Cer-L9-Cit-2His: A Ratiometric Intracellular FRET-Based Nickel Sensor

By

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Abstract

Nickel is a trace metal that plays an important role in plants and microorganisms as an enzyme cofactor. Several strains of pathogenic bacteria such as *Helicobacter pylori* and *Escherichia coli* require nickel. Monitoring the available intracellular nickel concentrations would shed light on the homeostasis of metal ions and the physiology of these infectious bacteria. The goal of this research is to develop an intracellular Förster resonance energy transfer (FRET)-based nickel sensor that can monitor the nickel concentrations in real time. In this study, Cer-L9-Cit-2His, a modified zinc sensor was characterized. The zinc affinity was determined to be in the low nanomolar range, but further experimentation is required to determine the nickel affinity. The sensor exhibited a change in FRET response in BL21(DE3) *E. coli* cells that reported on changes to intracellular nickel concentrations. Modifications to the sensor will be required to improved nickel selectivity and the FRET response to nickel.
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1. **Introduction**

1.1 **Nickel in Bacteria**

Nickel is an important transition metal in many microorganisms and is essential for the function of nickel-dependant metalloenzymes.\(^1\) Several nickel-dependant metalloenzymes provide alternate energy sources for bacteria: [NiFe]-hydrogenase, carbon monoxide dehydrogenase, and methyl-coenzyme M reductase.\(^2,3\) Another class of nickel metalloenzymes include urease and superoxide dismutase, which serve to help microorganisms adapt to the environment and are involved in bacterial pathogenesis. For example, the urease metalloenzyme is used by *Helicobacter pylori* (*H. pylori*) to neutralize the acidic environment of the human stomach during infections.\(^4,5\) Although nickel is an important metal, it could also be detrimental to the cell when the free intracellular nickel concentrations accumulate to cytotoxic levels. High levels of nickel could replace the transition metals in non-nickel metalloenzymes and alter the enzymes’ functions or activities.\(^6\) In addition, excess nickel could inhibit the function of non-metalloenzymes by binding to either catalytic or allosteric sites. Finally, nickel could indirectly cause oxidative stress within the cell, increasing DNA damage, lipid peroxidation, protein cleavage, and DNA interstrand cross-link formation.\(^7\) To prevent nickel toxicity, the free nickel concentrations are tightly controlled within cells by several metallochaperones, metalloregulators, and metal transport pumps.\(^1\)

Since nickel plays such a pivotal role in bacterial cell function and pathogenesis, measuring the change of free intracellular nickel concentrations would aid in the elucidation of both the regulation of nickel in cells and the model of pathogenesis. The total cellular nickel
concentrations for several microorganisms have been recorded, with *E. coli* containing a total concentration of roughly 30 μM. In contrast, measuring the available intracellular nickel concentrations is difficult, with no values determined for any microorganism. A method that has been used to indirectly estimate the range of free intracellular nickel concentrations was based on the dissociation constants of major nickel-dependant transcription factors. NikR is a nickel-dependent transcription repressor with a $K_d$ for nickel in the picomolar range and is found in both *E. coli* and *H. pylori*. RcnR is a transcription repressor responsive to nickel and cobalt found in *E. coli* and has a nickel $K_d$ in the range of nanomolar. The two $K_d$ for NikR and RcnR could then be used to estimate the free intracellular nickel concentrations within *E. coli*. However, this method is only an approximated calculation and cannot determine the intracellular nickel concentration in real time. A potential tool that could be used for detecting and measuring free intracellular nickel concentration in microorganisms is a nickel-responsive sensor.

### 1.2 Intracellular Nickel Sensor

There is currently only one sensor that has been reported to detect the free intracellular nickel concentration, Nickelsensor-1 (NS1). The sensor consists of a boron-dipyrrromethane (BODIPY) chromophore and a mixed N/O/S ligand set for binding nickel (Figure 1). The sensor is a water-soluble small molecule and is capable of permeating the cell membrane of microorganisms. Nickel binding to NS1 elicits an increase in fluorescence intensity at 507 nm and a Hill plot was used to determine a simple 1:1 binding model with an apparent $K_d$ of 193 ± 5 μM. The affinity towards nickel for NS1 is too weak for real-time monitoring in microorganisms since the expected free intracellular nickel concentrations lie within the nanomolar range or lower.
Therefore, an alternative sensor to measure free intracellular nickel concentrations must be developed.

![Nickel Sensor 1 (NS1)](image)

**Figure 1**: Nickel Sensor 1. The only intracellular nickel sensor reported in the literature. The sensor consists of a BODIPY chromophore and N/O/S nickel binding domain. Nickel binding to NS1 results in an increase of fluorescence at 507 nm. The sensor has a 1:1 nickel binding with $K_d$ of $193 \pm 5 \, \mu M$.

### 1.3 Cer-L9-Cit-2His

In this study the sensor, Cer-L9-Cit-2His was examined. It is a genetically-encoded Förster resonance energy transfer (FRET) sensor that contains two 6-histidine peptides (His-tags) as nickel-binding sites. There are several advantages to the development of genetically-encoded FRET-based sensors. The first advantage is the expression of the sensor from within the cell. Since the sensor is a genetically encoded protein, there is no further need to ensure that the sensor could penetrate the cell membrane and enter the cell. The second advantage involves the FRET-based system compared to a simple fluorescence intensity measurement. In a FRET system, two fluorophores participate in Förster resonance energy transfer (FRET) with one fluorophore...
as a donor and the other as an acceptor. The donor fluorophore is first excited with light of appropriate wavelength and then energy is transferred to the acceptor fluorophore through nonradiative dipole-dipole coupling. The acceptor fluorophore’s emission intensity depends on its’ distance from the donor fluorophore and this phenomenon has an effective physical distance of 10 nm or less.\(^{14}\) The FRET ratio is determined by measuring the ratio of the emission of the donor fluorophore compared to that of the acceptor fluorophore. In the design of the sensor, intramolecular metal binding to the two His-tags would induce a conformational change, bringing the two fluorophores closer together. The FRET ratio change can then be correlated to a metal binding calibration curve. The ratiometric analysis between the emission of the donor and acceptor fluorophores also eliminates the need to determine the sensor concentration within the cell because the measurement is based upon the change in the ratio rather than the change in a simple fluorescence intensity.\(^{15}\)

The sensor, Cer-L9-Cit-2His was derived from the zinc sensor CLY9-2His designed by the Merkx group and was previously optimized for FRET response and stability.\(^{12}\) The protein consists of the donor fluorophore, cerulean, and the acceptor fluorophore, citrine. The two fluorophores are connected together with a flexible linker of nine GGSGGS repeats. The N-terminus of cerulean is bound to a 6XHis tag with the three amino acid linker SGG. The C-terminus of citrine is bound to the second 6Xhistidine tag with the same three amino acid linker. The donor fluorophore, cerulean, has a maximum excitation wavelength of 434 nm and an emission wavelength of 475 nm.\(^{13,16}\) The acceptor fluorophore, citrine, has a maximum excitation wavelength of 515 nm and an emission wavelength of 525 nm. Therefore, we calculated the FRET ratio for this sensor based on the fluorescence intensities measured at 525 nm and 475 nm.
(I_{525}/I_{475}) upon excitation at 434 nm. As stated previously, the two fluorophores are brought closer in proximity upon intramolecular metal binding (Figure 1), which leads to an increase in the FRET ratio.

**Figure 2:** Cer-L9-Cit-2His Schematic. The protein sensor consists of cerulean (blue cylinder), citrine (yellow cylinder), and the metal-binding His-tag domains. In the absence of metal, cerulean is excited with 434 nm light and emits at 475 nm. Upon intramolecular metal binding, FRET occurs between the excited cerulean and citrine emits at a wavelength of 525 nm.

### 1.4 Purpose

The purpose of this project was to characterize the Cer-L9-Cit-2His sensor and correlate the FRET ratio response to the concentrations of either nickel or zinc through metal titration experiments. The binding stoichiometries for nickel and zinc were determined using the 4-(2-Pyridylazo) resorcinol (PAR) colorimetry method. The affinities of the sensor for nickel and zinc were also examined with metal titration experiments monitored by fluorescence. However, since it was determined that the protein binds to both zinc and nickel with affinities tighter than micromolar, more complex competition experiments were required to determine the metal...
affinities. In addition to protein characterization, the sensor proficiency *in-vivo* was determined with whole cell fluorescence measurements in *E. coli* BL21(DE3) cells by using a pET expression system. The fluorescence measurements from the whole cell assay were then correlated to inductively coupled plasma mass spectrometry (ICP-MS) experiments performed on the whole cell samples. Molecular cloning was utilized in an attempt to clone the gene into a different destination vector, so that whole cell fluorescence experiments could be performed with different strains of *E. coli*. We had previously assumed that the FRET change for the sensor was due to the intramolecular dimerization of the metal-binding domains; however, intermolecular dimerization between two or more proteins is also possible. To investigate this, gel filtration studies were performed with an analytical size exclusion chromatography (SEC) column. The results from these experiments could help to direct the project towards the final goal: the development of a sensor that could determine the free intracellular nickel concentrations in microorganisms.

