-7A) due to the presence of sulfur in cysteine and methionine residues. PAO^{III} (37 min) and its oxidation product, PAO^{V} (23 min), both of which are smaller molecules, are retained longer on the size-exclusion column. When PAO^{III} is bound to p53 (Figure 4-7B), the complex of arsenic with p53 elutes at the same retention time (11 min) as

free p53 (Figure 4-7A). The column is incapable of separating the arsenic-bound protein from the free protein due to the negligible differences in their size. The ICPMS monitoring of AsO^+ allows for the arsenic–protein complex to be distinguished from the unbound protein.

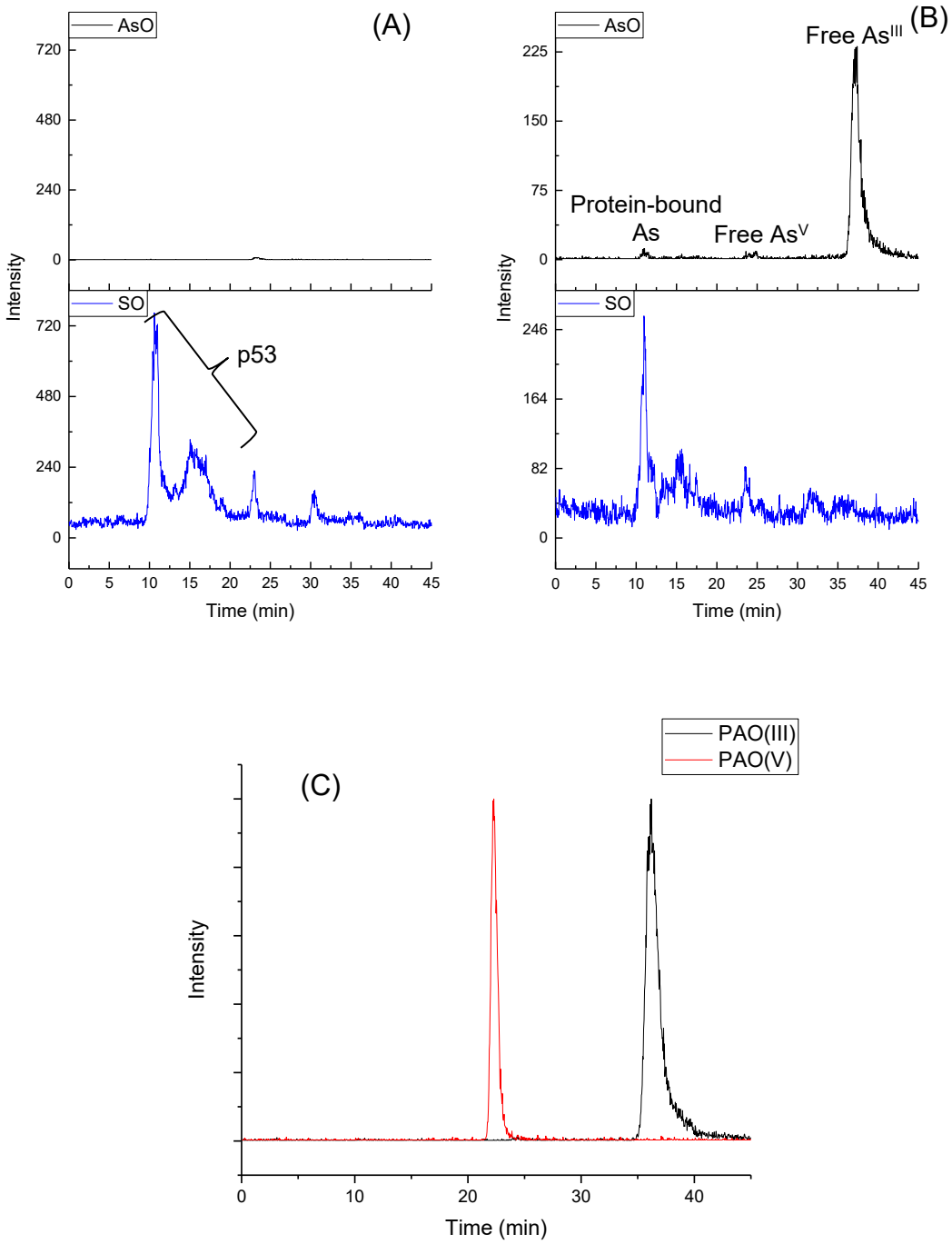

(A)
AsO
720
480
240
0
Intensity
SO
720
480
240
0
p53
0 5 10 15 20 25 30 35 40 45
Time (min)
(B)
AsO
225
150
75
0
Free As^III
Protein-bound
As
Free As^V
Intensity
SO
246
164
82
0
0 5 10 15 20 25 30 35 40 45
Time (min)
(C)
PAO(III)
PAO(V)
Intensity
0 10 20 30 40
Time (min)

Figure 4-7. Chromatograms showing PAO^{III} that is free or bound to p53 after being subjected to SEC separation coupled with ICP-DRC-MS to selectively detect two oxides, SO^+ at 48 m/z and AsO^+ at 91 m/z. Wild type p53 (50 μM) was incubated with PAO^{III} (5 μM) at 4˚C for 30 min in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis. (A) p53 only, (B) p53 incubated with 5 μM PAO^{III}, (C) PAO^{III} and PAO^{V} standards, only AsO^+ detection shown.

Minor interactions between p53 and MMA^{III} were observed. Figure 4-8 shows chromatograms obtained from the SEC-ICPMS analyses of (A) a mixture of 50 μM p53 and 5 μM MMA^{III} after incubation at 4˚C for 30 min and (B) of the AsO detection of MMA^{III} and MMA^{V} standards. The complex of arsenic with p53 elutes at 11 min (Figure 4-8A), which is the same retention time as the free protein. Figure 4-8B shows the elution of standard MMA^{III} (28 min) and its oxidation product, MMA^{V} (24 min).

