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Using allosteric transcription factor gated Cas12a activation for metal ion and small molecule biomarker detection

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USING ALLOSTERIC TRANSCRIPTION FACTOR GATED CAS12A ACTIVATION FOR METAL ION AND SMALL MOLECULE BIOMARKER DETECTION

by

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DEDICATION

To my parents who gave me the greatest opportunity to be the best person I can be

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ABSTRACT

Biosensors are pivotal in addressing global challenges in healthcare, environmental monitoring, and diagnostics by providing rapid, sensitive, and portable detection solutions. This thesis explores the integration of allosteric transcription factors (aTFs) and Cas12a to create a novel biosensing platform, leveraging the ligand specificity of aTFs and the robust signal amplification of Cas12a's trans-cleavage activity. The platform focuses on detecting biomarkers such as N-Acetylneuraminic acid (Neu5Ac) and heavy metals like lead, cadmium, and copper, using corresponding aTFs—NanR, CadC, and CsoR. Experimental evaluations address heavy metal interference on Cas12a activity, aTF gating efficiency, and variability among Cas12a orthologs. Our findings showed differences in heavy metal interference on different Cas12a orthologs and the ability for aTFs to gate Cas12a activation. Future development and expansion of the platform could lead to innovative solutions for precise diagnostics and environmental monitoring, addressing challenges in resource-limited and field-deployable settings.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aTF	Allosteric Transcription Factor
BU	Boston University
CDC	Centers for Disease Control and Prevention
COVID-19	Corona Virus Disease 19
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAILY	Disability-adjusted life years
DNA	Deoxyribonucleic acid
dsDNA	Double Stranded Deoxyribonucleic acid
FQ pair	Fluorescer Quencher Pair
ICP-MS	Inductively coupled plasma mass spectrometry
Neu5ac	N-Acetylneuraminic acid
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RFU	Relative Fluorescence units
ssDNA	Single Stranded DNA

Chapter 1: Introduction to Biosensors for Metal Ions and Small Biomolecules

Biosensors have emerged as essential tools for addressing critical challenges in environmental monitoring, medical diagnostics, and beyond. In this chapter, we explore the foundational principles and current advancements in biosensor technologies, focusing on their versatility and potential applications. Additionally, we discuss the importance of developing field-deployable biosensors that are portable, user-friendly, and effective in resource-limited settings.

1.1 What is a Biosensor?

Biosensors are devices designed to convert biological signals into readable outputs, providing valuable information to users. These devices integrate varying proportions of biological and electronical components but typically rely on a biological receptor that interacts with a specific target molecule. This interaction is transduced into an electronic signal, which is then processed and displayed for interpretation. [1]. Biosensors have found applications across a wide range of fields, including the food industry for quality control, the medical field for diagnostics, and the environmental sector for monitoring marine ecosystems and detecting pollutants. Their versatility and precision make them indispensable tools in both research and practical applications. [1], [2].

Synthetic biology biosensors leverage the inherent adaptability and specificity of biological systems to detect a wide variety of target analytes in a modular and systematic manner. These sensors can be constructed using diverse biological components, such as engineered bacterial cells, enzymes, aptamers, or proteins, each tailored to detect specific molecules or conditions. [3], [4], [5], [6]. A key advantage of synthetic biology biosensors is their ability to exploit natural evolutionary adaptations. Over billions of years, biological systems have developed proteins and other biomolecules capable of precisely sensing and responding to critical biomarkers. Synthetic biologists harness these naturally evolved mechanisms, optimizing and reconfiguring them to create biosensors that meet human needs.

1.2 Importance of Field-Deployable Biosensors

With growing medical and environmental challenges worldwide, the demand for biosensors continues to rise. To meet this demand, sensors must be highly selective, reproducible, stable, and sensitive while addressing the specific needs of their users [1]. Among these requirements, designing biosensors that effectively serve the end user is paramount. Consumers of biosensors, such as individuals testing water for contaminants or patients undergoing disease screening, often operate in remote regions with limited access to sophisticated laboratory equipment. To ensure usability in such contexts, biosensors must be portable, field-deployable, and capable of functioning reliably outside of controlled laboratory settings. These attributes make biosensors indispensable tools for tackling real-world challenges across healthcare, environmental monitoring, and beyond.

1.3 Current State of Metal Testing

The detection and analysis of metal ions in environmental and medical samples have become increasingly critical due to the impact of heavy metal contamination on ecosystems and public health [7], [8]. Metals such as lead, mercury, cadmium, and arsenic are toxic even at low concentrations and can accumulate in living organisms, causing severe health issues, including neurological damage, developmental disorders, and organ failure [9]. Additionally, heavy metals can persist in the environment, contaminating soil and water sources, disrupting ecosystems, and entering the food chain, where their effects can magnify through bioaccumulation. In medical contexts, metal ion imbalances, such as elevated levels of copper or iron, can signal underlying health conditions, including metabolic disorders or neurodegenerative diseases [10]. Current commercial tests for metal ions in water primarily fall into three main categories: electrochemical, optical, and spectrometry methods.

1.3.1 Electrochemical methods

Electrochemical methods such as potentiometry and anodic stripping voltammetry use electrodes that measure changes in either the electrochemical potential in comparison to a reference or reference/counter electrode pair [11], [12]. These methods often require careful calibration and maintenance of the reference electrode or reference/counter electrode pair to ensure correct readings. [11]. This can make them expensive to purchase and maintain. Electrochemical methods make up for this by being reusable and usable in continuous monitoring.

1.3.2 Optical Methods

Optical methods use changes in color of visible light, which is called colorimetry. They can also use change in emission of light due to excitation called fluorescence.

Colorimetry is one of the most widely used methods for detection of heavy metals due to low cost, ease of use and fast results. Detection uses a chromogenic material or dye that changes color from interacting with specific metals. [13]. These changes can then be seen by eye or quantified through use of a spectrophotometer. Many different kinds of chromogenic materials have been tested for metals including dithizone for lead and mercury, or ammonium purpurate for cadmium[13]. Colorimetry's main benefits are its easy portability and inexpensive nature. The methods though can lack sensitivity and can be affected by other substrates in the sample. Use of the spectrophotometer on samples increases sensitivity but also dramatically increases costs to perform tests.

Fluorescence testing measures the change in emission of light given an excitable photon. The binding of analytes to fluorescent compounds can decrease or quench the amount of fluorescence of the compound. This can be used to determine the concentration of an analyte in a system [11]. Fluorescence methods have unique advantages of being used in living systems for bioimaging and intracellular detection [14]. They can be influenced by the sample being tested and requiring expensive machines to increase measure small changes in fluorescence.

1.3.3 Spectrometry Methods

Spectrometry methods utilize an atom's unique response to electromagnetic radiation to determine the number of analytes in a solution. These methods are usually highly sensitive but require large expensive machinery to perform [15].

The most widely used spectrometry method being used today is Inductively Coupled Plasma Mass Spectrometry (ICP-MS). ICP-MS is considered the "gold standard" for metal detection for water and allows for parts per trillion levels of detection for many different metals [11]. ICP-MS works by first nebulizing samples and transfers them into argon plasma. The plasma ionizes the sample by removing one electron. The ions are then scanned using a quadrupole mass spectrometer which separates the ions based on their mass to charge ratio which is then measured by a detector [16]. This set up allows the device to analyze individual atoms as they go through the machine which gives it some of the best sensitivity of any metal ion detection. This sensitivity comes at the cost of large and expensive equipment that cannot be used in the field.

1.4 Current State of Small Biomolecule Testing

While metal ion testing primarily addresses environmental concerns, small biomolecule testing shifts focus toward human health and medicine. Small biomolecules are often prognostic markers to greater physiological and pathological issues. Detecting these molecules is crucial for diagnosing diseases, monitoring responses to medicines, and for investigating new ones. In this section we will give, reasons for, general techniques and examples of biosensors for sensing nucleic acids, lipids, polysaccharides, and proteins.

1.4.1 Nucleic Acid Testing

Nucleic acid testing focuses on detecting specific RNA or DNA sequences, leveraging the natural base-pairing properties of nucleic acids and their interactions with biological machinery. Scientists have developed numerous biological methods to identify and amplify these sequences, enabling precise and reliable diagnostics.