2. **Experimental**

2.1 Materials

OneTaq® Hot Start, DpnI, Quick ligase, NEB Turbo and BL21(DE3) *E. coli* competent cells were obtained from New England Biolabs. HisPur™ Ni-NTA resin, Mag-fura-2, Plasmid miniprep kit, Phusion® PCR components, PCR purification kit, and Quick gel extraction kit were obtained from Thermo scientific. Protease inhibitor tablets were obtained from Roche™. HEPES, glycerol, NaCl, KCl, EDTA, LB Broth Lennox, Arabinose, IPTG, and Tris were obtained from BioShop. Kanamycin and ampicillin were obtained from BioBasic. Chelex® 100 resin and Gel filtration
standards were obtained from Bio-Rad. The PD10 desalting columns, HiTrap™ Q and Superdex™
200 increase 10/300 GL columns were obtained from GE Healthcare. Spin concentrators were
obtained from Amicon and Standard RC tubing for dialysis was obtained from SpectrumLabs.
Sterile 0.45 µm MCE membrane filters were obtained from Millipore and Acrodisc® syringe filters
of 0.2 µm and 0.45 µm were obtained from Pall Corporation. PAR, EGTA, NTA, Imidazole, Tween,
and metal salts (trace metal grade) for metal stocks were obtained from Sigma Aldrich. The ICP-
MS multi-element metal standards were obtained from PlasmaCal and the Aristar® Plus nitric
acid was obtained from VWR Analytical. The RedSafe nucleic acid staining solution was obtained
from FroggaBio. Double deionized water was prepared on a Milli-Q filtration system with 18.2
MΩ.cm resistance and the water was used for all experiments.

2.2 Methods

Plasmid Preparation

The pET28a plasmid containing the Cer-L9-Cit-2His construct was provided by the lab of
Maarten Merkx. Plasmid stocks were created by transformation of NEB® Turbo competent
Escherichia coli (E. coli) cells and subsequent growth and plasmid extraction. Heat shock was
performed on the samples at 42°C for exactly 30 seconds and then they were placed on ice for 5
minutes. After the 5 minutes incubation, 900 µL of SOC media was added to each cell mixture.
The cell samples were then placed at 37°C for 1 hour rotating at 250 rpm. The samples were then
centrifuged at 12000 rpm for 10 minutes, the supernatant was removed to leave only a volume
of 100 µL. The pellets were then resuspended in the remaining supernatant. The resuspended
samples were then plated onto a 50 µg/mL kanamycin LB-agar plate and incubated at 37 °C
overnight. Single colonies were selected and inoculated overnight in 30 mL of LB-kanamycin media. The plasmids were extracted following a modified GeneJET™ plasmid mini Prep-kit Protocol.

The Plasmid concentrations were determined by using the nucleic acid option on the Nano Drop Spectrophotometer. Agarose gel electrophoresis was performed to determine the purity and relative size. The plasmid samples were prepared with 1.2 µg of purified plasmid and 2 µL of 6X gel loading dye. The GeneRuler™ 1kb Plus DNA ladder was prepared with 1 µL of the ladder, 9 µL of water, and 2 µL of 6X gel loading dye. The ladder and samples were loaded onto a 0.7 % gel with RedSafe dye which was run at 160 V for 25 minutes and imaged with UV-visible imaging instrument. The plasmids were submitted to ACGT Corp for DNA sequencing and the results were then compared with the original Cer-L9-Cit-2His construct DNA sequence.

**Protein Expression and Purification**

The pET28a plasmid containing Cer-L9-Cit-2His construct was transformed into *E. coli* BL21(DE3) cells using the heat shock method. The transformed *E. coli* cells were then plated onto 50 µg/mL kanamycin LB-agar plates and grown overnight at 37 °C. In a sterile environment, a colony from the overnight plate was inoculated into a flask containing 50 mL of 50 µg/mL kanamycin and LB mixture. The flask was then placed in an incubator at 37 °C and shaken at 250 rpm overnight. The overnight culture was then used as a starter culture for 1.0 L of LB media; 20 mL of the overnight culture was added to 1.0 L of 50 µg/mL kanamycin and LB mixture. The culture was then grown at 37 °C, shaking at 250 rpm, until the cells had reached an optical density (OD600) of 0.6 to 0.8. At this point, the culture was induced with 100 µL of 1.0 M isopropyl β-D-
thiogalactopyranoside (IPTG) for a final concentration of 0.1 mM IPTG. The culture was then grown overnight at room temperature (25 °C) shaking at 200 rpm. The cells from the overnight culture were separated into two 500 mL Nalgene bottles and centrifuged for 40 minutes at 4000 rpm. The supernatant was discarded and the pellets were resuspended with 30 mL of 20 mM Tris at pH 7.6 into a 50 mL Falcon tube.

Protein extraction was performed from the resuspended cells by sonication using a Branson Sonifier Cell Disruptor for 5 minutes with the following procedure: A cComplete™ protease inhibitor tablet from Roche was first added to the 30 mL of resuspended cells. Next, the cells were sonicated on ice 5 times for 1 minute. Before each sonication cycle, 30 μL of 1.0 M TCEP was added to the cells. The final concentration of TCEP in the cell solution after the 5 sonication cycle was 5 mM. After sonication, 3 μL of DNase 1 (V<sub>DNase 1</sub>: V<sub>cells</sub> was 1:10,000) was added to the mixture, which was incubated on ice for an additional 20 minutes. The cells were then centrifuged at 4°C for 1 hour at 12,000 rpm. After centrifugation, the supernatant was filtered through a 0.45 μm syringe and then loaded onto a Ni-NTA affinity chromatography column at 4 °C.

The Ni-NTA column was prepared by adding 10 mL of Ni-NTA slurry into a mini-column and washing with 30 mL of ddH<sub>2</sub>O. After the initial wash, the column was then equilibrated with 50 mL of Equilibration Buffer (50 mM Tris, 100 mM NaCl at pH 8). The filtered cell lysate was then loaded into the column with a peristaltic pump and washed with 75 mL of Wash Buffer (50 mM Tris, 500 mM NaCl at pH 8). The protein was then eluted with 30 mL of Elution Buffer (50 mM Tris, 100 mM NaCl, 200 mM Imidazole at pH 8) and collected into 2 mL fractions. After collecting the fractions, the column was washed with 50 mL of ddH<sub>2</sub>O and stored with 20% ethanol. The
fractions were then analyzed with UV-visible spectroscopy at 515 nm and the protein concentration was calculated using an extinction coefficient of 77000 M$^{-1}$cm$^{-1}$. The fractions containing high protein concentrations were pooled together and 10 μL of 0.5 M EDTA was added for each 2 mL fraction. The pooled fractions were then transferred to a clamp dialysis tube and sealed. The protein sample was then dialyzed overnight at 4 °C in 3 L of Dialysis Buffer (20 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$ at pH 8.4). After dialysis, the protein concentration was determined and a sample was submitted for ESI-MS analysis.

Thrombin cleavage was performed on the dialyzed protein to remove the extra N-terminal 6XHis-tag. A stock solution of 100 U/mL thrombin was prepared with thrombin cleavage buffer (20 mM Tris, 159 mM NaCl, 2.5 mM CaCl$_2$ at pH 6.5) and 1 mg/mL buffer of BSA. After the protein concentration was determined, 0.3 U of thrombin was added per 0.2 mg/mL of protein. This solution was incubated for 24 hours at room temperature followed by the addition of PMSF in DMSO for a final concentration of 1 mM PMSF.

The final purification step was anion exchange chromatography and it was performed on HiTrap™ Q HP column attached to a fast protein liquid chromatography (FPLC) instrument. The column was run with a salt gradient buffer system consisted of the two filtered Chelex® treated buffers: Buffer A (20 mM Tris pH 7.5) and Buffer B (1.0 M NaCl, 20 mM Tris pH 7.5). The column purification was performed overnight with 2 mL fractions collected at 4 °C.

SDS-PAGE was performed on the collected fractions. The samples and PageRuler™ Prestained protein ladder were loaded onto a 10% SDS-PAGE gel which was run with 1X SDS tris-glycine electrophoresis buffer at a constant voltage of 150 V for 45 minutes. The gels were then
stained with Coomassie dye and destained for gel imaging. Fractions containing protein with high purity as indicated by the SDS-PAGE were pooled together and a sample was submitted for ESI-MS analysis. The pooled protein then underwent buffer exchange with 25 mM HEPES, pH 7.6, 100 mM KCl and was stored at -80 °C for future experiments.

**Protein Characterization**

To determine the amount of metal loaded on the protein, a 4-(2-Pyridylazo)resorcinol (PAR) colorimetric assay was performed. A stock of PAR was prepared by dissolving 12.96 mg of 4-(2-Pyridylazo)resorcinol in 10 mL of ddH\textsubscript{2}O. Metals stocks of 100 µM zinc and nickel were prepared by dissolving zinc sulfate and nickel sulfate in ddH\textsubscript{2}O. Standard samples were prepared with 50 µM PAR and varying metal concentrations from 0-10 µM. A standard curve with a linear relationship between the metal concentrations and the absorbance at 500 nm were plotted. The 100 µL samples were prepared in triplicate with 50 µL of 8 M GuHCl, 1 µL of PAR, and 1 µL to 49 µL of protein; the sample was then topped up to 100 µL with Chelex® treated PAR buffer (50 mM Tris pH 8.5). A protein blank sample was prepared for each protein concentration measured. The protein blank consisted of the same volumes of 8 M GuHCl and protein; however, the protein blank did not contain any PAR. The electronic absorption of the standards and protein samples was measured on the UV-visible spectrometer at wavelengths of 412 nm and 500 nm.