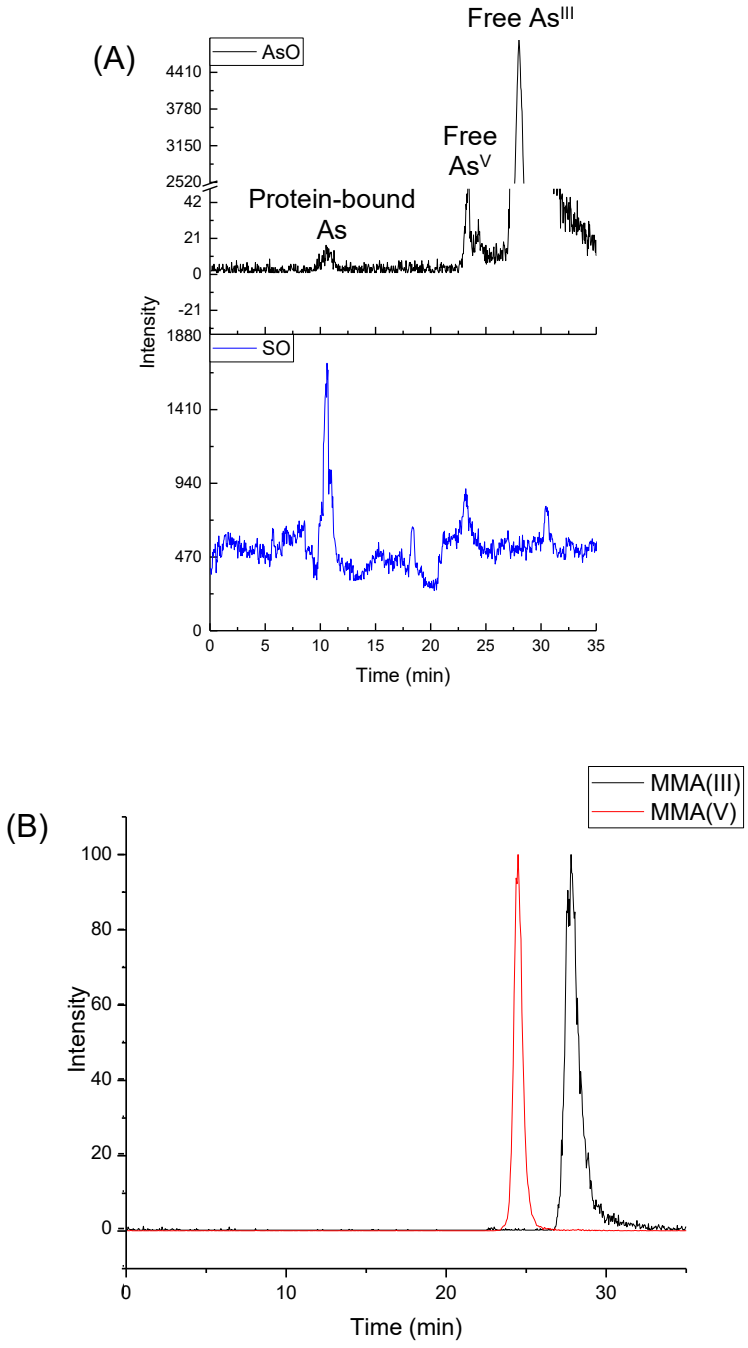


Figure 4-8. Chromatograms showing MMA^{III} that is free or bound to p53 after being subjected to SEC separation coupled with ICP-DRC-MS to selectively detect two oxides, SO^+ at 48 m/z and AsO^+ at 91 m/z. Wild type p53 (50 µM) was incubated with MMA^{III} (5 µM) at 4˚C for 30

min in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis. (A) p53 incubated with 5 μM MMA^{III}, (B) MMA^{III} and MMA^{V} standards, only AsO^{+} detection shown.

Substantial amounts of binding were observed between p53 and FlAsH. Figure 4-9 shows chromatograms obtained from the SEC-ICPMS analyses of (A) a mixture of 50 μM p53 and 5 μM FlAsH after incubation at 4˚C for 30 min and (B) of the AsO detection of a FlAsH standard. The complex of arsenic with p53 elutes at 11 min (Figure 4-9A), which is the same retention time as the free protein. Figure 4-9B shows the elution of standard FlAsH in multiple peaks at retention times later than 20 minutes. Further experiments are needed to identify the oxidation state of arsenic in each peak. The percent of bound arsenic was determined for PAO^{III}, MMA^{III}, and FlAsH, and the apparent binding constants (nK) for the arsenic–p53 complexes were estimated, as listed in Table 4-3, using the equation described in Chapter 2. Total elemental analysis by ICPMS was used to quantify arsenic concentrations, and the Bradford Assay was used to confirm the concentrations of the protein.

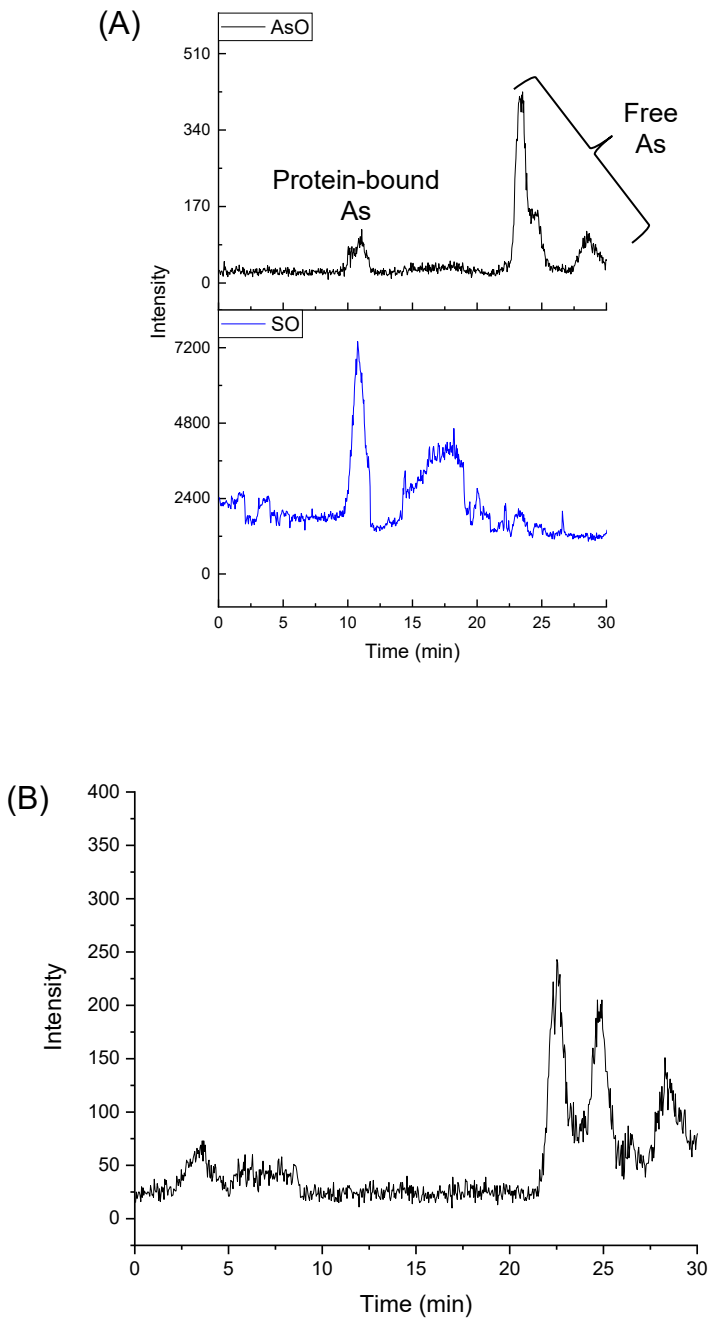


Figure 4-9. Chromatograms showing FlAsH that is free or bound to p53 after being subjected to SEC separation coupled with ICP-DRC-MS to selectively detect two oxides, SO^+ at 48 m/z and AsO^+ at 91 m/z. Wild type p53 (50 µM) was incubated with FlAsH (5 µM) at 4˚C for 30

min in 10 mM ammonium bicarbonate buffer (pH 7.4) prior to analysis. (A) p53 incubated with 5 μM FlAsH, (B) FlAsH standard, only AsO^+ detection shown.