Polymerase chain reaction or PCR is an amplification method for DNA that can be used to detect the presence of a specific sequence of DNA or RNA. PCR uses two short DNA primers that bind in reverse directions to the target nucleic acid [17]. The binding of the primers to the target nucleic acid gives a spot for either a DNA polymerase or reverse transcription polymerase, to bind and extend the primers to create a complimentary DNA copy of the target DNA or RNA respectively. The sample is heated to 95°C to break the hydrogen bonds between the target DNA and the new DNA copy and then cooled to allow the primers to once again bind to the target DNA and now the copy of the target DNA. The heating and cooling cycle is preformed 25-35 times to create billions of copies of the target DNA [17], [18]. After these copies are made, other machines such as a spectrophotometer can detect the efflux of new DNA that PCR created leading to detection of a specific sequence of DNA. While this method is very sensitive the results are binary due to the amplification nature of PCR. This makes PCR good for all or nothing tests for specific diseases such as COVID-19 but worse for when quantification of the amount of target nucleic acid is required.

Hybridization Sensors take advantage of the binding affinity of single stranded DNA and single stranded RNA to complimentary sequences of itself. Single stranded DNA that is complementary to the target nucleic acid is immobilized on a substrate. When the target nucleic acid meets the immobilized complimentary DNA, it binds forming a double stranded nucleic acid. This binding can then be measured through optical or electrochemical signals [19], [20].

1.4.2 Lipid Testing

Detecting lipids can be considered more difficult than nucleic acid detection because you can not exploit any base pair or complementary nature to detect specific lipids. Sensors instead try to utilize other properties of lipids such as hydrophobicity, amphiphilicity and any functional groups bound to the lipid. Methods for this include electrochemical, mass-sensitive and optical. Many tests utilize lipase or phospholipase enzymes as the actual sensing agent and then detect changes in pH or oxidation[21].

The most common lipid test in a clinical setting is the 'Lipid Panel'. The lipid panel is a group of tests that measure total cholesterol, low-density lipoprotein, and high density lipoprotein [22]. The test is used to help predict risk of heart disease and stroke.

1.4.3 Polysaccharide Testing

Polysaccharides or sugar molecules are used for structure, cell signaling and storage of energy in almost all living organisms. They are made of monosaccharides that are bonded and branched together in unique structures. These structures give rises to many variations in the properties of polysaccharides [23].

Glucose is one of most targeted monosaccharide for biosensors due to its importance in diabetes [24]. Most glucose biosensors use enzymatic reactions to determine the amount of glucose in a blood sample. These sensors, using a combination of enzymatic and electrochemical methods, determine the amount of glucose in a blood or intercellular fluid sample [25]. [26].

1.4.4 Protein Testing

Proteins play a vital role in almost every biological process serving as enzymes, structural components, signaling molecules and genetic regulators. Their presence, absence, or altered expression can serve as an indicator for many physiological problems and pathological conditions. These include cancer with specific protein biomarkers [27], [28] and heart disease with troponins [29]. Protein biosensors use a variety of methods for detection including optical, electrochemical and biomolecular.

Simple dye-based, such as the Bradford assay or biuret test, are often used for sensing the general concentration of protein in a sample. These assays use a dye reagent that interacts with all proteins and gives a colorimetric result that can be quantified by a spectrophotometer [30], [31]. These tests, while cheap and able to be performed quickly are limited to only detecting total amounts of protein.

Electrochemical sensors for protein can use amperometry and impedimetric techniques and have been used to detect specific proteins. The most widely being used for the detection of C-reactive protein, who’s concentration in blood has been shown to be a sign of cardiovascular diseases [32], [33].

Chapter 2: Allosteric Transcription and Cas12a for Biosensing

In this chapter, we delve into the mechanisms and applications of aTFs and the Cas12a enzyme, two pivotal tools in synthetic biology. We explore how aTFs regulate gene expression through ligand-specific binding and how Cas12a, a CRISPR-associated nuclease, enables precise DNA targeting and robust signal amplification.

2.1 Allosteric Transcription Factors and Cas12a

Transcription factors and CRISPR-associated nucleases represent two critical molecular tools that nature has refined over millennia, offering powerful mechanisms for regulating biological processes and defending against threats. Transcription factors orchestrate gene expression by interacting with DNA, while Allosteric transcription factors (aTFs) add an additional layer of control by linking environmental signals to gene regulation. In parallel, CRISPR-Cas systems, particularly the Cas12a nuclease, provide a sophisticated immune defense for bacteria and archaea, with unique DNA-cleaving capabilities that have been adapted for cutting-edge biosensing applications. Together, these systems highlight the ingenuity of natural molecular machinery and their transformative potential in synthetic biology and diagnostic technologies. The following sections explore the structural and functional attributes of transcription factors and Cas12a, emphasizing their roles in nature and their innovative applications in biosensing.

2.1.1 Transcription Factors in Nature and Biosensing

Transcription factors are proteins that regulate when and how genes are activated in a cell by controlling transcription. They typically have two key domains: a DNA binding domain and a regulatory domain. The DNA binding domain recognizes and attaches to a specific DNA sequence, often found in the promoter or enhancer regions of genes. Once bound, the transcription factor can either repress transcription by blocking RNA polymerase or activate transcription by recruiting RNA polymerase and other components of the transcriptional machinery [34]. Additionally, the regulatory domain may interact with other proteins to modulate chromatin structure and fine-tune gene expression [35].

aTFs are a specialized class of transcription factors that undergo conformational changes upon binding to a specific ligand. This structural change can either enhance or inhibit the DNA-binding domain's affinity for DNA, thereby modulating gene expression. By linking the concentration of a ligand to the activity of an aTF, cells can indirectly regulate gene expression in response to environmental signals.

In nature, bacteria make extensive use of a wide variety of aTFs to respond to changing environmental conditions. These aTFs allow bacteria to detect and react to diverse signals, such as the presence of nutrients, toxins, or changes in pH. Each aTF is finely tuned to recognize a specific ligand, enabling precise regulation of gene expression [34], [36]. For instance, the LacI family of transcription factors regulates lactose metabolism in *E. coli* by sensing lactose or related sugars [37]. This natural diversity of aTFs provides a rich toolkit for synthetic biology and biosensing applications [38].

2.1.2 Cas12a in Nature and Biosensing

Cas12a is a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) associated protein CRISPR is a collection of systems which is used by many bacteria and archaea to protect themselves from viral infections [39]. The system works by storing segments of viral DNA within the bacterial genome of past infections. When the same or similar virus attacks again, the bacteria use this stored viral DNA to produce RNA guides that direct nucleases such as Cas12a, to the matching viral DNA degrading the viral DNA and protecting the bacteria [39].

Cas12a targets double-stranded and single-stranded DNA by incorporating a guide called CRISPR RNA (crRNA), which contains a sequence complementary to the invading viral DNA. Upon binding to its target, Cas12a performs a staggered cut in the DNA (cis-cleavage), after which it enters an activated state and exhibits 'collateral cleavage' (trans-cleavage) activity. In this state, Cas12a nonspecifically cuts nearby single-stranded DNA. This unique trans-cleavage activity has been repurposed for biosensing by using fluorescence quencher probes linked by single-stranded DNA. When Cas12a is activated, it cleaves the single-stranded DNA linker, separating the fluorophore from the quencher, thereby producing a detectable fluorescence signal in response to specific DNA or RNA sequences [40], [41].

Many biosensing platforms have emerged from CRISPR-Cas systems. DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) and specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) are two innovative platforms that specifically utilize Cas12a to sense DNA and RNA [42], [43], [44]. The ability for Cas12a to target specific DNA sequences makes it attractive for biosensing applications.

