Novel bioreactor technology for real time in-cell NMR

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NOVEL BIOREACTOR TECHNOLOGY FOR
REAL TIME IN-CELL NMR

By
Leonard Breindel

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Preface

Parts of this dissertation have been published in peer review journals. The development of the bioreactor and the study of the ribosomal effect on quinary interactions (discussed in chapter 2) was published with the following citation: Breindel, L. M., DeMott, C. M., Burz, D. S., and Shekhtman, A. (2018) Real Time In-cell NMR: Ribosome Targeted Antibiotics Modulate Quinary Protein Interactions, Biochemistry 57, 540-546. Permission was grated to me, the dissertation author, by the American Chemical society to reproduce this publication in it’s entirety for inclusion in printed and electronic copy of this dissertation. In addition the study of the in-cell interaction between Pup and Mpa (discussed in chapter 3) was published with the following citation: Breindel, L., Burz, D. S., and Shekhtman, A. (2020) Active metabolism unmasks functional protein-protein interactions in real time in-cell NMR, Communications Biology 3. Permission was grated to me, the dissertation author, by the Springer Nature to reproduce this publication in it’s entirety for inclusion in printed and electronic copy of this dissertation. I served as the first author and primary research for these projects. These articles were adapted for this dissertation because the reflect the line of research conducted for this dissertation.
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Abstract

Long has it been the goal of biochemists to observe a protein within the cellular environment allowing the true nature of a protein function to be determined within a cell. To understand the nature of a protein within a cell, the role of the cellular environment must be considered when determining the function as a protein. The cellular environment is a crowded place filled with macromolecules such as DNA, RNA and proteins that all interact with one another affecting the function of a protein. Intracellular macromolecules can reach concentrations of 300 g/L and occupy 30% of the available space in a bacteria cell leaving minimal space with in the cells for protein migration. Hydrophobic and electrostatic interactions between macromolecules have been shown to have both positive and negative effects on a proteins function within the cell. These weak, transient interactions that play a function role in the cell have been coined quinary interactions.

Observing these interactions relies on a noninvasive technique that will not disturb the cellular environment. In-cell NMR is such a technique that allows for the protein to be observed within the cellular environment while maintaining the integrity of the cell. Doing so relies on incorporating stable isotopes such as $^{13}\text{C}$, $^{15}\text{N}$ and $^{2}\text{H}$ to aid the isolation of the NMR signal of the target protein from the complex cellular environment. With the addition of transverse relaxation-optimized spectroscopy, TROSY, and cross relaxation-induced polarization transfer, CRIPT, proteins previously too large to be observed by NMR have been able to be observed.
Bioreactor technology has expanded the scope of in-cell NMR studies. We developed a bioreactor that is able to stabilize the metabolism and remove waste during NMR acquisition. Using the bioreactor we investigated the role of quinary interaction. It is not well understood how ribosome-targeted antibiotics affect a wide range of metabolic pathways. Weak functional interactions between a target protein and the cellular environment named quinary interactions, mediated by RNA and proteins inside the crowded cytosol may provide one possible mechanism for this effect. We developed a flow in-cell NMR system that allows us to monitor both the spacial and temporal changes in protein quinary interactions that occur when ribosome antibiotics are added to living cells. We show that only antibiotics binding to the small ribosomal subunit cause a change in the quinary interactions of thioredoxin.

Further we improved upon the bioreactor design in hopes of allowing interactions depend on an active metabolic state to be observed by in-cell NMR. Protein-protein interactions, PPIs, underlie most cellular processes, but many PPIs depend on a particular metabolic state that can only be observed in live, actively metabolizing cells. Real time in-cell NMR spectroscopy, RT-NMR, utilizes a bioreactor to maintain cells in an active metabolic state. Improvements in bioreactor technology maintained ATP levels at >95% for up to 24 hours enabling protein overexpression and a previously undetected interaction between prokaryotic ubiquitin-like protein, Pup, and mycobacterial proteasomal ATPase, Mpa, to be detected. Singular value decomposition, SVD, of the NMR spectra collected over the course of Mpa overexpression easily identified the PPIs despite the large variation in background signals due to the highly active metabolome.
Lastly we applied bioreactor technology to trace metabolic pathways. By means of a pulse-chase experiment, metabolic pathways were traced as $^{13}\text{C}$-glucose was digested by cardiomyocytes. Doing so allowed for the changes in the metabolism of glucose to be observed in the presence and absence of diaphanous homolog 1 (Diaph1). Diaph1 is a member of the Rho family of GTPases that has been hypothesized to slow the TCA cycle and decrease ATP production in the heart.
List of Abbreviations

ADP  Adenosine diphosphate
AMP  Adenosine monophosphate
ATP  Adenosine triphosphate
BSA  Bovine Serum Albumin
CRINEPT  cross-correlated relaxation-enhanced polarization transfer
Diaph1  Diaphanous homolog 1
DMEM  Dulbecco’s modified Eagle’s medium
DTT  Dithiothreitol
EDTA  Ethylenediaminetetraacetic acid
FBS  Fetal bovine serum
GTP  Guanosine triphosphate
HMQC  Heteronuclear multiple-quantum correlation spectroscopy
HSQC  Heteronuclear Single Quantum Coherence
IDP  Intrinsically disordered protein
INEPT  Insensitive nuclei enhanced by polarization transfer
IPTG  IsoPropyl 1-Thio-β-D-Galactopyranoside
LB  Luria-Bertani medium
M9  Minimal medium
Mpa  Mycobacterial proteasomal ATPase
mRNA  messanger RNA
Mtb  Mycobacterium tuberculosis
NADH  Nicotinamide adenine dinucleotide (reduced)
NADPH  Nicotinamide adenine dinucleotide phosphate
NMR  Nuclear Magnetic Resonance
PBS  Phosphate Buffered Saline
PEG  Polyethylene glycol
PPI  Protein-protein interaction
PTFE  Polytetrafluoroethylene
Pup  Prokaryotic Ubiquitin-like Protein
REDPRO  Reduced Proton
shRNA  Short hair turn RNA
SOFAST-HMQS  Selective Optimized-Flip-Angle Short-Transient Heteronuclear Multiple Quantum Coherence
SVD  Singular Value Decomposition
SVs  Singular Values
TCA cycle  The citric acid cycle
Tris  Tris(hydroxymethyl)aminomethane
tRNA  Transfer RNA
TROSY  Transverse relaxation-optimized NMR spectroscopy
Trx  Thioredoxin
βME  Beta-mercaptoethanol
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CHAPTER 1: Introduction

1.1 Molecular Crowding:

The interior of a cell is a complex environment. Macromolecules like DNA, RNA and proteins increase the complexity of the environment within a single cell. With the bulk of space being occupied by macromolecules, the cellular environment affects the behavior of a protein differently from what is observed in aqueous buffers. To understand the influence macromolecules have on any particular protein within the cellular environment, molecular crowding should be considered. Molecular crowding is a general term that describes two different situations within a cell, crowding that results from other macromolecules occupying space and confinement that results from macromolecules conforming to fit within specific areas of a cell. The influence of crowding and confinement dictate the physical space a protein may occupy as well as the shape and resulting activity of a protein in the cellular environment.