**Fluorescence Analysis and Metal Titration**

The fluorescence response of metal binding to the protein was measured with a metal titration experiment. Two metals were examined in the metal titration experiments: zinc and nickel. The metal titration buffer consisted of 20 mM Tris, 100 mM NaCl, 10% glycerol, and 0.01%
Tween at pH 7.5 and was treated with Chelex® before filtering into a clean bottle for storage at 4 °C. The purified protein was thawed on ice and the concentration was determined by using the absorption at 515 nm and an extinction coefficient of 77000 M⁻¹cm⁻¹. Then it was diluted to 2 μM in metal titration buffer. For each metal, 16 titration samples (150 µL) were prepared with varying metal concentrations from 0 to 33 µM. The 16 samples were then incubated at room temperature for 2 hours. After incubation, 40 µL of each sample was pipetted in triplicate into a black 384 micro-well plate. The fluorescence measurement was performed on the CLARIOstar plate reader with an excitation wavelength of 434 nm. The endpoint fluorescence spectra from 460 nm to 560 nm were recorded for each sample.

**Metal Binding Stoichiometry**

The stoichiometries of nickel and zinc binding to the protein were determined using the PAR assay method. Nickel and zinc PAR standard samples, ranging from 1 µM to 10 µM, were prepared with 50 µM PAR. A 150 µL sample of 2.0 µM apo-protein sample was prepared in the metal titration buffer. The protein aliquot was then treated with 10 molar equivalents of either nickel or zinc and incubated at room temperature for two hours. During the 2 hour incubation, a PD10 desalting column (GE Healthcare) was equilibrated with three column volumes of metal titration buffer in the cold room (4 °C). The metal-treated protein sample was then loaded onto the PD10 column to remove excess unbound metal from solution. Five 1.0 mL fractions were collected. The fractions were analyzed by UV-visible spectroscopy at 515 nm to determine the presence and concentration of protein. Fractions with concentrations lower than 5 µM were spin-concentrated to achieve higher concentrations for further experimentation. A 150 µL sample containing 2 µM of metal treated protein was prepared for fluorescence analysis. Three 100 µL
PAR samples were prepared with 50 µL of 8 M GuHCl, 1 µL of 5 mM PAR, and 49 µL of metal-treated protein. A protein blank was prepared with 50 µL of GuHCl, 49 µL of metal treated protein, and 1 µL of PAR buffer. The standards and protein samples were measured on the UV-visible spectrometer at absorbance wavelengths of 412 nm and 500 nm. The ratio of metal concentration to protein concentration could then be determined. The fluorescence of the apo-protein sample and the metal-treated sample were measured with the same procedure outlined for the metal titration experiments.

**Metal Affinity Equations**

The affinities for zinc and nickel were determined with the data obtained from the metal titration experiments and the stoichiometry of binding. The first step was to convert the FRET ratio ($R$) response to represent the fraction of protein-bound ($r$).\(^{18}\)

\[
r = \frac{R - R_{\text{min}}}{R_{\text{max}} - R_{\text{min}}}
\]

The minimum FRET ratio ($R_{\text{min}}$) was expected to be the value from the apo protein, while the maximum FRET ratio ($R_{\text{max}}$) was expected to be the value of fully loaded protein. The fraction of protein bound would then be used to determine the concentration of protein bound to metal [PM].

\[
r = \frac{[PM]}{[P]_T}
\]

The free metal concentration was calculated by subtracting the product of the stoichiometry of metal binding ($x$) and concentration of metal bound protein from the total metal concentration.

\[
[M]_{\text{Free}} = [M]_T - x[PM]
\]
The fraction of protein bound was plotted against the concentration of free metal and analyzed using the Hill equation on the Prism® 7 software to determine the $K_d$ for each metal:

$$r = \frac{[M]^n}{K_d^n + [M]^n}$$

The units of the dissociation constant $K_d$ are in molar concentration (M) and $n$ is the Hill coefficient.

**Metal Competition**

Competition assays were performed to determine the binding affinities for zinc and nickel. The experiments were performed with Chelex®-treated 25 mM HEPES, pH 7.6, 100 mM KCl buffer containing metal chelators. The first chelator used was EDTA, samples were prepared with 2 µM protein, 1 mM EDTA, and 1 mM CaCl$_2$ or 10 mM CaCl$_2$. These samples consisted of varying total nickel and zinc concentrations ranging from 0 to 1 mM. Samples were incubated overnight at 4 °C and then equilibrated to room temperature for 5 minutes. The samples were pipetted in 40 µL triplicates into a black 384 micro-well plate and fluorescence signals were measured on the CLARIOstar plate reader. Other competitors used were EGTA, NTA, and imidazole. Competition samples were prepared with 1 mM of competitor and 2 µM protein. The dissociation constant for nickel and the respective competitors are: $\text{Ni}^{2+}$/EDTA ($K_D = 4.0 \times 10^{-19}$ M), $\text{Ni}^{2+}$/EGTA ($K_D = 3.1 \times 10^{-14}$ M), $\text{Ni}^{2+}$/NTA ($K_D = 1.8 \times 10^{-11}$ M), and $\text{Ni}^{2+}$/Imidazole ($K_D = 9.8 \times 10^{-4}$ M).$^{19}$

A Mag-fura-2 (MF2) competition assay was used to examine the tight nickel-binding affinity of the protein. Stocks of MF2 were prepared with Milli-Q water and quantified by measuring the absorbance at 369 nm with an extinction coefficient of 22,000 M$^{-1}$ cm$^{-1}$.\textsuperscript{20} Competition experiments were prepared by incubating 2 µM of Cer-L9-Cit-2His protein with 2
µM of MF2 and varying amounts of nickel(II). The samples were incubated overnight at 4 °C and allowed to equilibrate to room temperature before measurement. The metal bound to the dye was determined either by monitoring the absorbance of MF2 at 366 nm or the decrease in fluorescence emission at 510 nm with excitation at 340 nm. The data were analyzed using DYNAFIT and an equilibrium equation between the protein, MF2, and nickel(II).

A PAR competition assay was also used to examine the nickel-binding affinity of the protein, CerL9Cit-2His. Apo-protein (0-50 µM) was titrated into 50 µM of PAR and 10 µM of NiSO₄ in 25 mM HEPES, pH 7.6, 100 mM KCl and incubated overnight at 4 °C to reach equilibrium. Samples were then analyzed with electronic absorption spectroscopy and the signal at 515 nm was monitored for nickel bound PAR.

Whole Cell Fluorescence Assay and ICP-MS

Whole cell fluorescence was performed with transformed E. coli BL21(DE3) cells expressing the protein in LB media. First, stock Cer-L9-Cit-2His plasmid was transformed into E. coli BL21(DE3) cells with the heat shock method and plated onto 50 µg/mL kanamycin LB agar plates. The plates were incubated overnight at 37 °C and single colonies were inoculated into 50 mL of kanamycin LB media. The cultures were then incubated at 37 °C and the OD600 was measured until it reached 0.6 to 0.8. The cultures were then induced with 5 µL of 1 M IPTG for a final concentration of 0.1 mM IPTG. The cultures were then grown overnight at room temperature. Fresh LB media was prepared to dilute the overnight culture to an OD600 of 0.4. The diluted cultures were then separated into ten 500 µL aliquots, one aliquot for no metal, one aliquot for 1 mM EDTA, four aliquots for nickel treatment and four aliquots for zinc treatment.
The four aliquots were treated with: 0.5 mM of metal, 1 mM of metal, 2 mM of metal, and 3 mM of metal. LB media containing no bacteria cells were pipetted into a black 96 well plate in 125 μL triplicates along with the cell aliquots. The fluorescence of the plate was read at 3 different time intervals: immediately after metal addition, 2 hours after metal addition, and overnight. The plate was read on the CLARIOstar plate reader with an excitation wavelength of 434 nm.

Inductively coupled plasma mass spectrometry (ICP-MS) was used to quantify the total amounts of metals in the cells. Following the same procedures outlined above for cell growth and protein expression, the induced cells were then diluted to an OD600 of 0.4 and separated into 2 mL aliquots. These aliquots were then treated with the desired metal concentrations and EDTA concentration and then were incubated at room temperature for 2 hours. Following the 2 hour incubation, the cells were spun down and washed 3x with 20 mM Tris, 1 mM EDTA (pH 7.6) to remove metal from the surface of the cells. After the wash step, the cells were pelleted and acid digested in 57 μL of 70% nitric acid (HNO₃) and incubated at room temperature for 30 minutes. After 30 minutes, the cells were then topped up to 2 mL with ddH₂O to achieve a final nitric acid concentration of 2% and heated overnight at 60 °C. A standard calibration curve from 0 ppb to 500 ppb was prepared from a multi-element standard solution. The samples and standards were then loaded onto the iCAP Q ICP-MS equipped with an autosampler and an inline internal standard was included before injection to the nebulizer. The internal standards selected were Indium-115, germanium-73, and scandium-45. The instrument provided raw counts for the standards and samples, which allowed for the quantification of nickel and zinc for each sample.