2.2 Goals for Combining Allosteric Transcription Factor and Cas12a for Biosensing

The development of field-deployable biosensors demands innovative strategies that integrate sensitivity, specificity, and portability to meet the needs of diverse users in real-world environments. Combining aTFs and Cas12a presents a promising avenue for creating biosensors capable of addressing these requirements. Leveraging the unique properties of aTFs for ligand-specific detection and Cas12a for signal amplification and transduction provides great opportunity for advancing biosensor design. The goals of this approach are outlined as follows:

2.2.1 Enhanced Specificity and Selectivity

A key requirement for field-deployable biosensors is the ability to distinguish target analytes from complex backgrounds. Allosteric transcription factors excel in this domain due to their ligand-specific conformational changes, enabling precise recognition of target molecules such as environmental contaminants or biomarkers.

2.2.2 Signal Amplification and Sensitivity

Biosensors designed for on-site use must detect low concentrations of analytes, often present in trace amounts in environmental or clinical samples. Cas12a's collateral cleavage activity provides a powerful mechanism for signal amplification, translating molecular interactions into detectable outputs. When an aTF detects its ligand and allows the activation of Cas12a the resulting transcleavage can be read out by single stranded probe.

2.2.3 Modularity and Versatility

The modular nature of both aTFs and Cas12a enables the design of biosensors that can be easily adapted to detect a wide range of analytes. Natural and engineered aTFs can be tailored to recognize specific ligands, while Cas12a would be able to target operator sequence of these aTFs. This flexibility allows for the development of customizable biosensors suited to diverse applications.

2.2.4 Stability and Portability

Field-deployable biosensors must be robust and capable of functioning outside controlled laboratory settings. Proteins such as aTFs and Cas12a are inherently stable and can be further engineered to withstand variable environmental conditions. Cas12a read outs can also be used outside of a laboratory using single stranded DNA to link two affinity moieties to be read out on lateral flow. This stability, coupled with the minimal equipment required to operate lateral flow, can ensure portability and accessibility for users in remote or resource-limited areas.

Chapter 3: Development of Allosteric Transcription Factor Gated Cas12a Activation Biosensors

This chapter focuses on the integration of aTFs and Cas12a to construct a novel biosensing platform. Building on the foundational principles explored in earlier chapters, we outline the methods and strategies for combining these components to achieve ligand-specific detection with enhanced sensitivity and adaptability. The chapter delves into the selection of target analytes, including biomarkers such as N-Acetylneuraminic acid (Neu5Ac) and heavy metals like cadmium, lead, and copper, alongside their corresponding aTFs—NanR, CadC, and CsoR. Furthermore, it addresses potential challenges, such as heavy metal interference with Cas12a activity, by evaluating the performance of various Cas12a orthologs.

3.1 Combining Allosteric Transcription Factors with Cas12a for biosensing

aTFs ability to bind and unbind to select sequences of DNA, usually called their operator, in response to a change in concentration in a ligand gives an opportunity for use in biosensing. The amount of transcription factor bound to DNA can be correlated to the amount of ligand for a sample. Cells normally use this to turn off and on genes by the transcription factors repressing or promoting transcription of a nearby gene. However, this binding-unbinding activity can be used instead to block or ‘gate’ the activation of the Cas12a enzyme.

In this design, Cas12a will target the operator sequence of an aTF using crRNA specifically complementary to that sequence. When the aTF is bound to its operator—which depends on the presence of a small-molecule ligand—Cas12a is blocked from accessing the target DNA and remains inactive. Conversely, if the aTF is unbound, the Cas12a-crRNA complex can recognize the operator sequence and activate its nonspecific trans-cleavage activity. This activity will cleave single-stranded DNA (ssDNA) probes, such as fluorescence-quencher probes (FQ probes), generating a fluorescence-based readout that provides a measurable signal linked to the ligand concentration. The platform also allows the use of other ssDNA probes for different readout mechanisms such as fam-biotin probe for lateral flow assays, which can allow for a visual reading of the system. This approach combines the specificity of aTFs with the sensitivity of Cas12a to create a powerful biosensing platform. Previous work has been done on similar systems for sensing of small biomolecules using immobilized aTFs. Other work in this lab has used free floating aTFs to sense for antibiotics in a pending manuscript. We plan to expand this work to other small biomolecules and metals with the end goal of using lateral flow assays to have a sensitive, field deployable platform.

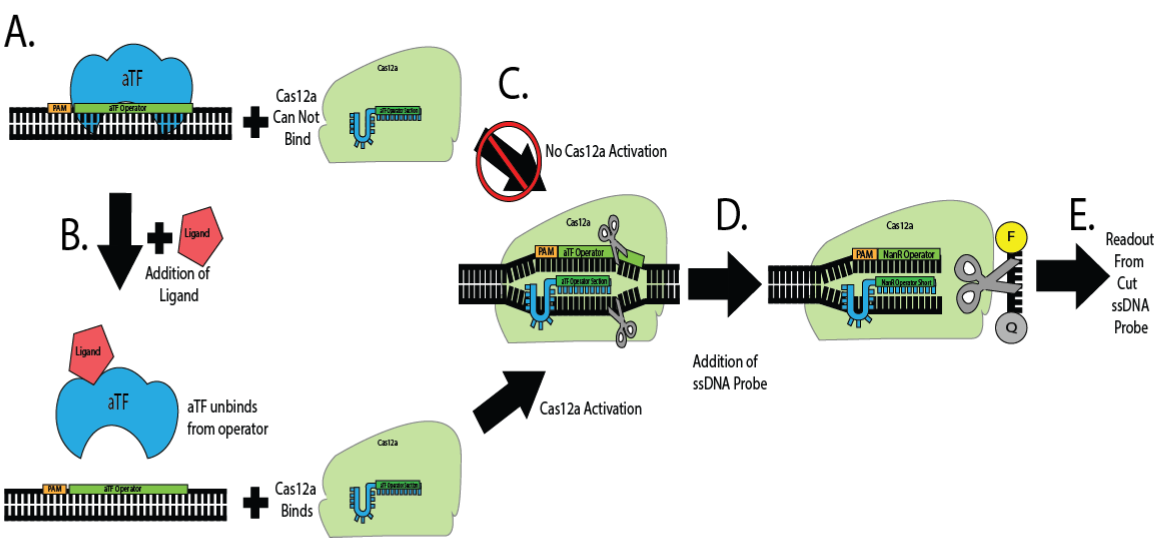

Figure 3.1 Diagram of aTF Gated Cas12a Activation

A) aTF bound to operator site of double stranded DNA. PAM site included before aTF operator site. Cas12a with gRNA for aTF operator site cannot bind due to bound aTF at same location. B)

Addition of ligand unbinding aTF from operator site. Cas12a with gRNA for aTF operator site able to bind to aTF operator site. C) Cas12a unbinds double stranded DNA with operator site and activates its cis and trans cleavage only if aTF is unbound from the operator site. D) single stranded DNA (ssDNA) probe is added and will be cut only by activated Cas12a. E) Cut ssDNA probe can be read out in either florescence plate reader for florescence-quencher probe or lateral flow assay for fam-biotin probe.

3.2 Identifying Targets for Testing

A wide range of allosteric transcription factor research was undertaken to look for suitable targets for the biosensor that had also had aTFs that could be used in the platform. For aTFs to be used in the platform we need them to have a known DNA operator sequence and respond to ligand by binding or unbinding to that sequence. We separated our targets into two categories: biomarkers for disease and heavy metals.

For biomarkers N-Acetylneuraminic acid (Neu5ac) was found as a promising candidate biomarker when paired with the aTF NanR. Neu5ac is seen as a potential biomarker for cardiovascular disease and rheumatoid arthritis [45], [46] and NanR had a known operator sequence. It’s interaction with Neu5ac had also been well characterized [47]. For heavy metals Cadmium and Lead were selected with the aTF CadC and Copper with aTF CsoR respectively. Each of these metals have been found by the Environmental Protection Agency to be harmful and regulated by the Clean Water Act [48].

3.2.1 Biomarkers

N-Acetylneuraminic acid (Neu5ac) is a widely expressed monosaccharide that is part of the sialic acid family, typically found at the terminal ends of glycoproteins and glycolipids. Sialic acids play essential roles in processes such as cell-to-cell signaling, cellular aggregation, reproduction, and more. [49]. Neu5ac specifically has been seen as a potential biomarker for cardiovascular disease and rheumatoid arthritis [45], [46]. Cardiovascular disease in particular is one of the leading causes of death in the United States according to the Centers for Disease Control and Prevention (CDC) [50].