Intracellular macromolecules can reach concentrations of 300 g/L and occupy 30% of the available space in a bacteria cell\textsuperscript{1, 2} and can be as high as 200-300 g/L of protein and 75-150 g/L of RNA in mammalian cells\textsuperscript{3}. The term molecular crowding was first used to describe the effect large macromolecules have on equilibria and the kinetics of other macromolecules within an occupied space\textsuperscript{4}. The high concentration of macromolecules has the ability to affect the stability and interactions that a protein experiences within the cellular environment and is an important aspect to consider when conducting structural and interaction studies.
Figure 1.1 The cellular environment is a very crowded place. A illustration of the complex cytoplasm of eukaryotic (left) and E. coli (right). Replicated with permission from ³

Volume exclusion limits the movement through the cellular environment as two macromolecules cannot occupy the same space. Cellular components feel the effect of molecular crowding differently; smaller molecules and metabolites that can easily fit between the macromolecules have more available space to occupy and do not experience crowding. On the other hand, the large hydrated radius of macromolecules strongly influence the diffusion of a protein as the available space is significantly reduced. Considering the influence of volume exclusion has allowed for the characterization of nonspecific interactions⁵ exerted on macromolecules by the cellular environment.

Molecular crowding can be described as the electrostatic and hydrophobic interactions between macromolecules that affect the diffusion of macromolecules in the cellular environment, weak interactions that can stabilize and destabilize a protein⁶, ⁷. Weak interactions, often described as the stickiness of the cellular environment⁸, are the electrostatic, hydrophobic, and hydrogen bonds that a macromolecule experiences as it conforms to the crowded cellular environment.
Figure 1.2 The volume exclusion effect. Small molecules (left) have more room to diffuse though the cellular environment as the available space (Blue) is greater that for macromolecules as two macromolecules cannot occupy the same space. Replicated with permission from

Electrostatic interactions have a significant effect on the diffusion of proteins through the cellular environment. Interactions that are diffusion dependent such as, protein-protein and protein-ligand interactions are limited by the encounter rate in the cellular environment. As molecular crowding in a cell increases, the diffusion of proteins are inhibited due to an increase in electrostatic interactions, both repulsive and attractive. Impeding the movement of a protein within a cell reduces the overall reaction rates because the reactants are less likely to find one another. Minton showed that the crowded environment greatly affects the rate of diffusion, lowering the encounter rate of the reactants. Minton also showed that an enhancement of reaction rates can occur from an increase in active concentrations that results from crowding, promoting the decay from transition state to product, but ultimately decrease as the transition state complex is depleted and the diffusion rate once again governs the kinetic rate.

Confinement of macromolecules has an entropic effect on the folding of proteins in the cellular environment. Because of the limited available space within cells, the cellular environment promotes the folding of a protein that favors the native state of a
protein over the unfolded state.\textsuperscript{7, 9, 12} Volume exclusion encourage proteins to fold into a state that occupies less space in the cell since they have a smaller molecular radius than unfolded proteins allowing folded proteins favored diffusion through the cell.

Mimicking the cellular environment is a complicated task. Although ionic strength, pH, redox potential can be recreated to simulate a cellular environment, molecular crowding is much harder to recreate\textsuperscript{13}. Synthetic polymers such as such as dextran, ficoll, and polyethylene glycol (PEG)\textsuperscript{14} are commonly used crowding agents. The use of crowding agents allows for the influence of volume exclusion to be studied as they can mimic the cellular environment were both size and concentration of the crowding agent can be controlled. However synthetic polymers do not have the ability to induce steric reputation and chemical interactions between macromolecules\textsuperscript{15}. For this cell lysate and protein crowding agents such as bovine serum albumin (BSA) and lysozyme\textsuperscript{1} are used. Although the use of crowding agents have shined light on the role of crowding, they are not able to mimic the unique interactions a protein experiences in the native environment.

Molecular crowding influences the nature of macromolecules in the cellular environment through volume exclusion and weak soft interactions that are vital for the cell’s survival. Some of the interactions that result from molecular crowding affect the function of a protein in the cell. This class of interactions is called quinary interactions and they represent important structural and functional changes to a protein in the cellular environment.
1.2 Quinary interactions:
Molecular crowding plays a significant role in understanding how a protein interacts with the cellular environment. Often interactions between macromolecules alter the function of a protein within a cell. These unique interactions are responsible for the difference between in vitro and in vivo studies of a particular protein. The functional interaction that arises as a result of the sticky cellular environment comprises a class of interactions that are called quinary and can be defined as the weak, transient interactions that can only be studied in the cellular environment. Evidence of quinary interactions can be seen in increase of the apparent molecular weight of proteins in-cell. Proteins with MDa apparent molecular weights in-cell often correspond to kDa proteins in vitro.

Proposed independently by Vainshtein\textsuperscript{16}, Edelstein\textsuperscript{17}, and McConkey\textsuperscript{18}, quinary interactions comprises the fifth level of a protein structure and can best be thought of along the same lines as traditional protein structure. Primary structure of a protein relates to the sequence of amino acids that constitute a protein, secondary structures are the $\alpha$-helices and $\beta$-sheets that form as a result of hydrogen bonding and repulsive forces. Tertiary structure is the functional shape that a protein takes when forming domains, and quaternary structure refers to the orientation of multiple monomeric units or subunits of a multimeric protein with respect to one another\textsuperscript{19}. Quinary structure describes an altered quaternary form of a protein as it interacts with the cellular environment. Quinary structure may alter the functional properties of the free quaternary state.
**Figure 1.3 Different levels of structure of a protein.** A. Primary structure consists of the amide backbone. B. Secondary structure are the α-helices and β-sheets that form by hydrogen bonding. C. Tertiary structure is the fully folded protein. D. Quaternary structure is comprised of several subunits to form a larger macromolecule. E. Quinary structure is a result of functional interactions within the cellular environment. Replicated with permission from 8

While Vainshtein\(^6\) and Edelstein\(^7\) used the term quinary structure to describe the aggregation of proteins within the cellular environment, forming functional structures such as hemoglobin S fibers found in sickle cell anemia\(^7\), as well as protein aggregates, chromosomes and the formation of protein-RNA complexes such as ribosomes\(^6\). Vainshtein and Edelstein’s definition does not describe influence of the crowded environment on a protein. McConkey’s definition of quinary interactions, on the other hand, described the conservation of the function of a protein as evolutionary changes occur. McConkey describes quinary structure as stabilizing interactions between macromolecules that conserve the function of a protein\(^8\). The stabilizing effect, found in homologous proteins when comparing HeLa cells to Chinese hamster ovary (CHO) cells, lead McConkey to propose that quinary interactions between
proteins in a common ancestor preserved function as evolution separated the two species.

To be considered a quinary interaction three requirements must be met: First the interaction must have low affinity, second the kinetics of association and dissociation must be rapid, and third the interaction should have some functionality. Having low affinity, \( i.e. \) \( K_d \) values >1 \( \mu M \), and low free energy barriers that allow for the transition between the bound and free states to be rapid allows quinary interactions to be transient as neither state of the protein is thermodynamically favorable.

Ribosomes, which comprise up to 40% of the dry weight of a cell, have been identified as one the main contributors to quinary interactions. Interactions between ribosomes and enzymes and metabolites have been shown to influence kinetic properties. As much as a 3.5 fold increase in catalytic rates were observed in enzymes that interacted with ribosomes when compared to \textit{in vitro} catalytic rates. Occupying a significant volume in the cells, ribosomes contribute to the volume exclusion effect allowing for the effective concentration of substances within non-excluded volumes to increase, further altering reaction velocities. Quinary interactions may vary the physicochemical properties of proteins as was seen for thioredoxin, ubiquitin, and FKBP, whose quinary interactions occurred within the active sites of proteins.