Table 4-3. Percent Bound and Apparent Binding Constants (nK) for 5 μM Trivalent Arsenicals Binding to 50 μM Wild Type p53

	Percent Bound	**nK (M^{-1})**
iAs^{III}	nd	nd
MMA^{III}	0.27 ± 0.03	54 ± 5
DMA^{III}	nd	nd
PAO^{III}	$1.7 \pm 0.4_5$	340 ± 90
FlAsH	32 ± 11	9000 ± 2000

4.3.4 Selection of Antibody for p53 Transcription Factor Assay

The primary antibody provided with the p53 transcription factor assay was mouse monoclonal with an unmapped epitope. To carry out the designed study, it was essential to use a primary antibody capable of binding to the 82–292 fragment p53 proteins. For this reason, in addition to the supplier provided antibody, two primary antibodies (pAb1620 and pAb240) were tested against full-length wild type p53 (both GST and His-tagged), 82–292 fragment, DNA domain mutant, FlAsH mutant, Surface mutant, and a positive control of Nutlin-3-stimulated MCF-7 nuclear extract. All antibodies tested were mouse monoclonal as no polyclonal mouse p53 antibodies are available commercially. We chose only test antibodies of mouse origin in order to not require substitution of the secondary antibody, which was goat anti-mouse. The 3D

locations of the epitopes of pAb1620, pAb240, as well as pAb246, which recognizes only mouse p53, are shown in Figure 4-10.

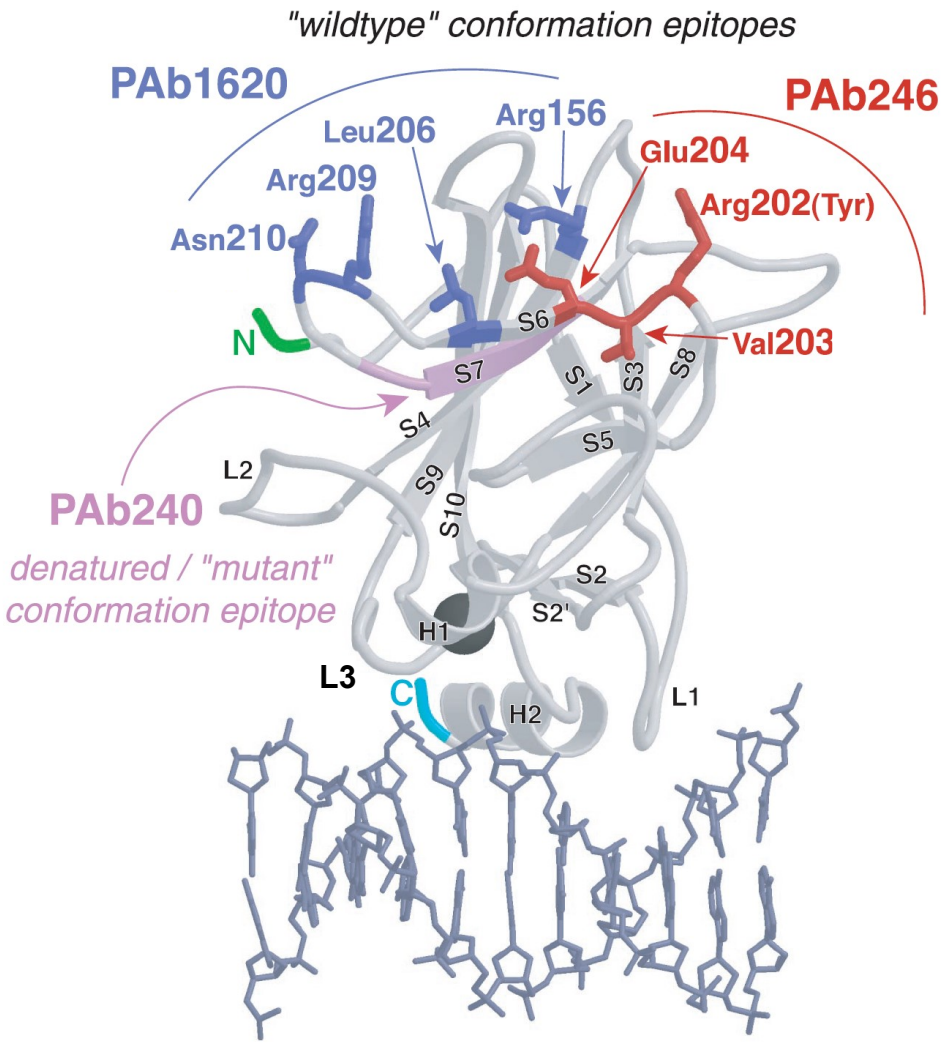


Figure 4-10. Three-dimensional locations of p53 epitopes. The main-chain ribbon and side-chains of key residues are shown. The loops (L), α helices (H), and β strands (S) are labelled. The pAb1620, pAb246, and pAb240 epitopes are blue, red, and violet, respectively; core domain N- and C-terminal residues (96–97 and 288–289) are green and cyan, respectively; DNA is blue-grey; and zinc is a dark grey ball. The core domain of human p53 PDB entry 1TSR was drawn using MOLSCRIPT[176] and Raster3D.[177] Reprinted with permission from Springer: Nature *Oncogene* "The 'wildtype' conformation of p53: epitope mapping using hybrid proteins" P. L. Wang, F. Sait, G. Winter, Copyright Nature Publishing Group (2001).