In several bacterial species, the aTF NanR is regulated by Neu5ac [47]. In bacteria, sialic acid serves as a vital energy source, and many species have evolved mechanisms to import and metabolize it efficiently. NanR plays a key role in regulating these pathways. In the presence of Neu5Ac and zinc, which acts as a cofactor, NanR binds to its operator region on DNA, controlling the expression of genes involved in sialic acid metabolism. Notably, NanR's operator site has been found to have three distinct operons that one NanR can bind to each. These operons allow NanR to exhibit cooperative binding, where the binding of NanR to one operon increases affinity of another NanR to bind to another operon, which enhances its regulatory capabilities [47]. This natural system provides a compelling foundation for engineering biosensors using NanR as a responsive element in conjunction with Cas12a. Due to NanR's need for zinc to undergo its conformation change other metals were investigated as other testing targets.

3.2.2 Heavy Metals

With rising concerns over heavy metal exposure, heavy metal sensing is a promising application for this biosensing system. While many metal sensing transcription factors have been previously characterized, two aTFs were selected as candidates for a biosensor development: CadC for Lead and Cadmium sensing and CsoR for Copper sensing due to the increasing concern over the presence of these metals in drinking water.

Lead is a toxic heavy metal that poses substantial risks to both human health and the environment. Even at low concentrations, lead can accumulate in the body over time, leading to severe health consequences, particularly in vulnerable populations such as children and pregnant women [51]. From an environmental perspective, lead contamination often arises from industrial activities, aging infrastructure, and improper disposal of lead-containing materials. Contaminated water supplies, such as those associated with corroding lead pipes, represent a significant public health concern, as exemplified by water crises in various regions [52]. According the Institute for Health Metrics and Evaluation lead exposure displayed a global health burden of 33.89 million disability-adjusted life years (DAILY) in 2021 [53]. One DAILY equaling the equivalent of losing one year in good health because of premature mortality or disability.

Cadmium is another toxic heavy metal that poses substantial health and environmental risks, with the two primary sources being occupational and environmental exposures. Occupational exposure to cadmium is most prevalent in industries such as plastics manufacturing, pigment production, metal refining, and the production of rechargeable batteries. The rising demand for electronics has further increased the use of cadmium in industrial settings, heightening the risk for workers in these fields [54], [55].

Environmental exposure to cadmium often stems from the use of phosphate fertilizers derived from sewage sludge that has not undergone proper heavy metal removal. This issue is particularly pronounced in underdeveloped regions, where these fertilizers are commonly used. Over time, the accumulation of cadmium in the soil can lead to its presence in water and food supplies, posing a widespread public health concern [8], [54]. Chronic cadmium exposure has been linked to severe health issues, including various cancers and toxic effects on the skeletal, urinary, reproductive, cardiovascular, nervous, and respiratory systems. These risks highlight the urgent need for effective monitoring and regulation of cadmium in both occupational and environmental contexts. CadC is an aTF that in response to lead or cadmium will bind to its operator sequence. It normally is used in bacteria to activate resistance genes to cadmium or lead.

Copper (Cu) is an essential trace element involved in various biological processes, particularly in oxidative metabolism. Its ability to alternate between two oxidation states, Cu (I) and Cu (II), makes it a key player in critical functions such as angiogenesis, the cellular response to hypoxia, and neuromodulation. Typically, copper is consumed through dietary sources, including food and drinking water. However, excessive copper intake can result in copper poisoning, posing significant health risks [56], [57].

Elevated copper levels in food and water often stem from environmental contamination. Sources include pollution from corroding pipes, discharge from mineral wastewater, and the widespread use of copper sulfates as herbicides and fungicides. Copper toxicity is especially prevalent in rural South Asian communities, where copper sulfate is frequently used in agricultural practices and sometimes burned during cultural or religious ceremonies. Acute copper poisoning can manifest as gastrointestinal symptoms, including nausea, vomiting with blood, diarrhea, jaundice, and intense thirst [57], highlighting the need for effective monitoring and mitigation strategies to protect public health. CsoR is a bacterial transcription factor that is known to bind to its operator in response to Cu(I).

3.3 Heavy Metal Interference on Different Cas12a Orthologs

During the development of a system targeting heavy metals, concerns arose regarding potential interactions between the metals and Cas12a's activation and trans-cleavage rates. Cas12a relies on magnesium ions (Mg^{2+}) for activation, raising the possibility that other divalent cations, such as zinc (Zn^{2+}), lead (Pb^{2+}), cadmium (Cd^{2+}), and copper (Cu^{2+}), could interfere with its function. To address this issue, research was conducted to identify Cas12a orthologs with enhanced resistance to heavy metal interference.

Previous studies have demonstrated that different Cas12a orthologs exhibit distinct properties and tolerances under varying conditions [58]. These differences come from slight changes in the structure of the Cas12a orthologs derived from different environmental pressures each bacteria species evolved from. This project focuses on testing three orthologs—Lb Cas12a, Fn Cas12a, and As Cas12a—sourced from New England Biolabs, MedChem Express, and Integrated DNA Technologies, respectively. By evaluating their performance in the presence of heavy metals, the goal is to identify an ortholog that maintains reliable activation and trans-cleavage activity, even in environments containing high concentrations of divalent cations. This selection process is critical for ensuring the robustness of the biosensor under real-world conditions.

Chapter 4: Testing of Allosteric Transcription Factor Gated Cas12a Activation Biosensors

In this chapter, we investigate the practical implementation of combining aTFs and Cas12a to create a robust biosensing platform. The focus lies on evaluating the effects of heavy metal interference on Cas12a's trans-cleavage activity and testing the gating capabilities of aTFs in regulating Cas12a activation. Through systematic experimentation, we aim to understand the impact of various metals, including zinc, copper, cadmium, and lead, on the performance of different Cas12a orthologs. Additionally, we explore the effectiveness of specific aTFs, such as NanR, CadC, and CsoR, in modulating Cas12a activity in response to target ligands.

4.1 Heavy Metal Interference of Cas12a Orthologs

In heavy metal interference tests, each heavy metal is prepared as a concentration series. Each concentration condition is then mixed with a double-stranded DNA sequence containing both the operator sequence recognized by the allosteric transcription factor (aTF) targeting the ligand and a protospacer adjacent motif (PAM) sequence required for Cas12a activation. A mixture of guide RNA (gRNA) and selected Cas12a orthologs are subsequently added to each concentration of ligand for a final concentration as outlined in Table 4.0.1.

The gRNA consists of two key components: a target-specific sequence complementary to the operator sequence of the aTF and a conserved loop region essential for Cas12a binding and activation. The combined mixture of ligand, DNA, and the Cas12a-gRNA complex is incubated at 37°C for 30 minutes to allow for proper activation of Cas12a. Following incubation, the mixture is transferred to a microwell plate preloaded with a fluorescence quencher single stranded DNA probe. Fluorescence measurements are then obtained using a plate reader to assess Cas12a activation by monitoring the cleavage of the probe. This setup enables the detection of Cas12a activation states and provides insight into the impact of heavy metal interactions on its trans-cleavage activity. We established a 50% decrease in fluorescence when compared to the baseline of no metal as being substantial enough to interfere with the readouts of the platform.