Metabolic pathways benefit from quinary structure by forming a metabolon that funnels intermediate molecules from one enzyme within a supramolecular complex, enhancing metabolic processes that are diffusion-limited. Without quinary structures to help funnel metabolites, the rate of metabolic reactions will be reduced as the
diffusion of metabolites through the cell is comparatively slow and would limit the ability of the cell to survive\cite{28} since many intermediates do not serve a function except to be funneled into the next reaction, and can be toxic\cite{29,30}. Evidence of quinary structure acting in this function can be seen in the TCA cycle\cite{31}, glycolysis\cite{32}, and amino acid metabolism\cite{33}.

Quinary interactions have both stabilizing and destabilizing effects on proteins\cite{12,34,35}. Studies into the destabilizing effect of quinary interactions have concluded that quinary interactions can promote intrinsically disordered regions, IDR’s\cite{11} in a protein structure that can be activated when specific function is needed, acting as a switch within a protein\cite{20,34,36}. Furthermore, destabilization of a protein through quinary interactions allows for fast degradation of proteins during cell maintenance as proteases act on unfolded proteins\cite{37,38}.

Quinary interactions is a result of the cellular environment influencing the function of a protein. Being that the cellular environment is highly diverse from organism to organism, it can be said that the influence of quinary interactions are specific within each organism and may influence a protein differently within homologs in different organisms. For this reason studies into the effect of quinary interactions on a target protein should be considered unique to each type of cell. \cite{}

Quinary interactions, unlike other protein-protein interactions, cannot be studied \textit{in vitro} as they result from the unique molecular crowding that can only be seen \textit{in vivo}. As a result studies into the effect of quinary structure are predominately done in living cells utilizing non-destructive techniques that maintain the whole cell and do not disrupt the cellular environment. Imaging methods such as fast relaxation imaging (FRel)\cite{39,40}
and real time Forster resonance energy transfer (FRET) lack the ability to provide amino acid resolution of quinary structure. In-cell NMR on the other hand, allows for the non-destructive probing of quinary structure while providing amino acid resolution and identifying quinary structural surfaces and quantifying the strength of quinary interactions.

1.3 In-cell NMR

Studying the behavior of a target protein in the cellular environment requires a technique that is non-destructive. In-cell NMR is the ideal analytical technique as it is non-invasive to the cell and can provide high-resolution structural information on a target protein within the cellular environment.

Through the use of in-cell NMR information about protein-protein, protein-ligand and protein folding can be investigated within living cells at the amino acid level. For in-cell NMR to be a viable tool in the investigation of macromolecules, NMR active isotopes such as $^{13}$C and $^{15}$N are integrated into the structure of a target protein to allow it to be distinguished from the cellular environment. Numerous methods have been utilized to integrate NMR active isotopes into target proteins. Most common is over-expression of protein in bacteria cells. Minimal medium allows for high control over the composition of the medium, by employing either $^{15}$N-NH$_4$Cl or $^{13}$C-glucose as the sole nitrogen or carbon sources respectively, NMR active isotopes can be incorporated into the target protein’s backbone during overexpression. The use of NMR active isotopes in the growth medium does provide the possibility that $^{13}$C and $^{15}$N are incorporated into metabolites and other macromolecules within the cellular environment.
that can complicate the resulting NMR spectra. However due to the high concentration of over-expressed protein, the presence of metabolites and other proteins have little influence on the resulting spectra\textsuperscript{43}. Selective labeling of individual amino acids can also be employed through over-expression, as long as the labels used are end products of metabolism. Selective labeling has been shown to fail when using amino acids that are also intermediates in metabolism, as they can be broken down and incorporated into other metabolites and not the target protein.

Proteins should be characterized in the cellular environment that matches the native one for a target protein. Because bacteria cells might not contain the correct mechanisms to properly fold a eukaryotic protein\textsuperscript{45}, methods are available to introduce a labeled eukaryotic protein into eukaryotic cells. Though there are several methods, all first require the over-expression of a target protein on labeling medium in bacterial cells that is then purified and concentrated. Microinjection has been used to introduce a target protein into \textit{Xenopus laevis} oocytes. Microinjection offers not only control of the final concentration of labeled protein in the cellular environment but the elimination of background NMR signals as the label protein is introduced to unlabeled cells. Cell-penetrating peptides (CPP) that can be cleaved in the reducing cellular environment\textsuperscript{46} have been employed to deliver labeled proteins to the cell. Reversible membrane pores have been exploited to deliver labeled protein through the use of streptolysin O. After pore formation labeled proteins can be introduced to the cell and the pores can be resealed with Ca\textsuperscript{2+}\textsuperscript{47}. Most commonly electroporation is used to transfect eukaryotic cells by applying a strong electric pulse to form pores in the cell membrane\textsuperscript{48}. Including labeled protein into the electroporation buffer will introduce the labeled protein into the
cell at the same concentration as in the buffer. Electroporation allows for a high diversity of cells lines to be used in different studies.

Figure 1.4 Isotopically labeled protein can be introduced to the cell by many methods. The most common method is protein overexpression but methods such as electroporation and microinjection are used on mammalian cells. Replicated with permission from 45

Interactions between labeled protein and the cellular environment can lead to significant broadening of NMR signals. Broadening of NMR signals is a result of slower tumbling rate of protein in the cellular environment due to the increased intracellular viscosity or quinary interactions, and results in an increase in the apparent molecular weight of the labeled protein. Early in-cell NMR studies were limited to proteins under 30 kDa as larger proteins were too broad to be able to make structural determinations. Signal loss during multidimensional NMR experiments are the result of transverse relaxation that occurs as polarization is transferred between nuclei and during signal evolution. The introduction of transverse relaxation-optimized spectroscopy, TROSY,
and cross relaxation-induced polarization transfer, CRIPT,\textsuperscript{49,50} has opened the door for in-cell NMR to investigate proteins >100kDa.

TROSY minimizes the broadening of signal resulting from transverse relaxation. Transverse relaxation occurs as coherence is lost during between dipole-dipole couplings, as photons are released at the Larmor frequency and absorbed by neighboring dipoles. Because transverse relaxation can also be caused by chemical shift anisotropy, which is proportional to the strength of the magnetic field, TROSY eliminates the effect of transverse relaxation by applying a magnetic field where the contribution from dipole-dipole coupling and chemical shift anisotropy cancel one another. Countering the effect of transverse relaxation increases the window of high molecular weight proteins that can be observed by in-cell NMR and increases the resolution of the resulting spectra.

The further addition of CRIPT negates transverse relaxation. Cross correlation between nuclei during the evolution of in-phase coherence signal as it is coupled to antiphase coherence by cross correlation relaxation between dipole-dipole coupling and chemical shift anisotropy in large proteins can be minimized by adjusting the transfer time between in-phase $^1$H and anti-phase $^{15}$N. The coupling of the evolution of in-phase coherence to the anti-phase coherence during polarization transfer is independent of molecular weight and allows the signals from large proteins to be resolved\textsuperscript{50}. Using both CRIPT and TROSY increases the scope of in-cell NMR as larger proteins can be studied in their cellular environment.