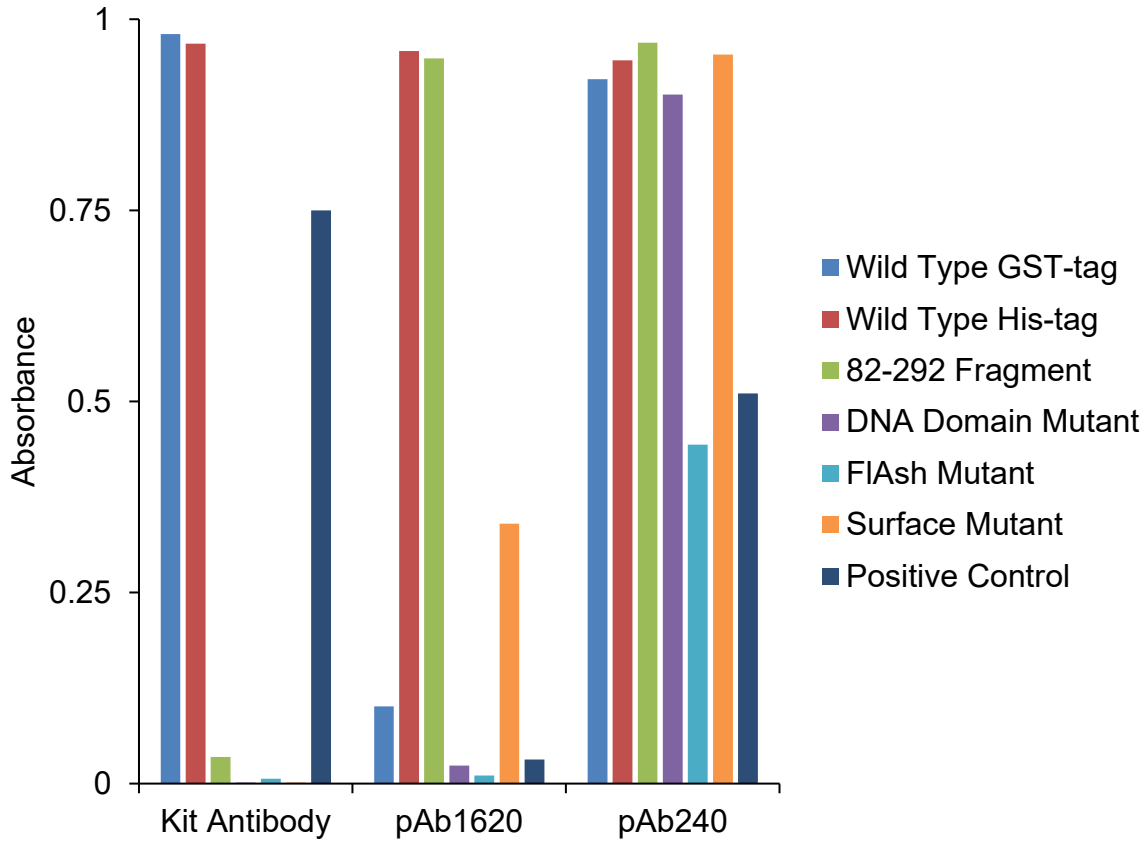


Figure 4-11. p53 transcription factor assay using different primary antibodies.

The primary antibody provided with the transcription factor assay kit only bound full-length p53, both GST and His-tagged, as shown in Figure 4-11. The inability of the kit antibody to bind to the 82–292 fragment p53 demonstrates that the epitope is likely located outside of the core domain and closer to the N or C terminus of the protein. Therefore, a different primary antibody is required for detection of the transcription factor DNA binding of the fragment p53 proteins.

The pAb1620 antibody binds to the core domain of p53 and is specific for the wild type conformation, meaning that it does not bind to denatured or mutant conformation p53.[178,179] pAb1620 bound to the His-tagged full-length wild type p53 and the GST-tagged 82–292

fragment p53, as well as pAb1620 only bound the Surface mutant to a minor extent. Interestingly, pAb1620 did not bind to the GST-tagged full-length wild type p53 or the Nutlin-3-stimulated MCF-7 nuclear extract positive control. Two possible reasons for these results are that either the GST-tagged wild type p53 and the nuclear extract did not display any DNA binding or that p53 is not in the wild type conformation in these samples. The assay using the kit antibody demonstrated that both the GST-tagged wild type p53 and the nuclear extract control are able to bind to the transcription factor DNA. In theory, denatured p53 cannot bind DNA, and the positive results seen for these samples using the kit antibody further confirm their wild type conformation. Lack of recognition of full-length human p53–DNA complexes has been seen,[180–182] and the conformation of p53 that binds to pAb1620 is extremely temperature sensitive.[183] Additionally, it has been observed that pAb1620 can disrupt the interaction between the core domain of human p53 and DNA.[184] Based on these findings and its inability to recognize fragment p53, pAb1620 was not used as the primary antibody in subsequent ELISA experiments.

Similar to pAb1620, pAb240 also binds to the core domain of p53, but it binds to mutant and denatured p53.[185–187] The epitope of pAb240 is near the 7th β strand of the core domain (S7) between residues 213–217, as shown in Figure 4-10.[188] The S7 region (residues 214–219) is hidden by another β strand, S6 (residues 204–207), and only exposed in mutant conformations or when p53 is denatured. As seen in Figure 4-11, pAb240 was capable of recognizing wild type, fragment, and mutant p53 proteins, as well as the positive control. The signal of the positive control was lower than when the kit primary antibody was used, but a strong signal was observed when using pAb240. The signal intensity from both the GST- and His-tagged wild type p53 is similar to that when the kit antibody was used. Additionally, the

signal intensity obtained with the use of the 82–292 fragment, DNA domain mutant, and Surface mutant are nearly the same as with the use of wild type p53, demonstrating that neither the truncation of the protein nor the mutations had a significant effect on their binding to the transcription factor DNA. Reduced signal was observed with the use of the FlAsH mutant, which has mutations at residues 241, 244, and 245 in the third loop (L3) of the core domain. As these mutations are far from the pAb240 epitope, the decreased signal is likely due to decreased DNA binding ability compared to the wild type versus a diminished ability of the primary antibody to recognize the mutant. pAb240 was found to be a suitable primary antibody for use in ELISA to investigate the effects of arsenic compounds on the transcription factor DNA binding of wild type and mutant p53.

4.3.5 Effect of Trivalent Arsenical on Transcription Factor Assay

Several additional experiments were needed to optimize the conditions of the assay for the arsenic studies. Dithiothreitol (DTT) was eliminated from the complete transcription factor binding assay buffer because DTT can bind trivalent arsenic and interfere with the arsenic−protein interactions. No major difference was seen in the signal from the nuclear extract positive control when running the transcription factor assay in the presence or absence of DTT.

To determine whether the presence of arsenic has any effect on the transcription factor assay, the assay was carried out using His-tagged wild type p53 in the presence of five trivalent arsenicals (iAs^{III}, MMA^{III}, DMA^{III}, PAO^{III}, and FlAsH). HPLC-ICPMS demonstrated that His-tagged wild type p53 was able to bind small amounts of MMA^{III} and PAO^{III} and did not bind iAs^{III} or DMA^{III}. FlAsH bound to wild type p53 in large amounts (32 ± 11%) as listed in Table

4-3. As the interaction between wild type p53 and four of the previously studied arsenicals is known to be minimal, the addition of arsenic into the assay should result in minimal perturbances to the protein's ability to bind DNA, therefore, little to no reduction in the absorbance signal from the assay. As shown in Figure 4-12, the addition of iAs^{III}, MMA^{III}, DMA^{III}, or PAO^{III} to the assay did not have a large effect on the output signal, although slightly more of a decrease in signal is seen when using pAb240 as the primary antibody compared to the kit antibody. A more substantial decrease in the signal is observed when wild type p53 is exposed to FlAsH prior to the assay, but as large amounts of binding was observed between wild type p53 and FlAsH by HPLC-ICPMS (Figure 4-9), it cannot be ruled out that the diminished signal is not a result of FlAsH's impact on the binding of p53 to its transcription factor DNA.