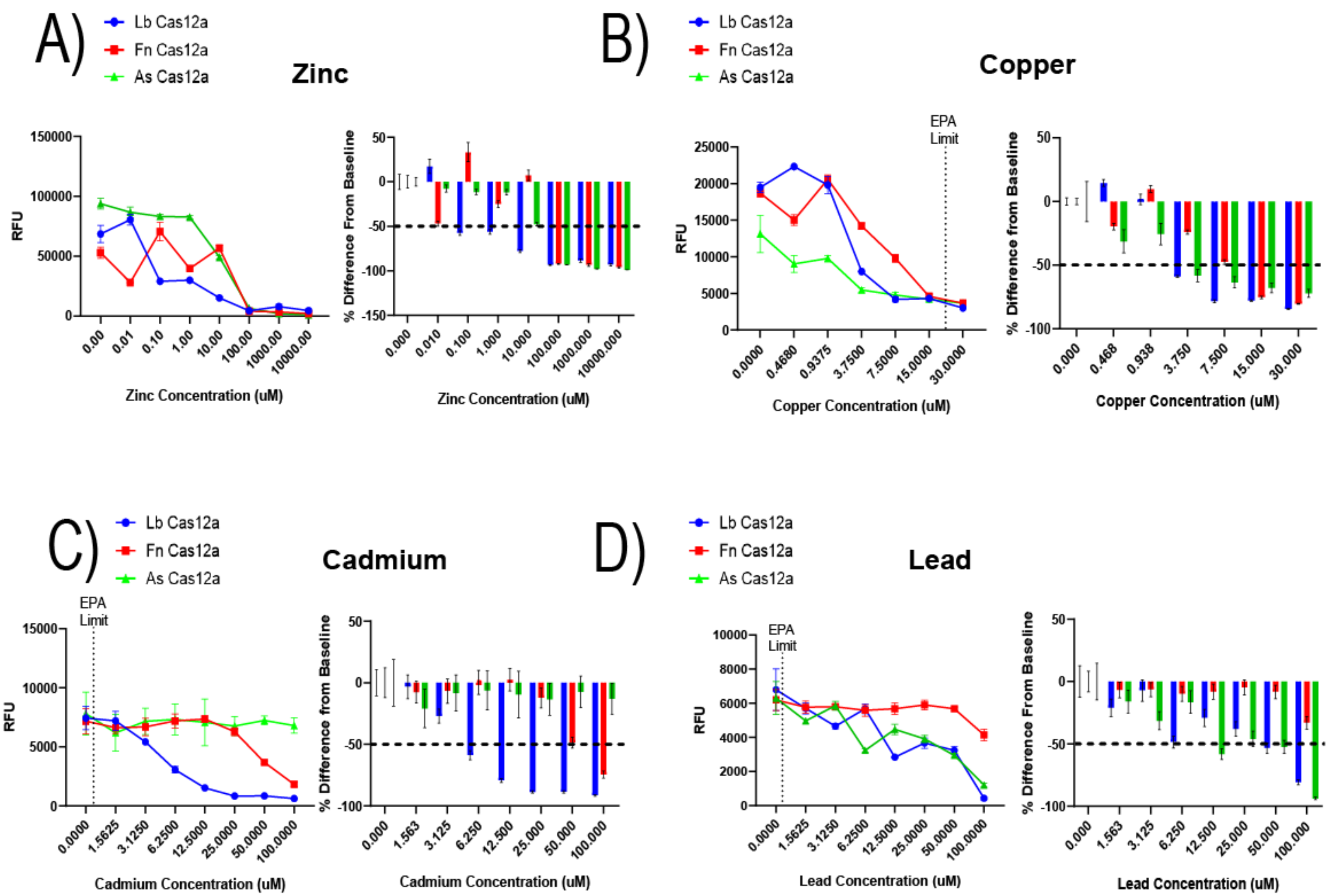

Figure 4.1 Heavy Metal Interference on Cas12a Trans Cleavage at 1 hour.

Fluorescence measurements taken 1 hour after plating with single stranded DNA florescence quencher pair. A) 0 – 10,000 µM zinc B) 0 – 30 µM copper, C) 0 – 100 µM Cadmium, D) 0 – 100 µM lead. N=3. Mean +/- standard deviation

Table 4.0.1 Substantial Metal Interference of Cas12a Orthologs

	EPA Limit	Lb Cas12a	Fn Cas12a	As Cas12a
Zinc	N/A	0.1 µM	100 µM	100 µM
Copper	20.47 µM	3.75 µM	15 µM	3.75 µM
Cadmium	0.0445 µM	6.25 µM	50 µM	**N/A**
Lead	0.072 µM	6.25 µM*	**N/A**	12.5 µM*

Substantial defined as a 50% decrease from base line. Data taken from Figure 4.1
*Percent difference from baseline recovers afterward

Results from the heavy metal interference study, as shown in Figure 4.1 demonstrate the effects of various metals on Cas12a activity 1 hour after the start of the addition of florescence-quencher probe. The screening revealed that all Cas12a orthologs experienced decreases in fluoresce at 1 hour when compared to baseline at the highest concentrations of metals tested.

For zinc in Figure 4.1.A, Lb Cas12a was the most affected, showing a 57% decrease from baseline starting at 0.1 µM. Fn Cas12a displayed irregular behavior across the tested concentrations dropping down a 47% decrease to a 33% increase from baseline from 0.01 µM to 0.1 µM zinc. This shows some unpredictability or noise in the system or protocol. As Cas12a showed the highest resistance to zinc interference remaining above the 50% decrease from baseline threshold until 10 µM of zinc was introduced.

In copper testing in Figure 4.1.B, both Lb and As Cas12a dropped below the 50% threshold at 3.75 µM and Fn Cas12a dropped below at 15 µM. All three orthologs saw substantial decrease before hitting the EPA limit of 20.47 µM.

For cadmium in Figure 4.1.C, Lb Cas12a was the most susceptible to interference dropping below the 50% threshold at 6.25 μM while Fn Cas12a dropped below at 50 μM. As Cas12a saw little interference and never dropped below the 50% threshold making it a promising candidate for cadmium sensing up to 100 μM.

With lead in Figure 4.1.D, some inconsistencies and noise are seen in the system between Lb and As Cas12a. Lb Cas12a first dropping below the threshold at 6.25 μM but then recovering and dropping below again at 50 μM. As Cas12a also showing this dropping below at 12.5 μM recovering and then going down again at 50 μM. Fn Cas12a showed the most resistance to lead never dropping below the 50% difference from baseline threshold. This would make it a suitable candidate for sensing lead.

These results highlight differences in heavy metal tolerance among the Cas12a orthologs, which can guide the selection of the appropriate aTF-Cas12a pair for specific metal targets. Notably, As Cas12a performed best by showing the least percent difference from baseline for cadmium, while Fn Cas12a stood out for lead, demonstrating superior resistance compared to the other orthologs tested.

4.2 aTF Gating of Cas12a Activation Testing

aTF tests are conducted similarly to the ligand interference tests, with additional steps to ensure proper binding of the aTF to its operator sequence. This section will include two different kinds of tests: DNA protection and DNA deprotection. DNA protection tests aim to determine whether the aTF can successfully block Cas12a from binding and activating its target. DNA deprotection tests will show that in the presence of the ligand the aTF will unbind from the operator sequence and allow Cas12a activation.

For DNA protection tests, aTF proteins are first produced in *E. coli* cells and purified using fast protein liquid chromatography (FPLC). After isolation, the aTF is prepared as a concentration series and mixed with a double-stranded DNA sequence containing both the operator sequence recognized by the aTF and the protospacer adjacent motif (PAM) sequence required for Cas12a activation. The mixture is allowed to incubate at room temperature for 15 minutes to enable aTF binding to the operator.

Next, a pre-made mixture of gRNA and selected Cas12a orthologs is added to the samples at the final reaction concentrations specified in Table B.0.2 in Appendix B. The combined mixture of aTF, DNA, and the Cas12a-gRNA complex is incubated at 37°C for 30 minutes to allow Cas12a activation where permitted. Following incubation, the mixture is transferred to a microwell plate preloaded with a fluorescence quencher single-stranded DNA probe. Fluorescence measurements are then taken using a plate reader, monitoring the cleavage of the probe to assess Cas12a activation and the ability of the aTF to inhibit this process effectively. If this test shows Cas12a gated activation a second test will be performed for DNA deprotection in response to the target ligand.

For DNA deprotection tests, aTFs will be diluted to specific concentrations determined from their performance in the DNA protection tests and then mixed with their corresponding double-stranded DNA operator sequences. The mixture is allowed to rest for 15 minutes to facilitate binding of the aTF to the operator. Subsequently, a concentration series of ligands is added to separate mixtures, followed by another 15-minute incubation to allow the ligands to interact with the aTF. This step simulates the introduction of a test sample containing the target ligand.