Molecular Cloning
Restriction Free Cloning is a molecular cloning technique based on a two-step PCR reaction that bypasses the need for traditional restriction enzymes. The first step consists of the synthesis of the megaprimer containing the gene flanked by 5’ and 3’ overhangs to the target destination vector. The second step consists of the insertion of the megaprimer into the target destination vector with PCR and the linear amplification of the target plasmid with the gene. The synthesis of the megaprimer was performed with the forward primer 5’GGGCTAGCAGGAGGAATCCatgggcagccatgctagcatgactg3’ and the reverse primer 5’ATGCCTGCAGGTCGACTCTAGATTAactcgagccgccgctttatcgag3’. The PCR reaction (50 µL) to prepare the megaprimer consisted of 10 ng of pET28a-CerL9-2His plasmid, 200 nM of both primers, 200 µM of mixed dNTPs, 2% DMSO (v/v), and 1 U of Phusion DNA polymerase in 1X HF buffer. The PCR cycle conditions were: initial denaturation (98 °C, 2 min), followed by 34 cycles of denaturation (98 °C, 20 sec), annealing (55-70 °C, 20 sec), and extension (72 °C, 2 min (30 sec/1 kb)). The final step is an additional extension at 72 °C for 5 mins. The target PCR product (megaprimer) was purified with a gel extraction kit. The second PCR reaction (50 µL) for RF cloning was performed with 200 ng of megaprimer, 20 ng of pBAD18-HypA plasmid, 200 µM of mixed dNTPs, 2% DMSO (v/v), and 1 U of Phusion DNA polymerase in 1X HF buffer. The PCR cycle conditions were: initial denaturation (98 °C, 2 min), followed by 17 cycles of denaturation (98 °C, 20 sec), annealing (55-70 °C, 20 sec), and extension (72 °C, 8 min (30 sec/1 kb)). The final step is an additional extension at 72 °C for 5 mins. The PCR product was treated with 1 µL of DpnI at 37 °C for 1 hour and transformed into NEB Turbo cells.
Exponential Megapriming PCR is similar to the RF method. The key difference is that the megaprimer consisted only of a 3’ end overhang and the second PCR step consisted of the megaprimer and an additional reverse primer to exponentially amplify the plasmid. Two sets of primers were used for different destination vectors. The primers used for the pBAD18-HypA plasmid consisted for forward primer 5’ggcagccatatggctagcatgactg3’, reverse primer 5’ATGCCTGCAGTCACTCTAGATTAAactcgagcggccgcttttagcagc3’, and 2nd reverse primer 5’catGGTGAATTCCCTCTGCTAGCCC3’. The primers used for the empty pBAD24 plasmid consisted of forward primer 5’atggctagcatgactgtgacagc3’, reverse primer 5’ACTCTAGAGGATCCCGGTACCACATcatagtgcgtgatggtgatgg3’, and 2nd reverse primer 5’GGTGAATTCCCTCTGCTAGCCC3’. The first PCR reaction (50 µL) was performed with 25 ng of pET28a-CerL9Cit-2His plasmid, 0.5 µM of both primers, 200 µM of mixed dNTPs, 1 U of Phusion DNA polymerase, and optional 2% DMSO (v/v) in 1X HF buffer. The PCR cycle conditions were: initial denaturation (98 °C, 30 sec), followed by 25 cycles of denaturation (98 °C, 10 sec), annealing (55-70 °C, 20 sec), and extension (72 °C, 2 min (30 sec/ 1 kb)). The final step is an additional extension at 72 °C for 5 mins. The target PCR product (megaprimer) was purified with a gel extraction kit. The second PCR reaction (50 µL) for EMP cloning was performed with 25 ng of megaprimer, 25 ng of desired destination vector, 0.5 µM of the appropriate 2nd reverse primer, 200 µM of mixed dNTPs, 1 U of Phusion DNA polymerase, and optional 2% DMSO (v/v) in 1X HF buffer. The second PCR cycle conditions were: initial denaturation (98 °C, 30 sec), followed by 25 cycles of denaturation (98 °C, 10 sec), annealing (55-70 °C, 20 sec), and extension (72 °C, 8 min (30 sec/ 1 kb)). The PCR product was phosphorylated with T4 PNK and ligated with Quick Ligase.
in vitro. The product was then treated with 1 µL of DpnI at 37 °C for 1 hour and transformed into NEB Turbo cells.

Restriction cloning is the classical method of molecular cloning which involves the use of restriction enzymes and restriction cut sites. The first step was identifying the restriction enzymes to be used: Ncol and Xbal. The restriction enzyme cut sites are not endogenous to the target gene and must be inserted via PCR cloning. The forward primer 5’TAAGCACCATGGatggctagcatgactggtggacagc3’ and reverse primer 5’TGCTTATCTAGAttagtcgacgtgatgatggtgatgg3’ were used to insert the restriction cut sites. The PCR reaction (50 µL) was performed with 40 ng of pET28a-CerL9Cit-2His plasmid, 1 µM of each primer, 250 µM of mixed dNTPs, 10% DMSO (v/v), and 1U of Phusion DNA polymerase in 1x HF buffer. The PCR cycle conditions were: initial denaturation (98 °C, 30 sec), followed by 25 cycles of denaturation (98 °C, 20 sec), annealing (55-70 °C, 20 sec), and extension (72 °C, 2 min (30 sec/1 kb)). The final step is an additional extension at 72 °C for 5 mins. The PCR product was purified with either PCR purification kit or a gel extraction kit. The purified PCR product and the target destination pBAD24 plasmid were cut with the 2 restriction enzymes in a double digest reaction. The double digest of the PCR product (50 µL) consisted of 40 µL of PCR product and 1 U of each restriction enzyme Ncol and Xbal in the 1x cutsmart buffer. The plasmid double digest reaction (30 µL) consisted of 2 µg of empty pBAD24 and 1 U of each restriction enzyme in the 1x cutsmart buffer. Both reactions were incubated at 37 °C for 1 hour. To prevent the plasmid from ligating, 1 µL of CIP was added to the plasmid reaction and incubated at 37 °C for 1 hour. The desired cut product was purified using either a PCR clean-up kit or a gel extraction kit. Varying amounts of
cut PCR product were incubated with the cut plasmid and quick ligase. Following ligation, the DNA was transformed into NEB Turbo *E. coli* cells.

Gibson assembly is a molecular cloning method that allows for the joining of multiple DNA segments into one product through a single reaction mixture. This method was used to separate the DNA sequence into 3 segments: Cerulean fluorophore, L9 linker, and Citrine fluorophore. The first segment Cerulean had a 5’ end overhang that complemented the Ncol site in the pBAD24 and was synthesized with PCR using the forward primer 5’ GTTTTTTGCGCGCTAGGAGGATCGACCATTCCATGGCTAGCATGACTGTGGG3’ and reverse primer 5’ cctcgaactgtacagctc3’. The L9 linker segment had a 5’ end overhang that complemented the 3’ end of the cerulean segment and a 3’ end overhang that complemented the 5’ end of the citrine segment. The L9 segment was synthesized with the forward primer 5’ gagctgtacaagttcgagccgcatccggcgaagcgccgcg3’ and reverse primer 5’tcctgcccttgctcaccataagacaccagacaccacccgg3’. The cerulean segment had a 3’ end overhang that complemented the XbaI cut 3’ end of pBAD24 and is synthesized with PCR cloning using the forward primer 5’ctggtggttctatggtgagc3’ and the reverse primer 5’TGCCGTGCAGTGACTCTAGAAttagctgcagtatgtgtgtggtcc3’. The PCR reactions (50 µL) for all segments were the same and contained 40 ng of pET28a-CerL9Cit-2His plasmid, 1 µM of each primer, 250 µM of mixed dNTPs, 10% DMSO (v/v), and 1U of Phusion DNA polymerase in 1x HF buffer. The PCR cycle conditions were: initial denaturation (98 °C, 30 sec), followed by 25 cycles of denaturation (98 °C, 20 sec), annealing (55-70 °C, 20 sec), and extension (72 °C, 1 min (30 sec/
The final step is an additional extension at 72 °C for 5 mins. The PCR products were purified with a PCR clean-up kit and analyzed on a 1.5% agarose gel.

Colon PCR was used to determine if the insert was incorporated into the plasmid. The primers used for the reactions were the same primers used to synthesize the gene insert. The PCR reactions (50 µL) were prepared with 0.1 µM of each primer, 100 µM of mixed dNTPs, and 1U of OneTaq Hot Start DNA polymerase in 1x OneTaq Standard Reaction buffer. A portion of a single colony was inoculated in each reaction mixture using the end of a pipette tip. The PCR cycle conditions were: initial denaturation and cell lysis (95 °C, 10 min), followed by 35 cycles of denaturation (95 °C, 1 min), annealing (58 °C, 1.5 min), and extension (72 °C, 5 min). The final step is an additional extension at 72 °C for 5 mins. The PCR products were then analyzed on an agarose gel.