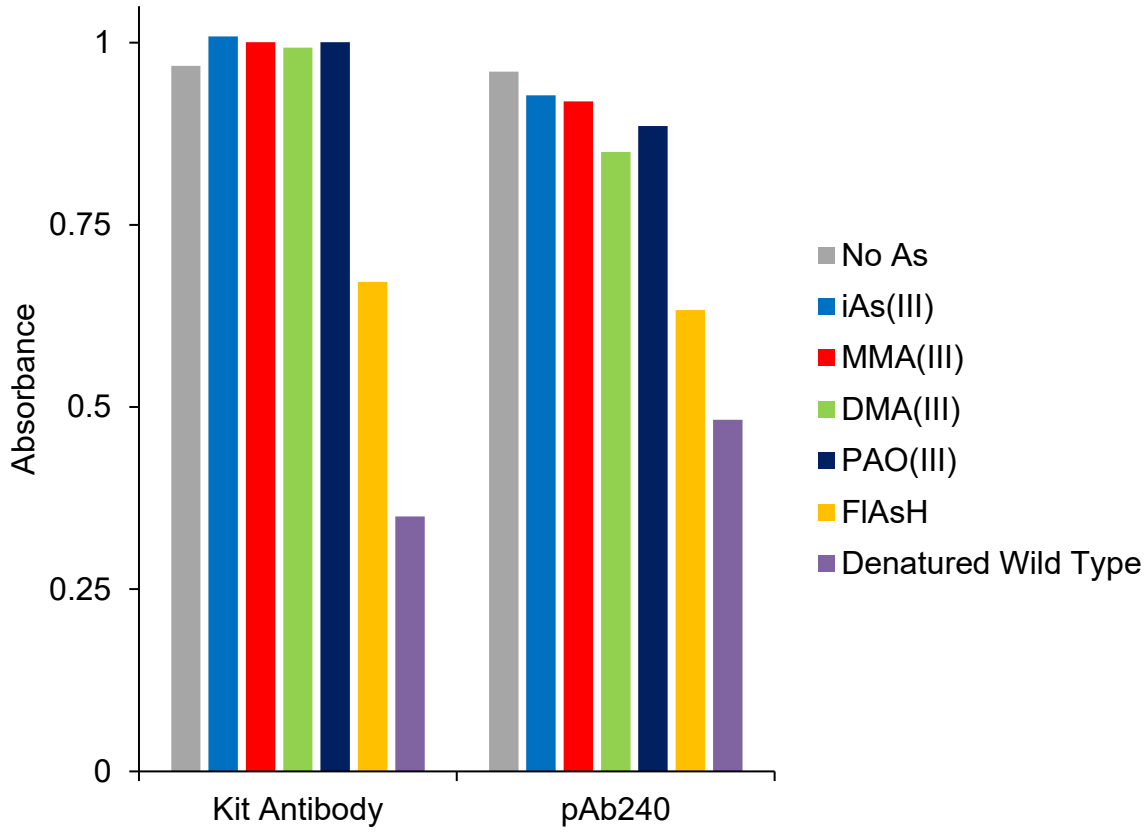


Figure 4-12. Absorbance measured from the transcription factor assay in the presence or absence of trivalent arsenicals. His-tagged wild type p53 and two primary antibodies, one from the assay kit and the pAb240 antibody, were used in the assay.

Finally, to determine whether non-specific binding contributes to the signal, the assay was run using denatured His-tagged wild type p53, which had been heated to 95ºC for 20 min. As shown in Figure 4-11, non-specific binding can contribute somewhat to the signal. To minimize the effect of non-specific binding on the arsenic studies, results are reported as relative absorbances compared to p53 binding to its transcription factor in the absence of arsenic.

4.3.6 Effects of Arsenic Compounds on the Binding of Transcriptional Factor to the Wild Type and Mutant p53 Proteins

Wild type and mutant p53 proteins were exposed to trivalent arsenic, by incubating 10 μM protein with 50 μM As^{III} at 4ºC overnight, prior to performing the transcriptional factor assay. Figure 4-13 summarizes the results obtained from the use of wild type and mutant p53 proteins (His-tagged full-length wild type; GST-tagged fragments, 82–292, DNA domain mutant, FlAsH mutant, and Surface mutant) after exposure to trivalent arsenicals (iAs^{III}, MMA^{III}, DMA^{III}, PAO^{III}, and FlAsH).

When the wild type p53 was used in performing the transcription factor assay, iAs^{III}, MMA^{III}, DMA^{III}, and PAO^{III} had negligible effect, as seen in Figure 4-13. However, when the p53 protein was mutated by introducing additional cysteines, all the tested trivalent arsenic species showed substantial effect. The strongest effects were observed with the introduction of cysteines into the DNA binding domain and the exposed surface region of the p53 protein. These effects could be due to arsenic binding to cysteines in those regions, altering the conformation of p53 and weakening the interaction between p53 with its transcription factor DNA.

As the DNA binding domain of p53 plays an important role in its interaction with the transcription factor DNA, it is conceivable that the binding of trivalent arsenicals to cysteines in this DNA binding domain could lead to the observed effect seen on the DNA domain mutant as seen in Figure 4-12. Because the exposed cysteines on the surface of a protein are more readily accessible for binding to trivalent arsenicals, the observed results of significant effect on the Surface mutant (Figure 4-12) are also understandable.

Note that the absorbance measured from the transcription factor assay, as depicted in Figure 1-5, is an indirect measure of the interaction between the transcription factor dsDNA and the p53 protein. Alterations to the p53 protein, e.g., due to arsenic binding, would reduce its interaction with the dsDNA and decrease the absorbance.