After ligand interaction, a prepared mixture gRNA and selected Cas12a orthologs is added to each sample at the final concentrations outlined in Table B.0.3. The combined mixture of aTF, DNA, and the Cas12a-gRNA complex is incubated at 37°C for 30 minutes to allow Cas12a activation. The combined mixture of aTF, DNA, and the Cas12a-gRNA complex is incubated at 37°C for 30 minutes to enable Cas12a activation. Following incubation, the samples are transferred to microwell plates.

4.2.1 NanR and CadC Testing

NanR was the one of the first aTFs selected for testing because of its interaction with the sialic acid Neu5ac, which has also been considered a potential biomarker for cardiovascular disease and rheumatoid arthritis [45], [46]. NanR on its own will bind to a specific DNA operator site but in the presence of Neu5ac will undergo a conformational change and unbind from this site. Preliminary testing was conducted using Lb Cas12a, on if the binding strength of NanR to its operator site was strong enough to block Cas12a from activation. as shown in Figures 4.2.1.A and B.

In Figure 4.2.1.A, DNA protection tests were performed to evaluate how NanR concentration gates Cas12a activation. If NanR can gate Cas12a activation, higher concentrations of NanR would be expected to show a slower rise in fluorescence compared to lower concentrations or the absence of NanR. This would be because more Cas12a would be activated and cleaving the single stranded florescence quencher probe. The DNA protection assay showed that at 1128.2 nM and 2256.5 nM of NanR, the fluorescence signal rose slower than at lower concentrations, supporting the hypothesis that NanR gates the activation of Cas12a at these levels. Based on these results, subsequent deprotection tests were initiated.

For the deprotection tests (Figure 4.2.1.B), 1000 nM NanR was used in combination with Lb Cas12a. However, these tests revealed no difference in trans cleavage activity, even with Neu5Ac concentrations up to 1 mM. This suggests that Neu5Ac did not induce the conformational change necessary to release NanR from the target DNA, thereby failing to trigger Cas12a activation.

DNA protection tests were then conducted to compare the performance of Cas12a orthologs with NanR (Figure 4.2.1.C). This graph provides a snapshot of fluorescence intensity at the one-hour mark across different orthologs and NanR concentrations. If NanR were effectively gating any of the Cas12a orthologs, we would expect to see a downward trend in fluorescence as the concentration of NanR increased. However, the data show minimal differences between NanR concentrations, suggesting that NanR was not gating Cas12a activation even at the 1000 nM concentration tested.

Even for Lb Cas12a, which had shown gating behavior in earlier tests, the expected trend was not observed. This inconsistency highlights potential variability in NanR's ability to gate Cas12a activation, possibly influenced by experimental conditions or intrinsic differences in the orthologs tested. These findings raise questions about the reliability of NanR as a gating aTF and emphasize the need for further investigation to understand the observed discrepancies.

CadC was selected to be a potential aTF to sense cadmium and lead. DNA protection tests were then conducted to compare the performance of CadC gating across different Cas12a orthologs (Figure 4.2.1.D). Similar to NanR, a snapshot of fluorescence data at one hour was plotted against CadC concentrations to compare gating of the

Cas12a orthologs. No ortholog saw substantial deviation from its baseline up to 100 nM of CadC suggesting it is not able to block the activation of any of the Cas12a orthologs. Some deviation from the baseline was seen by Lb Cas12a at 25 nM but not enough to confidently separate the no CadC baseline from the CadC sample.

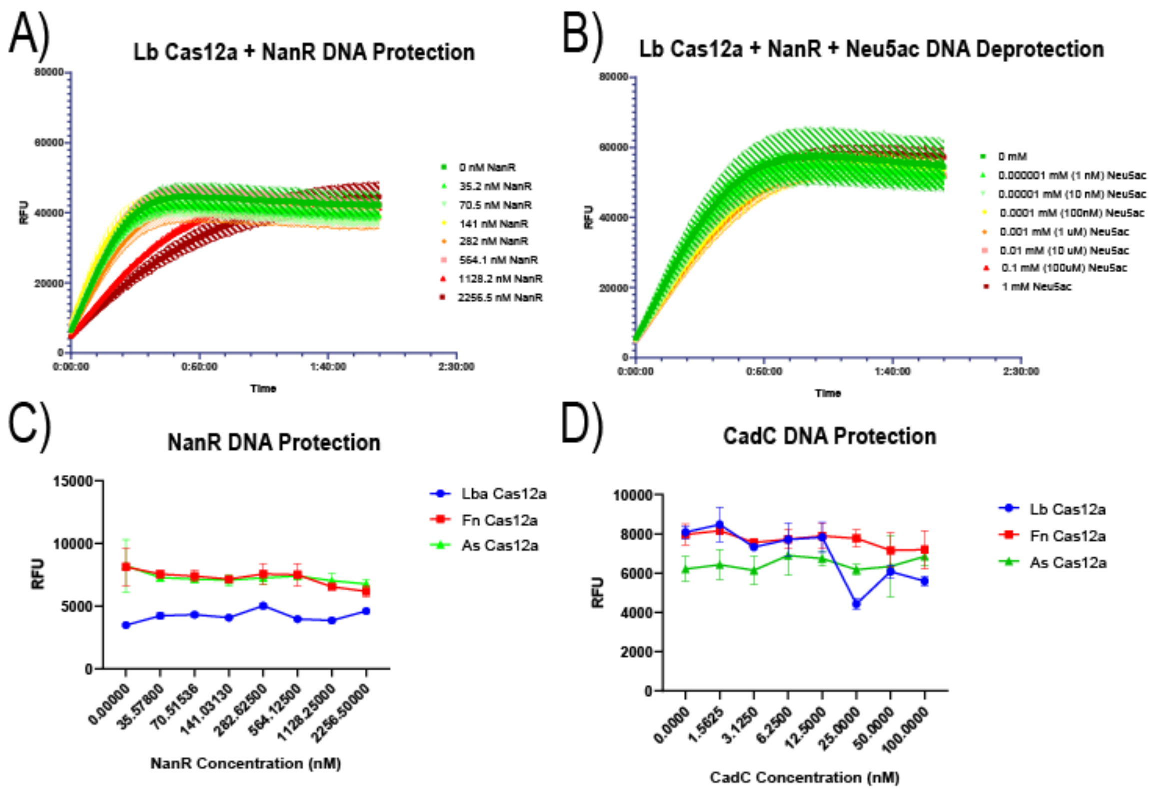

Figure 4.2.1 NanR and CadC DNA protection and Deprotection

A) DNA protection test with Lb Cas12a and range of NanR aTF concentrations B) DNA deprotection test with Lb Cas12a, 1000 nM NanR and Range of Neu5ac. C) DNA protection test with Cas12a Orthologs and range of NanR aTF concentrations at 1 hour D) DNA protection test with Cas12a Orthologs and range of CadC aTF concentrations at 1 hour. For A, B) N=3, dark line is mean with shaded region over 1 standard deviation. For C, D) N=3, mean +/- 1 standard deviation error bars.

4.2.2 CsoR Testing

CsoR was selected to be the aTF to sense copper. DNA protection tests were again conducted to compare how different Cas12a orthologs were gated by CsoR. The fluorescence readings at one hour are seen in Figure 4.2.2.A. Here we see a large drop between the fluorescence at one hour for no CsoR compared to adding 0.168575 µM CsoR suggesting that CsoR may be gating Cas12a activation leading to lower florescence at one hour.

A deprotection test was then conducted for all three orthologs with and without CsoR and at 30 µM, 0.94 µM and 0 µM of copper (Figure 4.2.2.B). The rate of change of fluorescence calculated as RFU / Minute was then calculated for each concentration of copper with and without CsoR.