As the door opened for observing larger molecular weight proteins, proton-proton dipolar interactions hampered in-cell NMR evolution as the spin relaxation between
protons decreased the efficiency of the polarization transfer resulting in spectral broadening. A reduced proton labelling strategy, REDPRO, overcomes the negative effect of proton-proton dipolar interactions on signal broadening, NMR-inactive deuterons can be used to replace non-exchangeable protons reducing spectral broadening\(^5\). Replacing water with 100% D\(_2\)O during protein over-expression results in a target protein with a reduced amount of protons limiting the proton-proton dipolar interactions that broaden spectra.

Smaller intrinsically disordered proteins, IDPs, that tumble freely in the cellular environment do not need CRIPT or TROSY to be detected but are negatively affected by the long acquisition times that multidimensional NMR requires to resolve a spectrum. The lengthy acquisition time limits the ability of in-cell NMR to acquire real-time data about protein dynamics. Selective Optimized-Flip-Angle Short-Transient Heteronuclear Multiple Quantum Coherence NMR, SOFAST-HMQC,\(^5\) shortens acquisition times and maintains an acceptable signal to noise ratio. Selectively pulsing on \(^1\)H while applying water suppression allows for a significant amount of relaxed water protons to remain in a relaxed state during acquisition. With an available pool of relaxed protons, relaxed protons can be exchanged with the excited labile amide protons along the backbone of a protein. This allows the protein to return to the relaxed state in between scans faster during the NMR experiment shortening the recycle time between scans. SOFAST-HMQC is an essential tool in in-cell NMR.

Protein structure, protein-protein interactions, protein-ligand interactions, and protein folding have been determined through the use of in-cell NMR. But as with any technique there are limitations. Collection of multidimensional NMR spectra can require
times that vary from several hours to days. As a result depletion of nutrients over time changes the metabolism of the cell and the cellular pH. Changes to the pH can result in varying degrees of changes in protein-protein interactions, protein-ligand interactions, and protein folding during spectral acquisition that may affect the resulting spectra. As nutrients are depleted the cells enter an intermediate energy state that ultimately leads to apoptosis and the release of the labeled protein out of the cell. Released protein negatively affects spectra as protein signals no longer arise from within the cellular environment but from a combination of free and in-cell protein.

During an in-cell NMR experiment the cells go through three distinct energy states that govern ability of the cell to carry out metabolism and vitality. In a high energy state, cell are producing ATP at a rate comparable to the depletion of ATP. Cell growth and high metabolism are achieved and cells are considered to be thriving. An intermediate energy state that often occurs as cell are starved of nutrients allows the cell to maintain viability but high energy interactions are halted as survival takes over. Cells are capable of forming colonies when plated on complete medium. Cells in the lowest energy state have depleted all ATP and are no longer able to support life and cell death follows.53

During in-cell NMR studies the cells are in an intermediate state. As a result plating after an in-cell NMR experiment is a common check of cell viability54. To achieve a high energy state that will allow for protein expression and high energy interactions to be studied by in-cell NMR, nutrients must be delivered to the cells for the duration of the experiment. Bioreactors allow for the exchange of nutrients and removal of waste so that cell can reach a high energy state during in-cell NMR.
1.4 Bioreactor

To understand the role the cellular environment plays on processes such as in protein-protein interactions, protein-ligand interactions, and protein folding and unfolding, maintaining a stable cellular environment is crucial. Fluctuations in the cellular environment may lead to false results as changes in NMR spectra resulting from the changing cellular environment may be misinterpreted as quinary interactions. Changes in the cellular concentration of ATP, pH, ionic strength, and redox state all influence the resulting in-cell NMR spectra of a protein. Eliminating the negative influence of the fluctuating cellular environment allows for in-cell NMR to accurately represent the nature of protein and its interactions in the cell.

Traditional in-cell NMR is conducted on a slurry of cells within an NMR tube. Due to the insensitivity of NMR spectroscopy a high density of cells is needed to produce an NMR signal. Typically ~10^8 cells are needed when doing mammalian in-cell NMR studies while ~10^8-10^9 bacterial cells are used. The result of the high density of cells needed to conduct NMR cell creates an anaerobic environment that negatively impacts cell health. Resulting changes in the cellular environment causes the cells to enter an intermediate energy state where many cellular processes are shut down as survival becomes the primary function. The fluctuating cellular environment plays an influential role in cellular function, as such it is vital to maintain the cellular environment in a steady state for the duration of the in-cell NMR experiment. Bioreactors allow for the exchange of medium facilitating a stable cellular environment for an extended time allowing the changing cellular environment to be negated.
Bioreactor technology has evolved from the early bioreactors that were used in NMR. Many of the first bioreactors relied on a variety of different tubes and sensors to report on the condition of the medium and to supply cell with nutrients requiring many of the early bioreactors to use wide bore 10 mm NMR tubes. Growth media in early bioreactors were often recirculated for the duration of the NMR experiment, returned into the NMR tube through the use of circulation methods such as stir rods, active exchange of media such as bottom up flow through NMR tubes, or a cyclone reactor to facilitate the even distribution of nutrients to cells. Resupplying oxygen to recycled media was often the job of oxygen pumps outside the NMR to alleviate anaerobic conditions within the bioreactor. With all of the probes and tubes needed to maintain life early bioreactors were quite complex in their setup and needed 10 mm NMR tubes to provide enough space, but the space came with a loss of sensitivity. Maintaining the homogeneity of the magnetic field is significantly tougher in wide bore NMR tubes leading to the loss of sensitivity as signal to noise is increased.

The sensitivity of the NMR is related to the amount of sample within the sampling region of the NMR tube. Because bioreactors rely on flowing medium to replenish nutrients and oxygen while removing waste, cells must be immobilized to prevent loss of sensitivity during the NMR experiment. Embedding cells in a porous material allows for the exchange of medium with the cells while preventing cell loss during the NMR experiment. The early bioreactors used agarose beads and threads, alginate, hollow fibers, and glass beads to immobilize cells within a bioreactor. Although hollow fibers and glass beads are sufficient ways to immobilize cells there are negative aspects. Cells are often in a monolayer limiting the number of cells in the
sample region of the NMR reducing the sensitivity and the supporting matrix occupies valuable space within the NMR tube. For these reasons agarose and alginate have become the preferred methods of immobilization in bioreactors.

Cell viability is assessed during an NMR experiment through the collection of $^{31}$P spectra. Observing the changes in the $^{31}$P spectra allows for ATP levels to be evaluated, the intensity of the phosphate peaks correlate to the changing ATP concentration in the cell proving information about the energy state of the cells during a NMR study. $^{31}$P spectra produces 3 distinct peaks; $\alpha$-ATP at $\sim$-9.9 ppm, $\beta$-ATP $\sim$-18.6 ppm, and $\gamma$-ATP $\sim$-4.9 ppm. Monitoring ATP provides insight into the energy state of the cell allowing researchers to assess cell viability within a bioreactor.

Bioreactors have been able to maintain healthy cells for several hours, allowing cells to maintain a high energy state. Implementation of a bioreactor into in-cell NMR studies will allow researchers to investigate high energy interactions and high energy cellular functions such as protein expression through in-cell NMR providing amino acid resolution to these events.