Analytical Size Exclusion Chromatography

Samples containing 90 µM of Cer-L9-Cit-2His protein were treated with one equivalent of metal or 3x nickel and 5x zinc, incubated overnight at 4 °C. All samples were prepared in Chelex® treated buffer consisting of 25 mM HEPES and 100 mM KCl (pH 7.6) and the Superdex 200 10/300 analytical gel filtration column (GE Healthcare) was equilibrated with the same filtered buffer. The column was calibrated with the Bio-Rad protein standard containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.4 kDa). The nickel-bound, zinc-bound, and apo-proteins were loaded onto the column in 100 µL sample sizes.
The elution profiles of the apo-protein and metal-bound proteins were compared to determine any changes between the different states of the protein.

3. Results and Discussion

3.1 Protein Purification and Initial Characterization

The pET28a Cer-L9-Cit-2His plasmid was successfully transformed into *E. coli* BL21(DE3) and induced for protein expression. The initial protein consisted of 3 6Xhistidine tags, two at the N-terminus and one at the C-terminus. The histidine tags allowed for the protein to be purified by using Ni-NTA chromatography. The elution step from the Ni-NTA column was simple to perform due to the visible yellow colour of the protein and fractions containing protein were easily identified by eye. The fractions were pooled together and dialyzed overnight with EDTA to remove any metal bound to the protein and to remove the imidazole used in the elution step. The protein was then cleaved with thrombin to remove one of the N-terminal 6XHis-tags, followed by anion exchange chromatography. The HiTrap Q HP anion exchange column served as the final purification step to remove the thrombin and other potential contaminants from the protein sample. The fractions containing protein were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, which displayed fractions containing pure protein (~66 kDa) and fractions with impurities (45 kDa ~ 35 kDa) (Figure 3). The theoretical protein weight was calculated from the translated protein sequence using the ProtParam software of ExPASy and was calculated to be 63022 Da. Based on the theoretical protein weight and the results from the SDS-PAGE, the band visible slightly below the 66.2 kDa molecular weight marker represents the target protein and the bands found between 45 kDa and 35 kDa are
unknown contaminants. The contamination could include thrombin, which has a molecular weight of approximately 37 kDa; however, ESI-MS performed on contaminated fractions did not detect the smaller contaminant and could not verify the molecular weight. An alternative identity of the contaminant is a partially degraded protein or perhaps an unknown protein complex that remains bound to the target protein until SDS denaturation. The pure protein fractions were combined and a sample was sent for ESI-MS analysis. The absorbance peak at 515 nm was used to quantify the protein concentration, with an extinction coefficient of 77000 M⁻¹cm⁻¹. The mass spectrum of the pure protein revealed a high abundance peak of 63023 Da corresponding to the theoretical mass of apo protein (Appendix 5.2).

![Protein Purification Analysis by SDS-PAGE](image)

**Figure 3:** Protein Purification Analysis by SDS-PAGE. SDS-PAGE performed on the fractions obtained from anion exchange column purification. Fractions 42 to 45 indicated a single band at approximately 66 kDa corresponding to the target protein. Fractions 46 to 50 showed two bands with one at 66 kDa and a second set of bands between 45 kDa and 35 kDa.
To determine the amount of metal bound to the purified protein, a PAR colorimetric assay for transition metals was performed. PAR (4-(2-Pyridylazo)resorcinol) is a small molecule dye that exhibits a change of absorbance from 412 nm to 500 nm upon binding a metal ion.\textsuperscript{17,22} Thus, by using PAR and a set of samples containing known metal concentrations, a linear standard calibration curve can be created that correlates the absorbance measured at 500 nm with the concentration of transition metal. The absorbance measured at 500 nm of a protein sample with PAR can then be fitted to the calibration curve to calculate the metal concentration in the protein. However, there exists a complication with the Cer-L9-Cit-2His protein, which is due to the yellow fluorescent protein, citrine. Citrine has a maximum absorbance wavelength at 515 nm and this peak interferes with measurements of the 500 nm absorbance of PAR. To correct for the inherent protein absorbance at 500 nm, a blank protein sample was prepared without PAR and the blank sample’s absorbance was subtracted from the PAR sample. The PAR samples had an average absorbance at 500 nm of 0.37 and the protein blank’s absorbance was 0.31. Thus, the corrected value for the protein samples resulted in an average absorbance reading of 0.06 and was used to determine the metal concentration from the standard curve, resulting in a zero value for the metal bound to the protein. This indicated that the purified protein was in its apo form.

3.2 FRET Metal Titration and Stoichiometry

The fluorescence of the protein and its ability to report on zinc or nickel loading was determined through metal titration experiments. As stated previously, the protein contains six-histidine metal-binding regions on both the N-terminus and the C-terminus, as well as two fluorescent domains, cerulean and citrine. The binding of metal to the two histidine tag will induce a change
in the protein structure and brings the two fluorescent domains closer in proximity if the metal bridges both tags. The decreased distance between the fluorescent domains would result in a greater FRET ratio. The initial experiments were performed with 2 μM of protein and metal concentrations ranging from 0 to 50 μM. Then the fluorescence spectra of each sample were measured after exciting at 434 nm light, which corresponds to the excitation wavelength of cerulean. The ratio between the fluorescence intensities of citrine (525 nm) and cerulean (475 nm) was used to determine the FRET ratio of each sample. The FRET ratios denoted by \( R \) were then normalized by subtracting the lowest ratio, \( R_{\text{min}} \) from each calculated ratio. The full fluorescence spectra of the samples at 4 different metal concentrations, 0 μM, 2 μM, 4 μM, and 10 μM were also analyzed to characterize the change in fluorescence upon metal binding (Figure 4). The zinc spectra showed that as the concentration of metal increases, the fluorescence intensity at 475 nm decreases and the intensity at 525 nm increases. A similar trend was observed with the nickel titration spectra and the trend is consistent with an increasing FRET ratio between citrine and cerulean. The results showed that metal addition to the protein caused an increase in the FRET ratio between the two fluorescent domains and this could be attributed to the decrease in distance between the two fluorophores.
Figure 4: Fluorescence Response. The fluorescence spectra of the protein in response to different concentrations of nickel and zinc are shown. For both metals, as the concentration increases the fluorescence intensity of the donor fluorophore decreases and the acceptor fluorophore’s emission increases.

To determine the stoichiometries of zinc and nickel binding to Cer-L9-Cit-2His, a PAR assay was performed. The stoichiometry binding experiments were performed with protein treated with 10x zinc or nickel and equilibrated overnight at 4 °C. After incubation, the samples were loaded onto a PD10 column to remove unbound metal and the protein concentration was determined with UV-visible spectroscopy. The metal concentration of each sample was determined with the PAR assay. Triplicates of the zinc stoichiometry experiments demonstrated a protein to zinc ratio of 1 to 3.1 ± 0.2. Triplicates of the nickel stoichiometry experiments demonstrated protein to nickel ratio of 1 to 1.9 ± 0.2.

Table 1: Metal-binding Stoichiometry

<table>
<thead>
<tr>
<th>Sample</th>
<th>[Protein]:[Metal]</th>
<th>$R_{\text{max}}$</th>
<th>$R_{\text{min}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Zinc</td>
<td>1:3.1 ± 0.2</td>
<td>3.57 ± 0.1</td>
<td>1.17 ± 0.01</td>
</tr>
<tr>
<td>10x Nickel</td>
<td>1:1.9 ± 0.2</td>
<td>2.17 ± 0.08</td>
<td>1.17 ± 0.01</td>
</tr>
</tbody>
</table>
The first attempt to determine the dissociation constant for zinc was by using a direct zinc titration performed with 2 μM CerL9Cit-2His protein. First, the FRET ratio response was converted to fraction bound and the concentration of free zinc was calculated. The $R_{\text{max}}$ corresponding to full saturation was 4.52 and the $R_{\text{min}}$ corresponding to apo-protein was 1.17. The data were then fitted to a Hill equation with a 3:1 binding model and resulted in a Hill coefficient of 4.5 ± 0.3 and a dissociation constant of 2.4 ± 0.1 μM (Figure 5). Given that 2 μM protein was used for these experiments, it is likely that there is a large error associated with the calculated dissociation constants. The results differ greatly compared to the unpublished experiments previously performed on the protein. In the unpublished experiments, the dissociation constant for zinc was determined with a competition assay at two different pH values, pH 7.1 and pH 8.0. At pH 8.0, the data were fitted to a 1:1 binding model with a $K_d$ of 17 ± 2 nM. At pH 7.1, the data were fitted to a 2:1 binding model of zinc to protein, with a $K_d$ of 33 ± 2 nM. The unpublished results suggests that the protein has a high affinity for zinc and would require a competition assay to accurately calculate the dissociation constant.
Figure 5: Zinc Titration. The binding curve for zinc titration performed on 2 μM protein. The FRET ratio was converted to fraction bound and the free zinc concentration was calculated based on 3:1 zinc to protein binding model. The fraction bound was plotted against the free zinc concentration and fitted with a Hill equation with a Hill coefficient of 4.5 ± 0.3. The dissociation constant was calculated to be 2.4 ± 0.1 μM with Prism®.