For Lb Cas12a in Figure 4.2.2.B top right panel, we see the addition of CsoR for 0 µM and 0.94 µM of copper leading to a decrease in RFU / Min. This would support CsoR gating the activation of Lb Cas12a as CsoR is bound to its operator at low copper levels. At 30 µM of copper we see the addition of CsoR lead to an increase in the RFU/min. This could show the unbinding of CsoR from the operator at high levels of copper leading to Cas12a activation. Notably for Lb Cas12a the 0.94 µM copper without CsoR was greater than our baseline of 0 µM copper without CsoR this could be from noise or inconsistency of the system or the addition of low amounts of copper benefiting the activation of Lb Cas12a

In Fn Cas12a in figure 4.2.2.B center right panel, a similar trend is observed where the addition of CsoR for 0 µM and 0.94 µM of copper leading to a decrease in

RFU / Min and an increase at 30 µM. Here for the no CsoR we see a steady decrease in RFU/Min as we increased copper following a similar trend as was seen in the metal interference testing in Figure 4.1.B.

For As Cas12a in Figure 4.2.2.B bottom right panel, for 0 µM we see a greater RFU/Min with the addition of CsoR compared to not this would suggest that CsoR is not gating As Cas12a successfully and show some inconsistency when compared to our results in Figure 4.2.2.A. At 30 µM as we see in the other graphs see the addition of CosR increasing the RFU/Min.

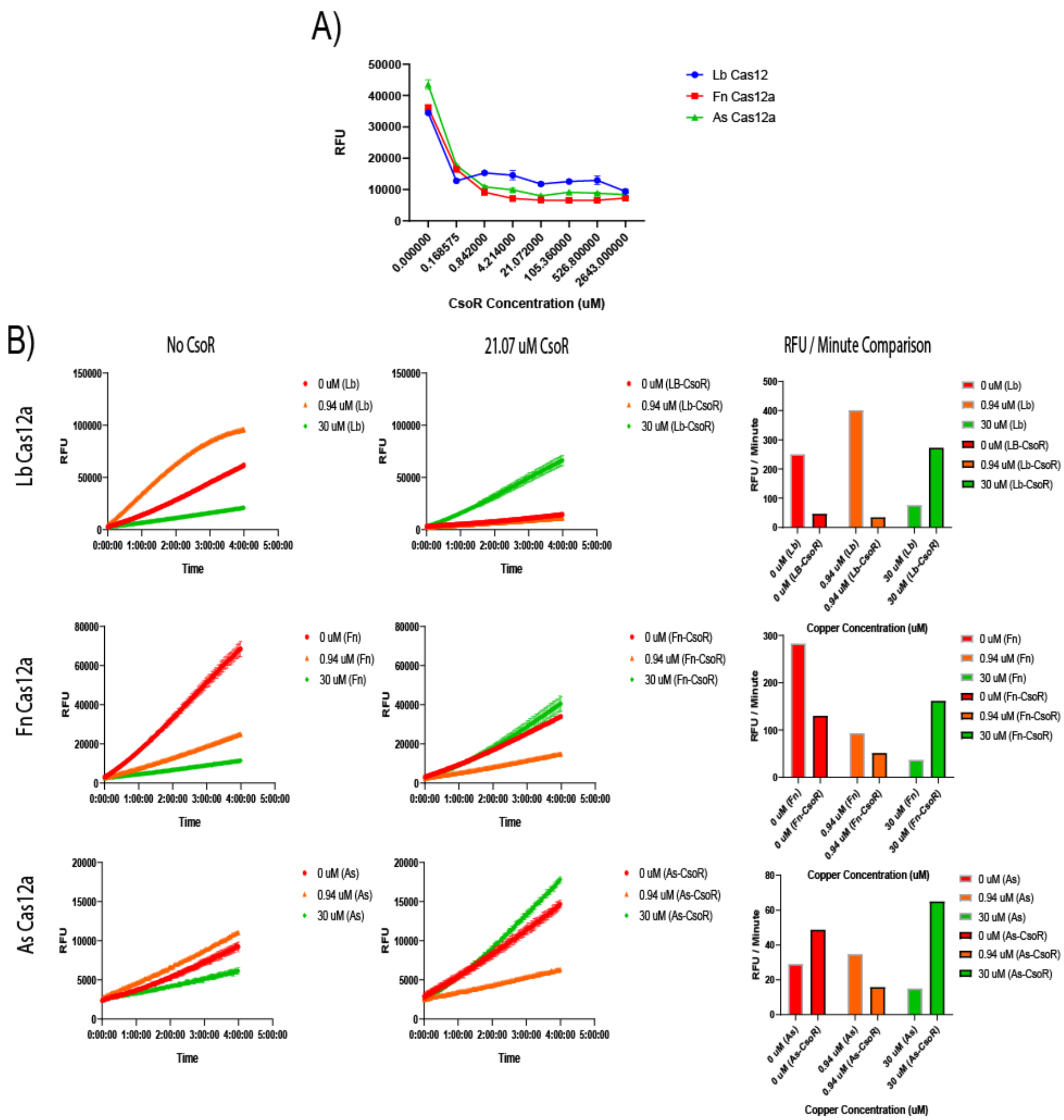

Figure 4.2.2 CosR DNA protection

A) DNA protection assay with range of concentration of CsoR with Cas12a orthologs at 1 hour. N=3. Mean +/- standard deviation B) Comparison of CsoR and lack of CsoR for 0 µM, 0.94 µM, and 30 µM of copper for Cas12a orthologs. N=3, dark line is mean with shaded region over 1 standard deviation. RFU/Min calculated as (Max-Min)/total time assuming a linear approximation.

It was interesting to see that for all three orthologs the 30 µM copper with the added transcription factor had a greater RFU/Min than with no transcription factor. One theory for this occurrence is that since copper concentration can play some effect on Cas12a trans cleavage rate as see in Figure 4.1.B the added CsoR is binding to the copper and lowering the free copper concentration in the sample reducing the effects of the copper on the Cas12a. This effect could compound with the gating to see the greater RFU/Min at the higher copper concentrations of copper with the added CsoR than with no CsoR added.

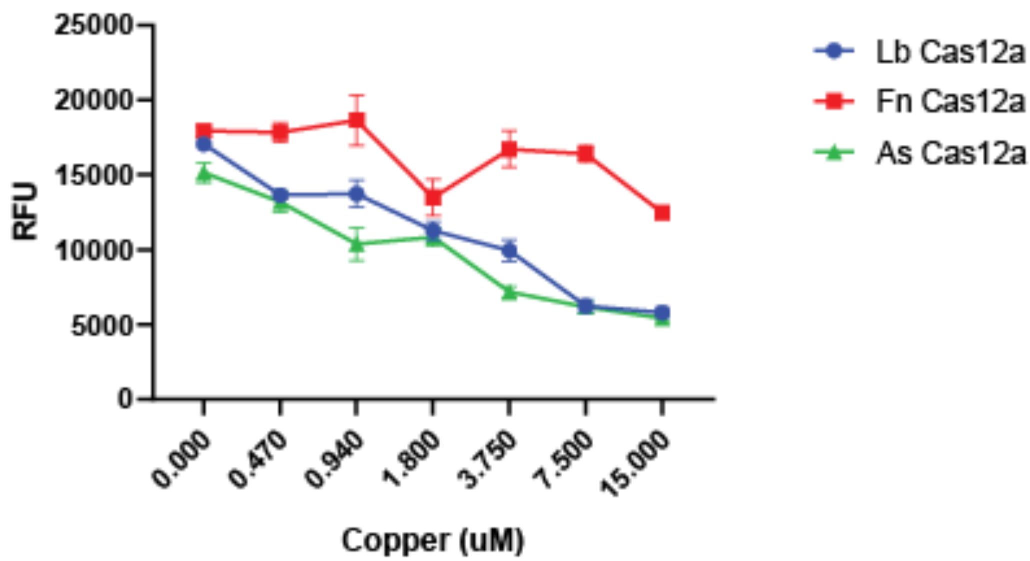

Figure 4.2.3 CsoR DNA Deprotection

DNA Deprotection with 21.07 µM CsoR and various amounts of copper. N=3, mean +/- 1 standard deviation error bars.

A concentration series of Copper with CsoR was then done to compare each ortholog (Figure 4.2.3.C) and to assess a detection range for copper. Fluorescence data was taken at one hour and graphed over each concentration of copper. From the trends we see an overall gradual slope downward as the concentration of copper rises. We do not see a rise in fluorescence at the high end of copper concentration which was observed in the previous graphs in Figure 4.2.1.B with Lb Cas12a and As Cas12a. This shows another inconsistency in the platform of its ability to show consistent protection and deprotection in the presence of an aTF and its ligand.