In designing a new bioreactor, limitations of previous bioreactors such as loss of sensitivity and the need for custom parts can be avoided while still maintaining a high energy state within the cells. In this dissertation I will show that our design for a new bioreactor can simplify the complexity of previous bioreactors. Through the use of our bioreactor, high energy events were investigated. First, quinary interactions that are mediated by ribosomes were investigated. Second, expression of proteins can be achieved within the bioreactor allowing for the high energy protein-protein interactions between prokaryotic ubiquitin-like protein, Pup, and mycobacterial proteasomal ATPase,
Mpa to be investigated. And third, through the use of a bioreactor, metabolic changes can be monitored and metabolic rates determined as cells respond to changing stimuli.
Chapter 2: Real-Time In-Cell Nuclear Magnetic Resonance: Ribosome-Targeted Antibiotics Modulate Quinary Protein Interactions

2.1 Introduction:

Part of the central dogma is protein synthesis. Ribosomes provide the only mechanism in the cell to facilitate protein production. Inhibition of protein production is a main target of antibiotics as halting protein production often leads to cell death. To what degree the ribosome is inhibited depends on the specific antibiotic used. Antibiotics that bind to the small 30S subunit of the ribosome such as tetracycline and streptomycin inhibit the interaction between the ribosome and tRNA. Antibiotics such as chloramphenicol bind to the 50S subunit of the ribosome and inhibit the peptidyl transferase activity of the ribosome inhibiting the growth of the nascent protein. Inhibition of the ribosome ultimately effects several different biological pathways that may result in cell death. The physical mechanisms of these effects are not well understood.

Previous studies have shown that weak, quinary interactions between proteins within the crowded cytosol of the cell are mediated by interactions with total RNA within the cellular environment. The ribosome represents 90% of the total RNA in the cell which leads the belief that quinary interactions are largely effected by interactions with the ribosome. Quinary interactions have been shown to affect a protein’s stability, activity, and ligand binding. It is possible that the action of antibiotic binding to the ribosome will alter the quinary interactions within the cell thus altering biological pathways. To test this idea real time in-cell NMR will allow changes in
a target protein’s quinary interactions to be gleaned from cells as stimuli are introduced to the cellular environment through the use of a bioreactor.

Thioredoxin (Trx), a 12 kDa protein that along with glutathione makes up the two major thiol-dependent antioxidant systems in mammalian cells makes a good protein to study by in-cell NMR. Because it is a highly structured protein it results in a well resolved spectrum. The apparent molecular size of Trx in-cell is ~1.1 MDa, leading to the conclusion that in-cell Trx is involved in many quinary interactions within the cell making it an appropriate protein to study when investigating quinary interactions.

Changes to the cellular environment in response to a stimulus may take several hours to manifest. Due to the extended time required to observe such changes, it is vital that cells are maintained in a steady-state of metabolism for the duration of the NMR experiment. Through the use of a bioreactor the viability of both bacteria and HeLa cells are able to be maintained for up to a 24 hour period allowing adequate time for a cellular response to be studied. Cell viability can be confirmed within the bioreactor through monitoring of phosphate-containing metabolites while medium is exchanged during a NMR experiment. Following inhibition of the ribosome by specific antibiotics, changes in a target protein’s quinary interactions can be followed. Results of this study show that as a response to ribosomal antibiotics, ribosome-mRNA interactions are perturbed altering Trx quinary interactions as Trx and ribosome compete for binding sites along mRNA.
2.2 Material and method:

Expression of \([U^{15}\text{N},2\text{H}]\) Trx

Expression of Trx was achieved by following a previously reported procedure\(^{24}\).

Expression of Trx ensued with a five milliliter aliquot of Luria broth (LB) medium that was inoculated with a single colony of BL21-DE3 *E.coli* that was transformed with pRSF-Trx plasmid, 75 μg/mL of kanamycin was added and allowed to grow overnight at 37 °C. Following the growth of the overnight culture, a secondary growth was started by transferring the overnight to a fresh 200 mL of LB with 75 μg/mL of kanamycin and growth of cells proceeded at 37 °C until an optical density (OD) at 600 nM of 0.7-1.0 was achieved. Once cells reached the appropriate OD\(_{600}\), cell were collected by centrifuging the medium at 200g for 20 min. The supernatant was decanted, and the wet cell pellet was washed twice with minimal medium (M9) to remove LB medium. Once washed the cell pellet was resuspended in 100 mL of deuterated M9 medium that contained 1.0 g/L of \([^{15}\text{N}]\) ammonium chloride as the sole nitrogen source and 0.2 % glucose as the sole carbon source. 1 mM of β-D-1-thiogalactopyranoside (IPTG) was used to induce overexpression of Trx over an 18 h period to achieve an in-cell concentration adequate for in-cell NMR. Once overexpression was complete, cells were collected by centrifuging the culture at 200g for 20 min and washed with M9 to remove M9 medium before casting for the bioreactor.

Preperation of HeLa cells:

HeLa cells were grown by following a previously reported procedure\(^{24}\). Each of five 150 cm\(^2\) culture dished (Corning) were seeded with 5 x 10\(^6\) HeLa cells (Sigma-Aldrich) and allowed to grow to 80% confluence in complete low-glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum
(FBS,Gilbco). To reach 80% confluence HeLa cells were incubated for 3 days in a 5% CO₂ incubator at 37 °C. Once confluence was reached cells were collected by exposing the cells to 0.25% trypsin/EDTA (Sigma-Aldrich) for 15 min in a 5% CO₂ incubator at 37 °C. After the incubation trypsin was neutralized with a 5-fold dilution in DMEM. To collect the HeLa cells, the medium was centrifuged at 200g for 10 min at 25 °C and the resulting pellet was washed with phosphate-buffered saline. The five 150 cm² culture dishes at 80% confluence resulted in 1 x 10⁸ HeLa cells for in-cell NMR study.

Preparation of Ribosomes:

Purification of ribosomes was modified from a previously reported procedure⁸⁸. E. coli cells were grown in 4 liters of LB medium to an OD₆₀₀ of 0.5-0.7 to allow for the maximum amount of ribosome to be purified and was centrifuged at 200g for 20 min to collect the wet cell pellet. The resulting wet cell pellet was resuspended at 1 g/mL in lysis buffer [20 mM Tris-HCl (pH 7.2), 100 mM ammonium chloride, 10 mM magnesium chloride, 0.5 mM EDTA, and 6 mM βME] and sonicated with a model 250 digital sonifier (Branson) to achieve cell lysis. Lysate was centrifuged at 30,000g for 45 min. to separate the cell debris from the supernatant. To separate the ribosome from the supernatant, the supernatant was layered onto lysis buffer that contained 37.7% sucrose and centrifuged at 280,000g for 18 h at 4 °C using a Optima LE-90K ultracentrifuge (Beckman Coulter) with a SW41 rotor. Following ultracentrifugation a clear pellet of purified ribosome was left in the centrifuge tube after decanting the liquid off. The ribosome pellet was washed several times with wash buffer [10 mM Tris-HCl (pH 7.4), 1 M ammonium chloride, 10 mM magnesium acetate, and 2.5 mM DTT] to remove all the ATPase activity from the purified ribosomes. The resulting prep was stored in 10 mM potassium phosphate (pH 6.5), 10 mM magnesium acetate, and 1 mM
The concentration of purified ribosome was assessed by the absorbance at 260 nM, using an ε₀.1% of 15 mg mL⁻¹cm⁻¹. Only solutions with a 260 nm/280 nm absorbance ratio at of 1.97-1.98 was used in the study.