The affinity for nickel was estimated with the data obtained from the nickel titration experiment performed on CerL9Cit-2His. The $R_{\text{max}}$ corresponding to full saturation was 2.51 and the $R_{\text{min}}$ corresponding to apo was 1.17. The converted nickel titration data were analyzed with a 2:1 binding model and resulted in a Hill coefficient of 6.1 ± 0.2 and a dissociation constant of 1.8 ± 0.2 μM (Figure 6). The Hill coefficient for nickel binding is greater than the Hill coefficient for zinc and the affinity of the protein for nickel was determined to be similar to zinc; however, since the $K_d$ determined for zinc differed from the unpublished results, a competition assay may be required to determine the $K_d$ of binding for both zinc and nickel. In addition, the amount of total metal added for both titrations suggest that there is close to quantitative binding. Therefore, the
$K_d$ determined from the metal titration experiments would not be an accurate representation for the protein’s affinity for either nickel or zinc.

![Figure 6: Nickel Titration](image)

**Figure 6**: Nickel Titration. The binding curve for nickel titration performed on 2 μM CerL9Cit-2His. The fraction bound was plotted against the free nickel concentration and fitted with a Hill equation with a Hill coefficient of 6.1 ± 0.2. The dissociation constant was calculated to be 1.8 ± 0.2 μM with Prism®.

### 3.3 Metal Competition Assay

The first competition assay was performed by using EDTA as a competitor for both nickel and zinc. The free metal concentrations were calculated using the MaxChelator program. The zinc competition data were then fitted to a Hill equation with a 1:3 binding model and resulted in a Hill coefficient of 1.3 ± 0.6 and a dissociation constant of 81.3 ± 1.3 nM (Figure 7). The $R_{\text{max}}$ was 4.51 and the $R_{\text{min}}$ was 1.17, which are similar to the values from the direct zinc titration experiments. The calculated dissociation constant was very similar to the reported $K_d$ of 33 ± 2 nM for the protein in a 2:1 binding model for zinc to protein (pH 7.1).
The nickel competition data were fit to the Hill equation with a 1:2 binding model and resulted in a Hill coefficient of 4.1 and a dissociation constant of 28.7 pM. The $R_{\text{max}}$ is 2.49 and the $R_{\text{min}}$ is 1.16, which are the same values observed during the direct nickel titration experiments. The calculated dissociation constant for nickel to protein suggests that nickel binds extremely tightly, even tighter than zinc; however, there are no previous studies reported on this protein binding to nickel. Moreover, the repetition of the same nickel EDTA competition experiments provided inconsistent results. Therefore, further competition experiments were required to accurately determine the binding affinity of nickel to the protein.

Three different metal competitors were used: EGTA, NTA, and imidazole. The fluorescence response from the nickel competition samples with EGTA and NTA were abnormal. For the initial few samples with increasing nickel concentration, the FRET response from the
protein decreased past the $R_{\text{min}}$ for apo protein. In the EGTA samples, the FRET response started at the initial 1.17 and dropped to a FRET response of 1.05 (Figure 8). The FRET response for the nickel NTA competition samples also started at 1.17, but dropped to a FRET response of 1.05 with increasing nickel concentrations (Figure 9). The reason for the decreasing FRET response with increasing nickel concentration is unknown; however, one hypothesis is that the decrease in FRET may be due to fluorescence quenching by nickel bound to a competitor. Nickel can quench fluorescence using photo-induced electron transfer and this could be a reason why the maximum FRET ratio observed in the nickel titrations are lower than the zinc titrations.\textsuperscript{24,25} In these competition experiments, the nickel bound to the competitor does not elicit an increase in FRET, but it does quench fluorescence.

**Figure 8:** Nickel EGTA Competition. The binding curve for nickel competition performed with 2 μM CerL9Cit-2His and 1 mM EGTA. The FRET ratio decreases as the free nickel concentration increases. Free nickel concentrations were calculated based on nickel bound to competitor.
Figure 9: Nickel NTA Competition. The binding data for nickel competition performed with 2 μM CerL9Cit-2His and 1 mM NTA. Similar to the EGTA competition, the FRET ratio decreased with increasing nickel. Free nickel concentrations were calculated based on nickel bound to competitor.

The nickel imidazole competition experiment did not reveal a decrease in the FRET response but rather responded normally to the increasing nickel concentration. The competition data were then fitted to a Hill equation with a Hill coefficient of 4 and a dissociation constant of 2.0 μM (Figure 10). The results from the competition with imidazole and nickel resembled the quantitative binding observed in the direct nickel titrations. The dissociation constant determined was not significantly different from the concentration of protein used in the competition experiment. This meant that the protein is outcompeting imidazole for nickel binding and has a lower dissociation constant than imidazole’s dissociation constant for nickel of 9.8x10^{-4} M.\textsuperscript{19} Since the protein out competes imidazole for nickel binding, a stronger competitor is required to determine nickel binding.
Figure 10: Nickel Imidazole Competition. Nickel competition with 1 mM imidazole and 2 µM CerL9Cit-2His. The fraction bound was plotted against the free nickel concentration and fitted with a Hill equation with a Hill coefficient of 4. The dissociation constant was calculated to be 2.0 µM with Prism®.

The stronger competitor used was Mag-Fura-2 (MF2), a fluorescent dye-based indicator that is generally used to quantify calcium and magnesium; however, MF2 can also be used as an indicator for other divalent metals, such as nickel and zinc. The dissociation constant of MF2 for zinc is 20 nM and the dissociation constant for nickel is 150 nM. Metal binding to MF2 can be monitored by using two methods: absorption spectroscopy and fluorescence spectroscopy. Utilizing absorption spectroscopy, metal binding to MF2 would result in a blue-shift of the absorbance from 366 nm to 325 nm. Utilizing fluorescence spectroscopy, metal binding to MF2 would result in a decrease in fluorescence emission at 500 nm using an excitation of 366 nm. In place of monitoring the FRET response of the protein, the change in MF2 response was used to determine the dissociation constant of nickel to the protein. The competition data were analyzed...
with DYNAFIT using a binding equilibria equation to determine the dissociation constant of the protein with nickel. The program reported an extremely small value for the dissociation constant with a high coefficient of variation. This suggests that the protein is outcompeting MF2 to bind nickel, which can be seen by overlapping the competition data with the standard MF2 nickel titration data (Figure 11). A 4 µM nickel offset was introduced to the competition data to account for 2:1 nickel to protein binding. This offset competition data perfectly aligns with the titration of MF2 alone, which further supports the theory that the protein is outcompeting MF2 for nickel and binds nickel with a 2:1 stoichiometry.

![Graph](image1.png)

**Figure 11:** Nickel Competition With 2 µM MF2. Superimposition of the MF2 nickel titration data in absence of protein and the MF2 competition data with a 4 µM nickel offset. The 4 µM nickel offset in the competition data is to account for the nickel tightly bound to the protein with a stoichiometry of 2:1 nickel to protein.

Another MF2 competition experiment was performed with a higher concentration of MF2 to increase the competitive environment for the protein. The samples consisted of 10 µM MF2 and the same 2 µM protein with varying concentrations of nickel. The decreased absorbance values at 366 nm in the competition samples were converted to fraction of bound MF2 and were
plotted against total nickel. Again, the competition data were superimposed onto the control MF2 nickel titration data with an offset nickel concentration (Figure 12). The offset nickel in this experiment was 2 µM, which only corresponds to a 1:1 protein-nickel complex. The nickel offset using a 1:1 binding model provided a better fit with the control titration compared to the offset of a 2:1 nickel to protein model. The difference between the two MF2 competition experiments could be due to the increased MF2 concentrations; however, this does not explain the complete disappearance of one nickel binding site. Another reason for the difference could be due to the different method used to monitor the change in MF2 binding. Further experiments would be required to determine the reason for the difference, but one similarity is the presence of the one tight nickel binding site. Thus, the protein must contain a minimum of one nickel binding site with greater affinity than MF2.

**Figure 12:** Nickel Competition With 10 µM MF2. Superimposition of the 10 µM MF2 nickel titration data and the 10 µM MF2 competition data with a 2 µM Nickel offset. The 2 µM nickel offset in the competition data is to account for the nickel tightly bound to the protein with a stoichiometry of 1:1 nickel to protein.
3.4 Analytical Size Exclusion Chromatography

One issue with the binding of metals to the protein is the possible formation of intermolecular bonds rather than the proposed intramolecular binding between two His tags on the same protein. Analytical size exclusion chromatography (SEC) was used to determine if any oligomers form during metal binding or any tertiary changes. A protein standard was first used to calibrate the column and create a calibration curve that corresponded the elution volume with the molecular weight. The first protein sample analyzed was apo Cer-L9-Cit-2His, with a protein theoretical molecular weight of 63 kDa. The chromatogram had a single peak corresponding to approximately 73 kDa (Figure 13). The difference in the calculated molecular weight from the SEC and the theoretical molecular weight is attributed to the low resolution of the SEC column.