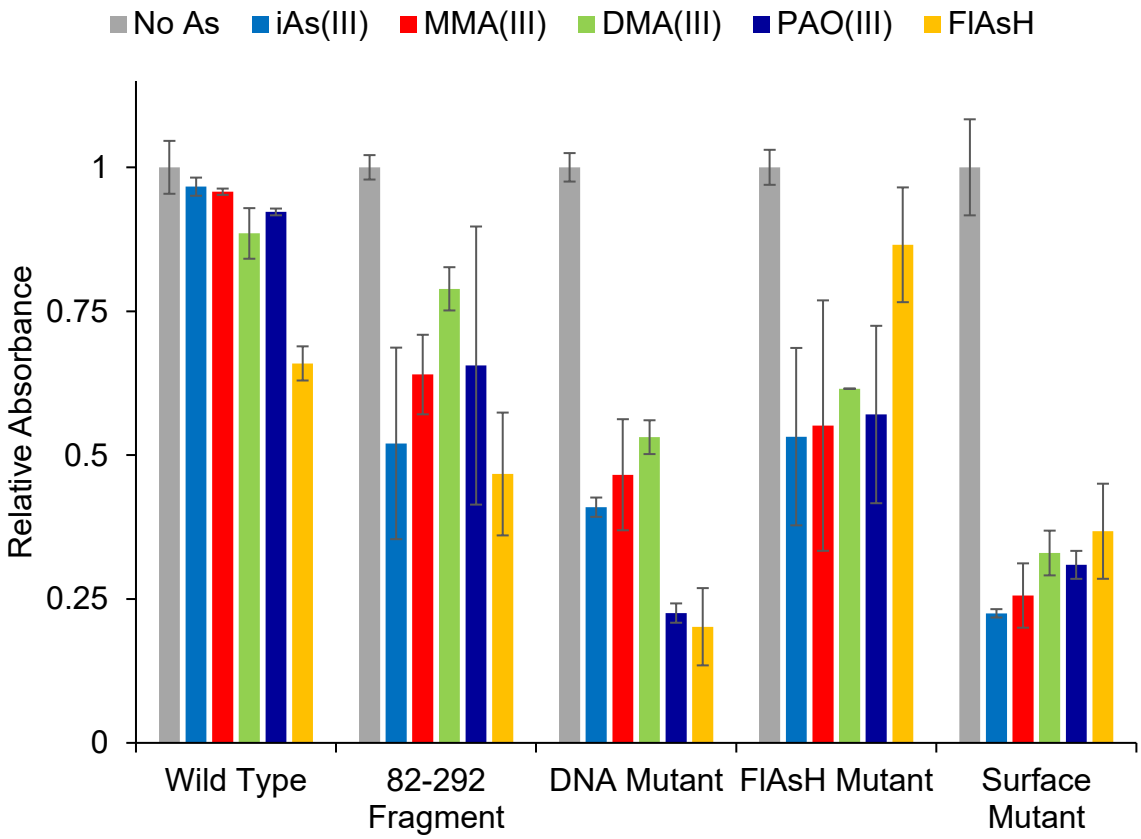


Figure 4-13. Binding of wild type and mutant p53 proteins to transcription factor DNA in the absence or presence of trivalent arsenicals. Absorbances for each protein relative to the arsenic-free signal.

4.4 Conclusion

The trivalent arsenic compounds PAO^{III}, MMA^{III}, and FlAsH were confirmed to bind wild type p53. There was no evidence for either iAs^{III} or DMA^{III} interacting with p53. PAO^{III}, MMA^{III}, and FlAsH are capable of binding 1 or 2 cysteine residues. 3D conformation data of p53 suggest that there are four possible arsenic binding sites: (1) binding to both cysteines 141 and 135; (2) binding to both cysteines 141 and 124; (3) binding to cysteine 182; and (4) binding to cysteine 229.

The interaction of the transcription factor DNA with the DNA core domain of p53 was found to be reduced significantly by five trivalent arsenic compounds (iAs^{III}, MMA^{III}, DMA^{III}, PAO^{III}, and FlAsH), while the full-length protein was affected only by FlAsH. As only minor binding was observed between wild type p53 and MMA^{III} and PAO^{III}, minimal disruption in the DNA-binding ability of the protein was expected. The ELISA style transcription factor assay was used to study the effect of trivalent arsenic on three specifically designed mutant p53 proteins (DNA domain mutant, FlAsH mutant, and Surface mutant). The addition of cysteine residues by substitution mutations resulted in decreased binding to the transcriptional DNA. The exposure to trivalent arsenic had a substantial effect on the ability of the studied mutant p53 proteins to bind to transcription factor DNA. Overall, the optimized ELISA proved to be a valid method for probing the effects of arsenic on the ability of p53 to bind to transcription factor DNA.

Chapter 5 Conclusion

The biological effects of arsenic are diverse, and the mechanisms of arsenic toxicity and carcinogenesis are complex and poorly understood. Trivalent arsenicals bind to thiols contained in proteins, causing a variety of outcomes, including changing the conformation and function of vital proteins and triggering cellular responses. The interactions between arsenic and sulfur are responsible for most of the health effects of arsenic. Given that over 170 million people are exposed chronically to elevated levels of arsenic in their drinking water, it is crucial to elucidate the mechanisms by which arsenic causes these adverse health effects in order to improve our ability to prevent and treat arsenic-related diseases.

5.1 Summary and Future Work for the Developed SE-HPLC-ICP-DRC-MS Method

The interactions between metals and proteins are extremely complicated, and a technique capable of separating high molecular weight proteins from small molecules is required to study and better understand the effects of arsenic on proteins. Size-exclusion high performance liquid chromatography (SE-HPLC) is biocompatible and can separate proteins rapidly and reproducibly. Inductively coupled plasma mass spectrometry (ICPMS) can detect elements sensitively, selectively, and quantitatively, and is an invaluable analytical tool for the analysis of metals and trace elements, such as arsenic. By hyphenating size-exclusion chromatography (SEC) with ICPMS, the advantages of both techniques are combined, providing a powerful method for the analysis of metal−protein interactions. Chapter 2 demonstrates the power of the dynamic reaction cell (DRC) for avoiding isobaric interference by using oxygen mass-shift.

By introducing oxygen gas into the DRC, the ions react with O_2 forming oxides, shifting their m/z by 16 amu (for singly charged ions). In this way, As^+ (m/z 75) is transformed into AsO^+ (m/z 91), and the isobaric interference caused by $ArCl^+$ (m/z 75) is eliminated. Similarly, this method enables the detection of proteins using ICPMS by monitoring SO^+ (m/z 48), which is not possible otherwise because of the substantial interference O_2^+ has on the S^+ (m/z 32) signal. Monitoring SO^+ as a means of detecting proteins is particularly beneficial as the other common elements found in proteins (hydrogen, carbon, nitrogen, and oxygen) exist in air and cannot be monitored because of the atmospheric pressure of the ICP interface. This method was validated using rat hemoglobin, which has a high affinity for trivalent arsenic, as a model system. It showed that SEC separation with ICPMS detection is an exceptional method for determining whether an arsenic species binds to a protein.

Chapter 2 also demonstrates the capabilities of ICP-DRC-MS for detecting $^{56}FeO^+$ (m/z 72) for the very first time. Oxygen mass-shift permits the detection of the most abundant isotope of iron, with high sensitivity and without isobaric interference from $^{40}Ar^{16}O^+$ and $^{40}Ca^{16}O^+$ (both m/z 56) that afflicts the detection of $^{56}Fe^+$. The detection of $^{56}FeO^+$ was extremely useful for the mass spectrometry studies of arsenic binding to hemoglobin from different animal species (Chapter 3). Results indicated that arsenic was not capable of dissociating or displacing the iron ion in the heme cofactor of hemoglobin. Therefore, all arsenic−hemoglobin binding occurs through cysteine residues in the globin chains of the protein. The Le group previously used the cysteine reactive reagent N-ethylmaleimide to block the free cysteines in rat hemoglobin. This resulted in complete inhibition of the arsenic−hemoglobin interaction, supporting the conclusion that arsenic−hemoglobin

complexes observed in HPLC-ICPMS are due to the binding of arsenic to the cysteine groups in hemoglobin.

The novel detection of $^{68}ZnO^{+}$ using ICP-DRC-MS also was attempted as it would allow for the monitoring of zinc cofactors, which are known to be displaced from zinc fingers by transition metals. Zinc detection would be invaluable for the study of metal interactions with zinc finger proteins, such as p53. $^{67}Zn^{+}$ has been detected previously using standard mode ICPMS, but this does not allow for simultaneous detection of proteins. Rather, by creating zinc oxide using the DRC, SO^{+} and ZnO^{+} could be monitored in unison, enabling simultaneous detection of zinc cofactors and proteins; this would be extremely valuable for the study of zinc finger proteins. Although ICP-DRC-MS can produce and detect $^{68}ZnO^{+}$, difficulties arose when separating zinc containing standards by SEC. Unfortunately, zinc chloride was retained completely on the cross-linked agarose and dextran SE column tested, and no zinc peak was observed from any of the p53 proteins. Further research and additional SE columns with different stationary phases need to be tested in order to determine the full capabilities of SEC-ICP-DRC-MS for studying zinc finger proteins.