_Casting of cells:_
The procedure for casting cells in agarose was modified from a previously reported method. Wet cell pellets of _E. coli_ containing overexpressed [U-¹⁵N, ²H] Trx (~350 μL) was mixed in a 1:1 ratio with 3% Cambrex Seaprep agarose in M9 salts. 3% agarose was chosen as it is the highest concentration of agarose that allowed for handling in a liquid state at 37 °C so as to not damage the cells. The final concentration of agarose was 1.5%. Casting HeLa cells differed from _E. coli_ only in agarose composition. 8 x 10⁷ HeLa were mixed at a 1:1 ratio with 3.0 % agarose in complete DMEM at 37 °C. The cell/agarose mixture was injected into 2 m of polytetrafluoroethylene (PTFE) tubing with an inner diameter of 0.5 mm. The PTFE tubing was wrapped around a 2.5 cm cylinder to allow for the 2 m of tubing to be submerged in an ice bath to facilitate the solidification of the agarose gel. The cell/agarose mixture was allowed to set for 15 min within the PTFE tubing before extruding the cell/agarose mixture into a 5 mm, 600 MHz screw cap NME tube with a PTFE/silicone septum (New Era). Cell/agarose threads were extruded from the tubing by applying positive pressure from M9 medium attached to the tubing by using a 3 mL syringe. At the bottom of the 5 mm NMR tube was 135 μL of 3% Cambrex Seaprep agarose in D₂O to facilitate locking of the NMR and removing the need to deuterate the fresh growth medium.

_Bioreactor setup:_
Description of the bioreactor set up follows the flow of fresh medium as it travels through the bioreactor. Fresh medium flowed into the NMR tube through 2 m of PTFE
(I.D. 0.5 mm) tubing that was placed into the fresh medium reservoir and weighted down to the bottom the reservoir with a Luer-lock connector. The opposing end of the PTFE tubing was passed through the PTFE/silicone septum of the NMR tube and thermally sealed. Eight 50 μm orifices were then made over a 3 cm length with 4 orifices on each side of the PTFE tubing allowing for an even distribution of fresh medium. Orifices were made with a 10 μm stainless steel microdissection needle (Roboz Surgical Instrument Co.) held by a microdissection needle holder (Roboz Surgical Instrument Co.). Waste medium was removed from the NMR tube in a second 3 m length of PTFE tubing passed through the PTFE/silicone septum that led to a reservoir placed in the floor. To facilitate the start of the gravity syphon that allowed medium to flow without the aid of a pump and to eliminate bubbles present in the tubing, a low-pressure tee with a syringe port (Idex Peek) was added half way into the fresh medium tubing. A flow rate of 100 μL/min was maintained by a height difference of 0.86 m between the two reservoirs. Before NMR studies a 30 min incubation of the sample within the bioreactor allowed the cells to return to equilibrium and functionality of the bioreactor to be assessed.

Extract of cellular metabolites:
Cellular metabolites were extracted following a procedure previously published. Extraction of metabolites was conducted on a 3 g wet cell pellet of E. coli. Five mL of water was added to the wet cell pellet along with 0.5 mL of 35% (v/v) perchloric acid. Metabolites were released from the cell through sonication and proteins were precipitated by the perchloric acid. The sample was centrifuged at 30,000g for 45 min and the supernatant was removed. Supernatant containing metabolites was neutralized with 2 M KOH and centrifuged again to remove potassium perchlorate salt. The solution
was lyophilized and reconstituted in 90% H$_2$O/10% D$_2$O supplemented with 5 mM EDTA.

**Cell viability in agarose:**
To assess the viability of cell in the agarose threads, $8 \times 10^7$ HeLa cells were packaged into agarose treads as previously described. The resulting cell/agarose threads containing HeLa cells were divided into two samples. The first sample was stained with trypan blue directly after casting that allowed the dead cells to be stained while leaving alive cells untouched. The second sample was placed in a 5% CO$_2$ incubator 37 ºC for 24 hrs. After incubation the second sample was stained with trypan blue to assess the viability. Images of threads were collected using a Nikon Eclipse TS100 inverted microscope that was fitted with a Canon EOS digital camera.

**NMR experiments:**
All NMR experiments were collected on a 600 MHz Avance III NMR spectrometer at 298 K (Bruker). The NMR spectrometer was equipped with a QCI-P cryoprobe (Bruker).

Collecting $^1$H-$^{15}$N CRINEPT-HMQC-TROSY in-cell spectra of [U-$^{15}$N, $^2$H] Trx allowed Trx to be monitored in real time as the cell responded to antibiotic stimuli. CRIPT was set with a transfer delay of 1.4 ms and a recycle time of 300 ms. Before the addition of antibiotics an in-cell spectra of Trx was collected for each trial individually to establish a reference spectrum. Upon completion of the reference spectrum tetracycline (15 μg/mL) was added to the fresh medium reservoir and five consecutive in-cell spectra were collected over an 18 h period. Following completion the experiment was repeated with chloramphenicol (33 μg/mL) and streptomycin (50 μg/mL). In-cell NMR spectra collected had a spectral width of 12 and 30 ppm in the proton and nitrogen
dimensions, respectively. 1024 and 128 points were collected in the proton and nitrogen dimensions, respectively.

Preparation of *in vitro* samples to study the interaction between ribosomes and Trx was done by mixing 10 μM ribosome with 50 μM [U-15N] Trx in NMR buffer (80 mM potassium phosphate (pH 6.5) in 90% H2O/10% D2O). A 1H-15N HSQC spectrum with Watergate water suppression allowed the interaction between ribosome and Trx to be assessed by monitoring changes in the protein’s chemical shifts in the presence of ribosomes. 1024 and 128 points were collected in the proton and nitrogen dimensions, respectively.

In-cell levels of metabolites were monitored by the collection of proton-decoupled 31P spectra. 31P spectra were collected for *E. coli* and HeLa cells in the bioreactor to judge the level of phosphorus containing metabolites. Each spectrum was collected over a 3.5 h period and the total time of acquisition was 25 h. To obtain the level of metabolites present over time, the 13P peak in the spectra at -11.5 ppm was integrated. The peak at -11.5 ppm represents not only the α-phosphate of ATP and ADP but also the diphosphate of NAD+ and NAD(H). To assess the error of integration of the phosphate peak the integration was repeated three times.

*Data Analysis:*
Analysis of NMR spectra was conducted following the procedure previously reported by the Shekhtman lab24, 90. Assuming that changes in the chemical shifts between *in vitro* and in-cell are minimal in both the hydrogen and nitrogen dimension91, in-cell peak assignments for Trx chemical shifts came from the *in vitro* spectrum of [U-15N]–Trx. Of the 102 peaks that were identified in the *in vitro* spectrum of Trx, 93 peaks
were identified in the in-cell spectra and used in the analysis. The changes in chemical shifts in response to antibiotics were calculated as \( \Delta \delta = \left[ \delta_H^2 + \left( \frac{\delta_N}{4} \right) \right]^{1/2} \), where \( \delta_H \) and \( \delta_N \) represent the changes in chemical shifts in the hydrogen and nitrogen dimension, respectively. The changes in intensity in the presence of antibiotics were calculated as \( \Delta I = \frac{(I/I_{\text{ref}})_{\text{bound}}}{(I/I_{\text{ref}})_{\text{free}}} \), \( (I/I_{\text{ref}})_{\text{bound}} \) represents the normalized intensity of Trx peaks in the presence of antibiotic and \( (I/I_{\text{ref}})_{\text{free}} \) represents the normalized intensity of Trx peaks in the absence of antibiotic. Normalization of each spectrum was accomplished by dividing each peak by \( I_{\text{ref}} \), a glutamine amide side chain at 7.49 and 112.4 ppm in the hydrogen and nitrogen dimension, respectively. The chemical shift of glutamine amide side chain was unaffected by the presence of antibiotic allowing it to be an appropriate peak of normalization. Upon analysis of the in-cell spectra, chemical shifts were found to be unaffected by the presence of antibiotics, ultimately leading to principal binding modes too small to properly resolve, so analysis was focused on changes in intensity.