Figure 13: Apo Protein SEC Chromatogram. Apo protein was loaded onto the analytical SEC column and the chromatogram displayed one single peak with a molecular weight similar to the theoretical molecular weight.
The subsequent protein sample was treated with 1 equivalent of zinc and the chromatogram again had one elution peak (Figure 14). The peak corresponded to monomeric protein with a molecular weight of 83 kDa. In contrast, when a protein sample with one equivalent of nickel was run through the column, the chromatogram contained multiple peaks indicative of oligomerization (Figure 15). The monomer protein peak was visible in the 1x nickel sample with a lower absorbance, which implied a decrease in the monomeric protein concentration. The 2 distinguishable peaks that eluted at earlier volumes corresponded to approximately 95 kDa and 171 kDa. This suggests that some of the protein may be forming dimers or trimers upon one equivalent of nickel binding.

Figure 14: Zinc SEC Chromatogram. A protein sample was treated with one equivalent of zinc and loaded onto the analytical SEC column. The chromatogram shows that the protein elutes with one single peak, similar to the apo protein elution profile.
Figure 15: Nickel SEC Chromatogram. A protein sample was treated with one equivalent of nickel and loaded onto the analytical SEC column. The chromatogram shows the protein elutes with multiple peaks. This indicates the formation of oligomers upon nickel binding.

Additional experiments were performed with the protein incubated with higher amounts of nickel or zinc. For the zinc samples, when the protein was treated with 3 equivalents of zinc, the chromatogram displayed two peaks (Figure 16). One peak corresponded to monomeric protein with an approximate size of 78 kDa and the earlier peak corresponded to oligomerization of the protein with an approximate size of 152 kDa. The nickel protein sample was treated with 2 equivalents of nickel and the chromatogram also displayed two peaks (Figure 17). Both peaks eluted at an earlier volume compared to monomeric protein, the two peaks corresponded to approximately 278 kDa and 117 kDa. These results support the formation of protein oligomers upon the binding of nickel or zinc. The formation of an intermolecular bond between the proteins upon metal binding could be a reason for the difficulty in determining the nickel-binding affinity.
Figure 16: Stoichiometric Zinc SEC Chromatogram. The protein was treated with 3 equivalents of zinc and loaded onto the analytical SEC. An earlier peak compared to the monomeric peak had eluted and indicated a larger size complex.

Figure 17: Stoichiometric Nickel SEC Chromatogram. The protein was treated with 2 equivalents of nickel and loaded onto the analytical SEC. Two peaks are observed indicative of protein oligomerization.
3.5 Whole Cell Fluorescence

To determine the protein’s efficacy in-vivo, a whole cell fluorescence experiment with *E. coli* BL21(DE3) was performed. The whole cells used in these experiments were newly transformed *E. coli* BL21(DE3) cells that were induced with IPTG to express the protein overnight, in order to adhere to two conditions. The first condition was to follow the same protein expression protocol performed for the in-vitro experiments and the second condition was to ensure that the fluorescent proteins were properly folded and functional. Fluorescent proteins require the completion of post-translational reactions to operate as properly functioning fluorophores and this process is known as chromophore maturation.\(^{27-29}\) For green fluorescent proteins and derivatives, the maturation step involves folding, cyclization, oxidation, and dehydration. Different fluorescent protein would require different amount of time to mature into functional chromophores. Thus, the overnight period was to ensure that all expressed proteins contained fully matured chromophores. After the overnight induction, the cells were diluted to an OD600 of approximately 0.4, this dilution step was performed to normalize the amount of *E. coli* cells in all samples. The diluted culture was then divided into aliquots and treated with metal concentrations from 0-3 mM for both nickel and zinc. An additional aliquot was treated with 1 mM EDTA to see how it would affect the cells metal uptake and protein response. The aliquots were incubated at room temperature for 2 hours and the OD600 of each sample was read prior to fluorescence measurement. There was no change in the OD600 of the aliquots after the 2 hour incubation period. After optical density measurements, the fluorescence of the whole cell samples was monitored with an excitation wavelength of 434 nm. In preliminary whole cell experiments, three time points were used: 0 hours, 2 hours, and overnight; however, the initial
time points were not sufficient incubation times and the last two time points resulted in similar FRET responses. Therefore, the incubation time chosen for these experiments was 2 hours at room temperature. The FRET ratios for the whole cell samples were plotted on a bar graph for EDTA, nickel, and zinc (Figure 18). The FRET ratio observed in the untreated cells was 1.9 and this was the same value as the aliquot treated with 1 mM EDTA. This suggests that treatment with 1 mM EDTA does not chelate the intracellular metal within the cells. The untreated cells FRET values are also different compared to the in vitro untreated protein FRET value of 1.17. This difference suggests the proteins in the untreated cells are partially loaded with metal. There was no change to the FRET ratio for samples that were treated with 0.5 mM of either nickel or zinc. This suggests 0.5 mM of metal in the environment does not significantly change the intracellular metal concentration of these E. coli cells. Upon treatment with 1 mM zinc or nickel, the FRET ratio increased with zinc samples reaching a FRET ratio of 2.6 and nickel samples reaching a FRET ratio of 2.4. The cells treated with 2 mM metal had a FRET ratio for both metals of 2.9, but the cells treated with 3 mM showed a difference between nickel and zinc. The cells treated with 3 mM zinc exhibited a slight increase, while cells treated with 3 mM nickel exhibited a decrease in FRET ratio. The reason for this decrease may be due to the fluorescence quenching properties of nickel or due to nickel toxicity. To correlate the FRET results of whole cell assay to metal concentration, ICP-MS was used to quantify the total intracellular metal concentrations.
Figure 18: Whole Cell Fluorescence of *E. coli* in LB media. The OD600 of each sample was 0.4. Cell samples were treated with different concentrations of nickel, zinc, or EDTA. The FRET ratio only changes upon treatment with metal concentrations greater than 1 mM and treatment with 1 mM EDTA resulted in a FRET ratio similar to the untreated cells.

3.6 Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the total nickel and zinc amounts in the whole cell assay samples. The preparation of the samples followed the same procedures as the whole cell fluorescent assay, except the minimum sample volume was 2 mL. This volume is due to the parameters set for the autosampler and the iCAP Q ICP-MS instrument that is used for these experiments. Three in-line internal standards were chosen as reference values for the samples and standards. Germanium-73 and scandium-45 were chosen as internal standards because they flank the first row transition metals. Indium-115 was also chosen due to its high stability, abundance and common usage as an internal standard. The intracellular nickel and zinc concentrations were calculated using the approximation that an
OD600 of 1.0 for *E. coli* corresponds to $8 \times 10^8$ cells/mL. Another approximation used was that the volume of each *E. coli* cell is $7 \times 10^{-16}$ L. Utilizing the volume of each cell and number of cells, the total intercellular volume for each sample could be calculated. The intercellular concentrations of nickel and zinc for the whole cell samples were calculated as summarized in Table 2. *E. coli* BL21(DE3) grown in LB media without any treatment of metal has an intercellular zinc concentration of $329 \pm 100$ µM and a nickel concentration of $55 \pm 10$ µM. Zinc supplementation of media resulted in an increase in the total zinc concentration and a decrease in the nickel concentration. For the nickel treated cell samples, increasing the nickel concentration in media resulted in an increase in cellular nickel concentration and the zinc concentration varied. The 1 mM EDTA treated sample did slightly affect the cellular metal concentration, the zinc increased and the nickel decreased. The change in the concentrations of both metals for the EDTA-treated sample and the 0.5 mM zinc sample are similar, but neither samples elicited a change in the protein FRET ratio compared to the untreated cell sample. The whole cell fluorescence data and the ICP-MS data show that treatment of *E. coli* with either zinc or nickel resulted in an increase in FRET and an increase in the respective metal uptake. However, these experiments are performed with proteins with an additional N-terminal His tag and an *E. coli* strain with deficient nickel uptake and homeostasis. Therefore, further experiments with whole cells were postponed until the gene was cloned into the pBAD24 plasmid so it could be transformed into different strains of *E. coli*. 
Table 2: Whole Cell ICP-MS Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nickel (μM)</th>
<th>Zinc (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>0</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>0 Metal</td>
<td>55 ± 10</td>
<td>330 ± 100</td>
</tr>
<tr>
<td>0.5 mM Zinc</td>
<td>12 ± 4</td>
<td>690 ± 80</td>
</tr>
<tr>
<td>1 mM Zinc</td>
<td>7 ± 2</td>
<td>940 ± 100</td>
</tr>
<tr>
<td>2 mM Zinc</td>
<td>7 ± 2</td>
<td>2200 ± 300</td>
</tr>
<tr>
<td>3 mM Zinc</td>
<td>4.9 ± 0.8</td>
<td>1300 ± 200</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>9 ± 1</td>
<td>580 ± 100</td>
</tr>
<tr>
<td>0.5 mM Nickel</td>
<td>160 ± 20</td>
<td>171 ± 20</td>
</tr>
<tr>
<td>1 mM Nickel</td>
<td>510 ± 30</td>
<td>350 ± 90</td>
</tr>
<tr>
<td>2 mM Nickel</td>
<td>1200 ± 100</td>
<td>180 ± 30</td>
</tr>
<tr>
<td>3 mM Nickel</td>
<td>1800 ± 200</td>
<td>290 ± 100</td>
</tr>
</tbody>
</table>

3.7 Molecular Cloning

To proceed onwards with the project the gene must first be cloned into a different plasmid so that it can be expressed in different *E. coli* strains and have the additional N-terminal His tag removed. Four different cloning techniques were attempted to clone the gene into an empty pBAD24 vector. Restriction-free cloning, Exponential Megaprimer PCR cloning, traditional restriction enzyme cloning, and Gibson assembly method all had issues with cloning CerL9Cit-2His gene into the target vector pBAD24. The gene with a size of 1826 bp was successfully amplified using all the methods; however, insertion of the cloned gene into the
target vector was unsuccessful. In addition, a second product with a smaller size between 1000-750 bp was routinely produced (Figure 19).