5.2 Summary and Future Work for the Interactions between Arsenic and Hemoglobin from Different Animal Species

The interactions between arsenic and hemoglobin were expanded upon in Chapter 3, where an additional six animal species (cow, guinea pig, human, mouse, pig, and rabbit) were studied using the developed method. Being the most abundant protein in blood, hemoglobin often is responsible for the transportation and retention of chemicals in the body. Investigating the

interaction between arsenic and hemoglobin from various animal species aims to address the interspecies differences observed in arsenic distribution, retention in the blood, and toxicity. The cysteine content of hemoglobin varies significantly between species, as demonstrated in Table 1-3. This, along with accessibility of cysteines, is responsible for the interspecies differences observed in the relative affinity of hemoglobin for trivalent arsenic. Results demonstrated that rat hemoglobin generally has the highest affinity for trivalent arsenic of all the species studied. Having ruled out the ability of trivalent arsenic to replace the iron atom in heme, and since cow hemoglobin only has one cysteine, it can be concluded that iAs^{III} likely is binding to cysteine 91 in the beta globin chain (Cys 91β). The Le group also has developed a methodology to characterize the binding of DMA^{III} to rat hemoglobin using ESI-MS and ESI-MS/MS, identifying Cys 13α as the residue responsible for the retention of arsenic in rat blood.[162]

Previous investigations into arsenic hemoglobin interactions give rise to additional questions regarding arsenic in the blood, which require further research. One such question is the fate of hemoglobin bound arsenic when RBCs are broken down at the end of their life cycle and hemoglobin is recycled.

One approach for studying arsenic−protein interactions is to use molecular dynamic simulations to examine the forces between arsenicals and the cysteine residues on proteins. These could provide information about the environment of the cysteines and the likelihood of the formation of an arsenic−sulfur bond. This approach has been used to study other metal protein interactions, and the reliability and consistency of the structural dynamic simulations have been validated experimentally.[189,190] Computer modeling of rat hemoglobin has shown that rat Cys 13α exists in an open hydrophobic pocket that is suited for binding with the more

hydrophobic DMA^{III} as opposed to the more hydrophilic iAs^{III}.[162] Molecular dynamic simulations could be used to support experimental studies of arsenic protein interactions and to improve our understanding of the nature of these interactions.

In addition to rats, cats have been shown to accumulate arsenic in the blood, although to a lesser extent.[191] Interestingly, cat hemoglobin also contains a Cys 13α, although it is not known whether a DMA^{III}–hemoglobin interaction is responsible for the accumlation.[7] There has not been an in-depth study of the toxicity of arsenic in cats. Given the major interspecies differences in the half-life of arsenic in the blood, additional research investigating the interactions between arsenic species and the blood proteins of different animal species is needed and could lead to the confirmation of an inverse relationship between arsenic accumulation in the blood and acute toxicity. As cat hemoglobin is extremely expensive commercially, I was not able to undertake this study. The purification of hemoglobin from blood is relatively simple and easily could be carried out in a lab equipped with the proper expertise and equipment. Using SE-HPLC-ICP-DRC-MS to study the interactions between trivalent arsenic and cat hemoglobin would provide valuable data that would complement the previous studies done in the Le group involving rat hemoglobin, as well as those discussed in Chapter 3.

HPLC-ICPMS analyses indicate that a fraction of the total arsenic is bound to the protein, but the information provided by this analytical technique is insufficient to identify what arsenic species is bound to what protein and the location of the binding site. Identifying the precise location of arsenic−protein binding sites is an essential next step in elucidating the full effect of arsenic on biologically relevant proteins. ESI-TOF-MS allows for the determination of the arsenic−hemoglobin binding ratio, using the accurate mass measurement

provided by the TOF, and the determination of the location of the arsenic binding site, using collision-induced dissociation (CID) and ESI-MS/MS. The Le group has demonstrated previously the feasibility of such a study using rat hemoglobin.[84,161,162] Employing ESI-MS and ESI-MS/MS to characterize arsenic−protein binding completely would not only be useful for hemoglobin but for all the proteins discussed in this thesis (peroxiredoxin-1, HMGB1, p53, and mutant p53).

Finally, an intensive literature search needs to be undertaken to compile the data available on the retention and accumulation in blood, the biomethylation capabilities, and the toxicity of different trivalent arsenicals in different animal species. This has proven difficult as very limited data is available for different animal species. Furthermore, the available literature demonstrated that comparing different animal species is more difficult than initially believed. Studies used different trivalent arsenic compounds and different routes of administration, and the majority of reported median lethal doses (LD_{50}), which were reported in units of mg/kg, did not specify whether the value reported was for the compound or for arsenic alone. The data reported in the literature for the distribution of arsenicals to various organs (tissue binding affinity constants) is provided in Table A2. The LD_{50} of trivalent and pentavalent arsenic and any information available on the biomethylation capabilities and retention is provided in Table A3. This information, as well as further toxicity studies using multiple arsenic compounds on various animal species are needed to evaluate confidently whether a negative relationship exists between trivalent arsenic's affinity for hemoglobin (Chapter 3) and the toxicity of the arsenic compounds for different animal species.

5.3 Summary and Future Work for the Effects of Trivalent Arsenic on the Interaction of Transcription Factor DNA with the Wild Type and Mutant p53 Proteins

Studying the interactions between arsenic and p53 proteins aims to determine how known amino acid mutations affect the ability of p53 to interact with its transcription factor DNA. The developed HPLC-ICPMS method confirmed the binding of small amounts of PAO^{III} and MMA^{III}, and larger amounts of FlAsH, to wild type p53. These three arsenicals can bind either one or two cysteine residues. Although 3D conformation data based on the crystal structure of p53 suggest that there are four possible arsenic binding sites, Cys 141 and 135, Cys 141 and 124, Cys 182, or Cys 229, carrying out ESI-MS and ESI-MS/MS experiments would confirm whether one or two cysteine residues are involved in the binding and would locate the exact binding site.

An objective was to employ the HPLC-ICPMS method to study the binding of iAs^{III}, MMA^{III}, DMA^{III}, PAO^{III}, and FlAsH to mutant p53 proteins, but this was more complicated due to the GST-tag and the use of glutathione (GSH) during the purification of the protein, which can bind to arsenic. A GST-tag was used instead of a His-tag to increase the solubility of the protein, as the addition of cysteine residues to the mutants can further decrease protein solubility. Moreover, when recombinant His-tagged 82−292 wild type p53 was desalted using dialysis following purification, the protein precipitated out of solution, and an optimal buffer was not found to maintain the solubility of the fragment protein. This is also the reason for using centrifugal filters to desalt the mutant proteins as opposed to dialysis. Mutations were

made to the DNA core domain, rather than full-length p53, as this was the domain of interest for the transcription factor assay. In future, the DNA domain, FlAsH, and Surface p53 mutants could be created in the full-length His-tagged protein. This would make it feasible to determine the percentage of bound arsenic and apparent binding constants for the five trivalent arsenicals to the mutant p53 proteins. Although the presence of GSH in the purified proteins impeded the quantitative ICPMS study, the majority of the GSH was removed by desalting the proteins; therefore, it would not have a large effect on the ELISA study as arsenic was in excess.