Chapter 5: Conclusions and Future Steps

In Chapter 1, we explored the critical need for biosensors in addressing modern challenges in healthcare, environmental monitoring, and beyond. We highlighted the versatility of biosensors, from detecting heavy metal contaminants to identifying small biomolecules as key biomarkers for diseases. We reviewed the current state of metal testing, emphasizing the limitations and advantages of electrochemical, optical, and spectrometry methods, and their essential roles in environmental and medical applications. Similarly, we discussed advancements in small biomolecule testing, delving into techniques for nucleic acid, lipid, polysaccharide, and protein detection. These methods underscored the importance of biosensors in enabling precise and rapid analysis, especially in scenarios where traditional laboratory-based equipment is unavailable. By bridging gaps in portability, specificity, and accessibility, biosensors continue to evolve as indispensable tools for improving global health and environmental safety, setting the stage for innovative approaches.

In Chapter Two, we examined the foundational principles and innovative potential of combining aTFs and Cas12a for biosensing applications. By leveraging the ligand-specific capabilities of aTFs and the robust signal amplification properties of Cas12a, this approach promises to address critical challenges in biosensor design. The chapter outlined key goals for this integration, emphasizing enhanced specificity, sensitivity, modularity and stability, all essential attributes for creating portable and efficient field-deployable biosensors.

In Chapter Three, we detailed the methodology for integrating aTFs with Cas12a to construct a versatile biosensing platform. This chapter explored the selection of targets, including biomarkers like N-Acetylneuraminic acid (Neu5Ac) and heavy metals such as lead, cadmium, and copper, using their corresponding aTFs (NanR, CadC, and CsoR). Additionally, we addressed the potential interference of heavy metals with Cas12a's enzymatic activity, evaluating various Cas12a orthologs for their tolerance to such conditions. By combining the ligand-specific properties of aTFs with Cas12a's collateral cleavage activity, this platform demonstrates robust potential for real-world applications.

In Chapter Four, we focused on the experimental evaluation of the proposed biosensing platform, investigating the impact of heavy metal interference on Cas12a's trans-cleavage activity and the gating capabilities of specific aTFs. The study revealed notable variations in the performance of Cas12a orthologs (LbCas12a, FnCas12a, and AsCas12a) under different heavy metal concentrations, with FnCas12a demonstrating superior resistance to lead and AsCas12a resistance to cadmium. Additionally, experiments with aTFs such as NanR, CadC, and CsoR highlighted their potential for gating Cas12a activation in response to specific ligands, though inconsistencies in gating efficiency suggest a need for further optimization.

Looking forward, further research is essential to address the inconsistencies observed in the gating efficiency of specific aTFs and their interactions with Cas12a orthologs. Understanding the underlying causes of these variations will be key to refining the platform's reliability and performance. Additional studies could explore alternative aTFs or modifications to the Cas12a system, such as introducing mismatches in the guide

RNA to reduce its affinity for the DNA target, potentially facilitating easier gating by aTFs. Furthermore, expanding the range of target analytes, optimizing ligand concentrations, and improving the scalability of this technology will be crucial for its transition to real-world applications. Specifically, the expansion of the platform to lateral flow assays will allow for portable testing of ligands important to meet the needs of users as discusses in Chapter 1. By addressing these challenges, this biosensing platform can achieve its full potential as a versatile tool for detecting biomarkers and environmental contaminants in diverse settings, ultimately contributing to advancements in both healthcare and environmental monitoring.

APPENDIX A: DNA and RNA Sequences Supplemental

Name	Sequence
Pam + NanR + Operator Forward	CAGTCGTTTATTGATCTAAGCGCACAGGTATAAAGGTATATCG TTTATGCCC
Pam + NanR Operator Reverse	GGGCATAAACGATATACCTTTATACCTGTGCGCTTAGATCAAT AAACGACTG
NanR gRNA Lb Cas12a	UAAUUUCUACUAAGUGUAGAUUUGAUCUAAGCGCACAGGUAU
NanR gRNA Fn Cas12a	UAAUUUCUACUGUUGUAGAUUUGAUCUAAGCGCACAGGUAU
NanR gRNA As Cas12a	UAAUUUCUACUGUUGUAGAUUUGAUCUAAGCGCACAGGUA
Pam + CadC + Operator Forward	CAGTCGTTTATCAAATAAATATTTGAATGTATGCCGC
Pam + CadC + Operator Reverse	GCGGCATACATTCAAATATTTATTTGATAAACGACTG
CadC gRNA Lb Cas12a	UAAUUUCUACUGUUGUAGAUUCAAAUAAAUAUUUGAAUGUA
CadC gRNA Fn Cas12a	UAAUUUCUACUGUUGUAGAUUCAAAUAAAUAUUUGAAUGUA
CadC gRNA As Cas12a	UAAUUUCUACUGUUGUAGAUUCAAAUAAAUAUUUGAAUGUA
Pam + CsoR + Operator Forward	CAGTCGTTTAATACCCTACGGGGGTATGGTATATGCC
Pam + CsoR + Operator Reverse	GGCATATACCATACCCCCGTAGGGTATTAAACGACTG

CsoR gRNA Lb Cas12a	UAAUUUCUACUAAGUGUAGAUAUACCCUACGGGGGUAUGGUA
CsoR gRNA Fn Cas12a	UAAUUUCUACUGUUGUAGAUAUACCCUACGGGGGUAUGGUA
CsoR gRNA As Cas12a	UAAUUUCUACUGUUGUAGAUAUACCCUACGGGGGUAUGGUA

APPENDIX B: Allosteric Transcription Factor Gated Cas12a Activation Supplemental

Table B.0.1 Cas12a Ortholog Metal Interference Protocol

Component	Final Concentration	Volume
NF-Water		4 µL
NEBuffer 2.1r	1x	2 uL
gRNA	200 nM	4 uL
Cas12a Ortholog	30 nM	2 uL
Metal	Variable	2 uL
dsDNA with aTF operator	10 nM	2 uL
DNAse Alert FQ	100 nM	4 uL
Total		20 uL

Combine dsDNA, Metal, NF-Water and NEBuffer 2.1r and incubate at room temperature for 15 minutes.
Add Cas12a and gRNA to mixture and incubate at 37c for 30 minutes
Place DNAse Alert FQ in Wells of plate
Add Cas12a-Metal mixture to plate

Table B.0.2 Cas12a aTF DNA Protection Protocol

Component	Final Concentration	Volume
NF-Water		4 uL
NEBuffer 2.1r	1x	2 uL
gRNA	200 nM	4 uL
Cas12a Ortholog	30 nM	2 uL
aTF	Variable	2 uL
dsDNA with aTF operator	10 nM	2 uL
DNAse Alert FQ	100 nM	4 uL
Total		20 uL

Combine dsDNA, aTF, NF-Water and NEBuffer 2.1r and incubate at room temperature for 15 minutes.
Add Cas12a and gRNA to mixture and incubate at 37c for 30 minutes
Place DNAse Alert FQ in Wells of plate
Add Cas12a-Metal mixture to plate

Table B.0.3 Cas12a aTF DNA DeProtection Protocol

Component	Final Concentration	Volume
NF-Water		2 uL
NEBuffer 2.1r	1x	2 uL
gRNA	200 nM	4 uL
Cas12a Ortholog	30 nM	2 uL
aTF	Variable	2 uL
Ligand	Variable	2 µL
dsDNA with aTF operator	10 nM	2 µL
DNAse Alert FQ	100 nM	4 µL
Total		20 µL

Combine dsDNA, aTF, NF-Water and NEBuffer 2.1r and incubate at room temperature for 15 minutes.
Add Ligand and incubate at room temperature for 15 minutes
Add Cas12a and gRNA to mixture and incubate at 37c for 30 minutes
Place DNAse Alert FQ in Wells of plate
Add Cas12a-Metal mixture to plate

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CURRICULUM VITAE

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