![Figure 19: PCR Product Agarose Gel. The PCR products from the restriction cloning method were analyzed on a 0.7% agarose gel. Samples are labelled by the annealing temperature used during the PCR reaction. Clear products in each reaction were identified and correspond to the desired size of 1800 base pairs. A smaller PCR product at approximately 850 base pairs was also observed.](image)

Only the smaller PCR product was inserted into the pBAD24, as observed by colony PCR (Figure 20). The primers used for colony PCR were the same forward and reverse primers used in the PCR reaction to amplify the gene. One of the colonies with the smaller insert was chosen for a plasmid miniprep and the DNA was sent for sequencing at ACGT. The sequencing revealed that the smaller gene aligned perfectly with the second fluorophore citrine; however, the second fluorophore should not have been amplified alone because the forward primer was designed to anneal to the region between the two N-terminal His tags. This suggests that there may be an issue with tertiary structure in the gene. This could be due to the high similarity between the two
fluorophores and a number of repeats in the protein gene sequence. To test this theory, the
target PCR product 1826 bp was purified with gel extraction and a PCR reaction was performed
with the PCR product as the template. The same primers were used as well as the same thermal
cycling conditions, but the resulting PCR products only consisted of the smaller insert (1000 bp ~
750 bp) (data not shown). This result supports the theory that there is a tertiary DNA structure
formation in the target gene preventing it from being inserted into target vectors. A reason why
the target gene could be amplified during the first PCR reaction is that any tertiary structure is
minimized when the gene is flanked by the rest of the plasmid. The last method used to
accomplish this task was to outsource the work to a company for gene synthesis.

Figure 20: Colony PCR. The colonies from NEB turbo E. coli cells transformed with the ligated
pBAD24 PCR product were analyzed on a 0.7% agarose gel. Colonies were screened and none
contained the desired insert of 1800 base pairs, but some colonies have the smaller 850 base
pair insert.
4. Conclusion and Future Work

4.1 Summary

The protein CerL9Cit-2His was successfully expressed and purified. The purified protein was apo and ESI-MS analysis showed the protein has a single high abundance peak at 63023 Da. The stoichiometry experiments for both zinc and nickel consistently measured 3:1 zinc to protein ratios and 2:1 nickel to protein ratios. In the metal titration experiments the protein exhibited a change in FRET ratio when the metal concentrations were increased; however, the dissociation constants determined from these experiments were indicative of quantitative binding. Therefore, competition experiments with EDTA were performed and the zinc competition data were fitted to the Hill equation with a Hill coefficient of 1.3 ± 0.6 and a dissociation constant of 81 ± 1 nM. The nickel competition with EDTA resulted in irreproducible data and the dissociation constant could not be determined. Other competitors (EGTA, NTA, and imidazole) were used in order to determine the nickel affinity, but these competition experiments were unsuccessful. The nickel competition with MF2 did provide a clue that the protein has one nickel binding site tighter than MF2, which has a dissociation constant for nickel of 150 nM. Size exclusion chromatography with the protein revealed oligomerization upon binding of either zinc or nickel. The formation of intermolecular complexes provides an explanation for the difficulty in determining the dissociation constant of nickel binding. The whole cell fluorescence assay was performed with E. coli BL21(DE3) cells and ICP-MS was used to correlate the FRET signal with the total metal concentrations in the cells. The FRET signals of the cells only increased when they were treated with metal concentrations greater than 1 mM of either nickel or zinc. The ICP-MS data showed that cells increased metal uptake in response to an increase in the respective metals in the
environment. Interestingly, cells treated with zinc showed a decrease in total cellular nickel compared to the untreated cells and cells treated with 1 mM EDTA had a slight increase in cellular zinc and decrease in cellular nickel. Future experiments with whole cell fluorescence and ICP-MS are planned when the gene CerL9Cit-2His is cloned into pBAD24. In terms of molecular cloning, 4 different cloning methods were attempted. However, none of the cloning methods were successful and the cloning was outsourced to Bio Basic for their gene synthesis service. The failure in cloning was hypothesized to be due to the formation of tertiary DNA structures preventing the insertion of the gene into the vector.

4.2 Future Work

The protein’s dissociation constant for nickel has yet to be determined. Future experiments would have to be performed to determine the protein binding affinity for nickel. One possible option is to use isothermal titration calorimetry (ITC) to monitor the change in thermal energy when nickel is bound to the protein. As mentioned previously, upon successful cloning of the CerL9Cit-2His gene into pBAD24 more whole cell fluorescence and ICP-MS experiments could be performed. The protein could be transformed into different strains of *E. coli* that have intact nickel uptake system and homeostasis. Additional experiments could also be performed with *E. coli* strains with knockout genes involving metal uptake and homeostasis.

The ultimate goal of my research was to produce an efficient sensor to measure intracellular free nickel concentrations. Therefore, it is important for the sensor to show selectivity towards nickel over other transition metals. To achieve a higher selectivity towards nickel, mutations in the metal-binding domain of Cer-L9-Cit-2His could be introduced. There are
several mutations that could affect the binding of metal. Since the binding domain consists of two 6XHis-tags, many variations of mutations could be introduced. Some examples of proposed mutations include truncating the 6XHis-tags into 3XHis-tags or 2XHis-tags, or a single substitution of a cysteine residue to any of the histidine residues. Other amino acid residues that could be used as a substitution for histidine include glutamic acid and aspartic acid. The desired mutations would elicit a higher selectivity towards nickel and could then be used as an intracellular free nickel sensor.

5. **Appendix**

5.1 Dynafit Script

```plaintext
(task)
data = equilibria

(task = fit)

[mechanism]
M + MF2 <=> M.MF2 : Kd1 dissoc.
M + CLC <=> M.CLC : Kd2 dissoc.
M + M.CLC <=> M2.CLC : Kd3 dissoc.

5.2 Representative ESI-MS Spectrum of Cer-L9-Cit-2His
```
5.3 Cer-L9-Cit-2His DNA Sequence

```
1 M G S S H H H H H H H S G L V P R G S H
2 atgggcagcagccatcactcatcatcactcatcactcactcagcgcggctgtgcgcgcggcagccat
3 M A S M T G G Q Q M G R G S E F H H H H
4 atggctagcactgctggtggacagcaaatgggtcgcggatccgaattccatcatcatcactac
5 H H S G G M V S K G E E L F T G V V P I
6 catcacagcggtggcatggtgagcaagggcgaggagc
tgttcaccggggtggtgcccatc
7 L V E L D G V N D G H K F S V S G E G E
8 ctggtcagctgacgcgcagctgaacgccaacaagttcagctgcgtcgcgagggcgag
9 G D A T Y G K L T L K F I C T T G K L P
10 ggcgatgccacctagcgcagctgacccctgaagtccactctgacccacccaggttaagctgccc
11 V P W P T L V T T L T W G V Q C F A R Y
12 gtcgccctgcccacctctgacccctctgcctgcaattggcctgtgctctgacctccgctac
13 P D H M K Q H D F F K S A M P E G Y V Q
14 cccgaccacatagcagcagcagctcttttcaagtccgcctagccggaaggctagtcag
15 E R T I F K D D G N Y K T R A E V K F
16 gacgcaccatcttttctcaagagcagcgcaactacaagacgaccgggctagttcagtc
17 E G D T L V N R I E L K G I D F K E D G
18 gaggccgacaccttgggtgacccgcatccgagctgagggcgcacctgacccgaggggagcgc
19 N I L G K E Y N A I S D N V Y I T A
20 aacatctggcacaagctgagttaccaacgcctacagcgaacgtctatattacacgccc
21 D K Q K N G I K A N F K I R H N I E D G
22 gacaagcagaaaaggccacactcagccacacagcaggtcggagccgagcccacacagcagggagggcc
22 S V Q L A D H Y Q Q N T P I G D G P V L
```
6. **References**


(18) Hessels, A. M., Chabosseau, P., Bakker, M. H., Engelen, W., Rutter, G. A., Taylor, K. M., and