An ELISA style transcription factor assay based on a kit was modified and optimized to study the effects of trivalent arsenicals on the DNA binding ability of p53. The ability of wild type p53 to bind its transcription factor DNA was impacted only significantly by FlAsH. FlAsH was found to bind to wild type p53 in moderate amounts; this is likely responsible for the decrease in the protein's ability to bind its transcription factor DNA. As only minor binding was observed between wild type p53 and MMA^{III} and PAO^{III} using HPLC-ICPMS, minimal disruption in the DNA-binding ability of the protein was expected. The results indicated that the addition of cysteine residues in the three specifically designed mutant p53 proteins caused a decrease in the DNA binding ability of p53. Furthermore, the exposure to trivalent arsenic had a more substantial effect on the ability of mutant p53 to bind to DNA compared to wild type particularly for mutations in the DNA core binding domain or on the surface of the protein. Overall, the modified ELISA was a valid method for investigating the effects of arsenic on the interaction between p53 and its transcription factor DNA. p53 is a protein of great significance to human health and is easily inactivated by interactions with xenobiotics or from single nucleotide mutations to the *TP53* gene. In future, the modified assay could be used to probe

the effects of various mutations and other non-arsenic compounds on the DNA binding ability of p53.

5.4 Overall Impact of Thesis

The developed SE-HPLC-ICP-DRC-MS method can be used to detect other arsenic interactions with proteins quantitatively. The modified p53 transcription factor assay is a valuable tool to study the effects of mutations and xenobiotics on the function of this biologically important protein. Overall, the work described in this thesis has provided significant insight into previously unstudied arsenic−protein interactions, and the developed analytical technique will prove useful for future studies.

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Appendix

Table A-1. Apparent Binding Constants (nK) for 5 μM Trivalent Arsenicals Binding to 20 μM Hemoglobin

	nK (× 10^3) (M^{-1})						
	Cow	**Guinea Pig**	**Human**	**Mouse**	**Pig**	**Rabbit**	**Rat**
iAs^{III}	3.4 ± 0.2	nd	1.84 ± 0.06	nd	2.8 ± 0.4	nd	2.5 ± 0.2
MMA^{III}	nd	nd	5.0 ± 0.9	1.2 ± 0.2	nd	nd	166 ± 3
DMA^{III}	nd	nd	11 ± 3	5.0 ± 0.2	nd	nd	288.1 ± 0.3

Table A-2. Tissue Binding Affinity Constants (K_i) for Arsenic Compounds in Rabbits and Hamsters[192]

Tissue	Tissue Binding Affinity Constant (K_i)			
	As^V	As^{III}	MMA	DMA
Liver	1	200	10	1
Kidneys	40	20	100	5
Lungs	1	1	1	20
Skin	1	60	50	1
Red blood cells	0.2	1.5	0.2	0.2
Bone and muscle	10	40	1	1

Table A-3. Median Lethal Dose (LD_{50}) for Trivalent and Pentavalent Arsenic for Different Animal Species

Animal Species	Arsenic Species	Arsenic Oxidation State	Median Lethal Dose LD_{50} (mg As/kg unless otherwise specified*)	Exposure Method	Biomethylation Capacity of Animal Species (if known)	Reference
Cat	AsH_3	+3	2.0−2.5 mg compound	Intraperitoneal	DMA	193
Cow	As^{III}	+3	1−25*	Oral	DMA	194
	As^{V}	+5	30−100*	Oral		194
Dog	$Ca_3(AsO_4)_2$	+5	38*	Oral	DMA	195
Earthworm	$NaAsO_2$	+3	0.191*	Injection		196
	Na_3AsO_4	+5	0.5194*	Injection		196
Goat	$NaAsO_2$	+3	25*	Oral		197
Guinea Pig	$NaAsO_2$ and $KAsO_2$	+3	9*		None	198
Hamster	MeAsO	+3	2	Intraperitoneal	TMAO	199

	$NaAsO_2$	+3	8	Intraperitoneal		199
Honeybee	$Ca_3(AsO_4)_2$	+5	39*	Oral		200
Human	As_2O_3	+3	1−2.6* estimated	Oral		201
	Na_3AsO_4	+5	10−20	Oral	DMA	202–204
	MMA^V	+5	700−1,600	Oral		202–204
	DMA^V	+5	700−2,600	Oral		202–204
Mallard	As_2O_3	+3	323	Oral		205
Mouse	AsH_3	+3	3.0 mg compound	Intraperitoneal		193
	$NaAsO_2$	+3	15−22	Oral		206,207
	$NaAsO_2$	+3	8	Intramuscular		208
	As_2O_3	+3	36-39, 35	Oral	TMAO	206,209,210
	Na_3AsO_4	+5	14	Intramuscular		208
	$Ca_3(AsO_4)_2$	+5	20-800	Oral		206
	MMA^V	+5	700-1800	Oral		206,209
	DMA^V	+5	1200-2699	Oral		206,209

	$TMAO^V$	+5	10,600	Oral		209
	Arsenobetaine	+5	≥10,000	Oral		206
	Arsenocholine	+5	6,500	Oral		206
Pheasant	As_2O_3	+3	386	Oral		205
Quail	As_2O_3	+3	47.6	Oral		205
Rabbit	AsH_3	+3	2.5 mg compound	Intraperitoneal	DMA	193
	$NaAsO_2$	+3	7.6 mg compound	Intravenous		211
	CH_4AsNaO_3	+5	102*	Oral		212
	$Ca_3(AsO_4)_2$	+5	50*	Oral		213
Rat	AsH_3	+3	15	Oral	TMAO	214
	As_2O_3	+3	4.5*	Intraperitoneal		215
	$NaAsO_2$	+3	24	Oral		216
	$NaAsO_2$	+3	4−5 (LD_{75})	Intraperitoneal		217
	Na_3AsO_4	+5	14−18 (LD_{75})	Intraperitoneal		217
	$Ca_3(AsO_4)_2$	+5	53	Oral		218

	$Ca_3(AsO_4)_2$	+5	>400	Dermal	218
	$PbHAsO_4$	+5	231	Oral	218
	$PbHAsO_4$	+5	>500	Dermal	218
Sheep	AsH_3	+3	3.0 mg compound	Intraperitoneal	193

*Not specified whether LD_{50} reported as mg of arsenic or mg of compound per kg.

List of Collaborative Peer Reviewed Publications

Popowich, A.; Cleland, T.; Solazzo, C. Proteomics composition of protein membrane metal threads used in historical textiles. *J. Cult. Herit.* Published online March 23, 2018. DOI: 10.106/j.culher.2018.03.007.

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