Using Excel (Microsoft, Inc.) changes in intensity were compiled into a matrix, \( M \) and exported as an ASCII text file which was read by MATLAB (Mathworks, Inc.). Using the \([U,S,V]=\text{svd}(M)\) function on MATLAB, singular value decomposition (SVD) was conducted on matrix \( M \). SVD analysis generates an output that consists of three different matrices, \( U \), which contains the left singular vectors, \( S \), which contains the singular values, and \( V \), which contains the right singular vectors. The \( S \) matrix singular values were plotted to form a Scree plot, a visual representation of the contribution of each binding mode to the \( M \) matrix. Fitting Scree plots with a linear regression produced a coefficient of determination, \( r^2 \). Poor linear fits of the singular values in a Scree plot are indicative as a single binding mode. Scree plots also determined the experimental
noise which is represented as the second and higher order binding modes present in the Scree plot. Bar plots illustrate the contribution of the first and second binding modes for each amino acid. Because the second binding mode signifies the background noise in the spectra a threshold of 1.5 times the strongest contribution of the second binding mode was set as the determining point to distinguish the amino acids involved in quinary interaction.

2.3 Results:
Real time in-cell NMR

Implementing a bioreactor for in-cell NMR studies minimizes stress caused by an unstable metabolic environment within the cell. Because the cellular response to the presence of antibiotics may take up to several hours to appear, maintaining a stable active metabolic environment ensures that the signal collected is representative of the antibiotic response and allows for spectra to be obtained at a signal to noise ratio of 3:1, as spectra that take several hours to collect will not be limited by the viability of the cells in the NMR tube. The design of the bioreactor for real time, RT in-cell NMR simplifies the complicated flow of bioreactors that were previously published, and standardizes the construction as all components are commercially available and requires no custom components or extensive modifications.

The bioreactor setup can be seen in Figure 2.1. The pump that is commonly used in bioreactors to recirculate medium was replaced with a passive flow that was sustained by a gravity siphon. By not recycling medium means that the medium is only used in a single pass through the bioreactor effectively eliminating the need to resupply medium with nutrients and oxygen as the NMR experiment proceeds, a process that
increased the complexity of early bioreactors\textsuperscript{64, 70}. Early in-cell NMR was hampered with leakage of the target protein from the cells as the experiment proceeded\textsuperscript{94}, exchanging the medium within the NMR tube eliminated this problem as leaked target protein is exchanged out of the bioreactor with the medium. To simplify the NMR tube within the bioreactor, a commercially available 5 mm screw-cap NMR tube equipped with a PTFE/silicon septum was used. The PTFE/silicon septum allowed for the inlet and outlet tubing to be sealed in place and maintain the closed system required to flow the medium in the gravity siphon. The reservoir of fresh medium was placed below the NMR spectrometer as a preventative measure should the closed circulation system within the NMR fail, the gravity siphon will flow back into the fresh medium reservoir and not into the bore of the NMR spectrometer, causing costly damage.
Figure 2.1: RT in-cell NMR. A gravity siphon drives the continuous flow of fresh medium through the cells for the duration of the experiment. Antibiotics are introduced through the inlet reservoir.

Embedding the cells in an agarose matrix ensured that a consistent quantity of cells containing the target protein remained in the NMR active region of the bioreactor. It has been shown that properties of agarose are appropriate to maintain the life of cells\textsuperscript{95}, an embedded cell can survive for an extended time while suspended in an agarose matrix. For these reasons cells containing \([U^{15}\text{N}, {2}\text{H}]\text{Trx}\) were embedded in 1.5\% agarose. Evenly distributing the medium over the NMR active region of the bioreactor was facilitated through a horizontal “drip irrigation” system that was developed to deliver fresh medium through eight orifices, four on either side of the inlet tubing (Figure 2.2). By increasing the area the fresh medium is distributed in the bioreactor the upward drag that is associated with the exchange of medium is limited is
significantly reduced ensuring that cells do not flow out of the bioreactor thus increasing the quality of the in-cell NMR spectra.

**Figure 2.2 Orifices on inlet tubing.** (A) Magnified image of an inlet tubing horizontal drip irrigation orifice. (B) Schematic of a horizontal drip irrigation orifice showing the formation of the irrigation jet.

Cell viability

Confirming that the use of horizontal drip irrigation to supply cells with a continuous flow of fresh medium maintained cells in a physiologically active state for the entirety of the NMR experiment, cellular levels of phosphate containing metabolites, ATP, ADP, NAD⁺, and NAD(H) were monitored by collecting in-cell °P spectra with and without the flow of medium from the bioreactor. Without the exchange of medium during the NMR experiments, cells are significantly affected by the changes in the cellular energy state. Phosphate containing metabolites were monitored for both *E. coli* and HeLa cells, and fresh M9 medium and DMEM was flowed through the bioreactor for each cell type respectively, (Figure 2.3 A,B) The addition of the bioreactor was able to maintain both *E. coli* and HeLa cells in a metabolically active state for 24 h without a
major, >40%, decrease in metabolite concentration. Cell viability within the agarose threads was established by staining HeLa cells with trypan blue that selectively stains dead cells. HeLa cells were able to maintain 99 ± 1% viability with in the threads after 24 h (Figure 2.3 C,D). HeLa cell viability further confirmed that cells are in a metabolically active state inside the threads of the bioreactor.

Horizontal drip irrigation was optimized to supply an adequate flow of medium to support a metabolically active state. The number of orifices and length of the irrigation jets produced were adjusted to achieve a flow rate of 100 μL/min of medium preventing a rapid decrease in cellular phosphate-containing metabolite concentration for both E. coli and HeLa cells. Each orifice along the inlet tubing measured 50 μm in diameter, the length of the subsequent jet of medium produced from each orifice can be calculated assuming that for a laminar flow of viscous fluid the hydrostatic force, \( \rho g H \times \pi dL \), is equal to the frictional force \( \mu \left( \frac{dv}{dy} \right) \times \pi dL \), where \( \rho \) is the fluid density, \( g \) is the acceleration of gravity, \( H \) is the height difference between the inlet and outlet reservoirs (0.86 m), \( d \) is the diameter of the orifice (50 μm), \( \mu \) is the coefficient of water viscosity (≈10⁻³ kg m⁻¹ s⁻¹), \( (dv/dy) \) is the gradient of the flow velocity across the orifice opening (≈2[(2gH)1/2/d]), and \( L \) is the length of the jet (Figure 2.2 B). The resulting ~1 mm jet of medium from the 50 μm orifice is able to achieve sufficient mixing of medium with the 5 mm NMR tube without disturbing the cell/agarose threads.
Figure 2.3. RT in-cell NMR maintains *E. coli* and HeLa cells in a physiologically stable state for ≤24 h. (A) Signal intensity of cellular $^{31}$P-containing metabolites, including ATP, ADP, NAD+, and NAD(H), over time for HeLa cells with (square) and without (circle) a flow of DMEM. (B) Signal intensity of cellular $^{31}$P-containing metabolites over time for *E. coli* cells with (square) and without (circle) a flow of minimal medium. Error bars give the standard deviation of each data point. (C) HeLa cells are 99 ± 1% viable immediately after casting. (D) HeLa cells are 99 ± 1% viable 24 h after casting; the increase in cell density is consistent with ongoing metabolic activity. White dashed lines outline the threads. HeLa cell samples contained $8 \times 10^7$ cells. *E. coli* samples contained cells from 100 mL of a culture suspended in ∼0.7 mL.

Analysis of in-cell NMR spectra
Placing cells in a bioreactor to achieve RT in-cell NMR ensures that cells are in a metabolic steady state permitting for the changes in quinary interactions to be monitored as the cell responds to internal and external stimuli. Changes to the chemical environment over time that result from changing levels of metabolite concentrations during normal cellular function can lead to changes in the NMR spectra.\textsuperscript{97}

Single value decomposition (SVD) is a linear algebra technique that is capable of reducing an arbitrary matrix to its principal components which display the greatest fluctuation in its elements.\textsuperscript{98} Over the course of the real-time in-cell NMR experiment successive NMR spectra of Trx were collected and compiled into matrix M, which consists of the Trx spectra peak intensities versus antibiotic exposure time. SVD distinguishes the systematic fluctuations in peak intensity that arise as a result of the antibiotic addition as opposed to the random fluctuations in intensity that arise from interactions with the cellular environment over the course of time. Determining the contribution of each amino acid to the principal component allows amino acids that exhibit the strongest changes in response to the antibiotic to be identified.\textsuperscript{90} Previously SVD analysis has been applied to identify protein-protein\textsuperscript{90} and protein-drug\textsuperscript{92} interactions in cells using in-cell NMR data.

In-cell spectra of target proteins are innately noisy, labeling target proteins with NMR active nuclei through overexpression will cause the background to incorporate the nuclei as well. SVD identifies the residues that exhibit the most noticeable changes in chemical shift and intensity that result from the changes in quinary interactions and not from the background noise. Scree plots of the singular values illustrate the contribution of each binding mode by the changes in chemical shifts and intensities. An abrupt drop
in singular values after the first binding mode indicates that changes in the NMR spectra are a result of changes in quinary interactions. A gradual decrease in singular values indicates no changes in quinary structure and that the changes in the spectra are a result of noise$^{90, 92}$. Analysis of the cellular response to antibiotics showed that changes in the chemical shifts were too small to resolve a first binding mode, as a result only peak intensities were used for further analysis of changes of quinary structure. SVD analysis identified the amino acids in the target protein involved in the first binding mode that change as a result of the addition of antibiotics.

Testing whether antibiotics influence the quinary interactions within living cells, it was established that the target protein *E. coli* Trx a 12 KDa oxidoreductase enzyme containing a dithiol–disulfide active site, does not interact with ribosomes (Figure 2.4) or antibiotics (Figure 2.5) directly. Changes in the quinary structure of Trx have been studied and found to be mediated by interactions with mRNA within the cell$^{24}$. Because Trx does not directly interact with the ribosome, it is expected that Trx will not display changes in quinary structure over a time course in the absence of antibiotic. Although because ribosomal inhibition affects the mRNA-ribosome interaction directly by competing for binding sites and indirectly by decreasing the ribosome concentration, the quinary structure of Trx could be affected by ribosomal antibiotics.
Figure 2.4 Trx does not interact with ribosomes. Ribosome do not induce changing the Trx spectra indicating that no interaction between Trx and ribosome occurs. Overlay of Trx $^{15}$N-HSQC spectra without (black) and with (red) ribosomes.
Figure 2.5 Trx does not interact with antibiotics. Addition of antibiotics showed to have no effect on the structure of TRX. No changes in peak position or intensity were observed as a result of the antibiotic addition. Overlay of Trx $^{15}$N-HSQC spectra without (blue) and with (red) antibiotic. A. Pure Trx B. Addition of 1 mM Tetracycline C. Addition of 1 mM Streptomycin D. Addition of 1 mM Chloramphenicol
SVD analysis of in-cell Trx showed no changes in the quinary structure in the absence of antibiotics. Five RT in-cell NMR spectra of Trx were collected, each spectrum required 3 h to acquire and were collected consecutively over a 15 h period. The scree plot of singular values exhibited a monotonic decrease in values with a linear correlation fit of $r^2$ of 0.84 indicating no changes in quinary structure. (Figure 2.6 A,B)

**Figure 2.6.** SVD analysis of in-cell NMR spectra of Trx in the absence and presence of chloramphenicol. A. Distribution of singular values for each dataset index (binding mode) for Trx residues in the absence of chloramphenicol. B. The weighted contribution of each amino acid is shown for the first (blue) and second (red) binding modes. C. Distribution of singular values for each dataset index (binding mode) for Trx residues in the presence of chloramphenicol. D. The weighted contribution of each amino acid in response to adding chloramphenicol is shown for the first (blue) and second (red) binding modes. In B and D, the largest weighted contribution from the second binding mode was used as a threshold to highlight the amino acids with the strongest effect on quinary structure.
Trx in-cell spectra were collected for each sample before antibiotic was introduced to the system to establish the reference. Antibiotics were introduced into the medium through the inlet reservoir and another series of RT in-cell NMR spectra were collected. The addition of tetracycline or streptomycin, both ribosomal antibiotics that inhibit function of the 30S, caused sharp drops in the scree plots of the singular values (Figure 2.8 A,C) with correspondingly poor linear fits with $r^2$ values of 0.67 and 0.66, respectively, indicating that changes in Trx quinary structure occurred.

Figure 2.7. Binding of tetracycline to ribosomes changes the quinary structure of Trx in *E. coli*. (A) Overlay of the in-cell $^1$H–$^{15}$N CRINEPT–HMQC–TROSY spectra of Trx without (red) and with (blue) tetracycline. The insets show overlays of the boxed regions of the in-cell spectrum (red) and the in vitro $^1$H–$^{15}$N CRINEPT–HMQC–TROSY spectrum of purified Trx (black). The intensities of the peaks of G52, V56, D62, and E102, residues involved in quinary interactions, are broadened out in the presence of tetracycline. Single and double asterisks indicate peaks from metabolites and unassigned side chain protons, respectively. The overlaid spectra are at the same contour levels. The reference peak used for peak intensity normalization is indicated by RP. (B) Changes in peak intensities of L95, I42, and K53 with
time. K53 shows no change; L95 exhibits a drop, and I42 exhibits an early rise and then a drop in peak intensity. Errors were determined from the signal-to-noise ratio of the in-cell NMR spectra.

Extensive broadening of the in-cell NMR spectra is further evidence that the addition of an antibiotic that targets the 30S subunit of the ribosome modifies the quinary structure of Trx. (Figure 2.7). Alternatively, the addition of chloramphenicol, which binds to the 50S ribosomal subunit\textsuperscript{99}, resulted in a linear decrease in singular values with an $r^2$ of 0.94, indicating that no changes in Trx quinary structure occurred. (Figure 2.6 C, D) As expected, since chloramphenicol does not interfere with the mRNA-interaction but instead inhibits ribosomes by inhibiting the peptide transferase activity\textsuperscript{76}, it is doubtful that the addition of chloramphenicol will result in changes to Trx’s quinary structure.

Addition of tetracycline and streptomycin lead to changes in the quinary structure on the surface of Trx similar to one another. Changes in quinary structure can be seen over three connecting patches on the surface of Trx (Figure 2.8 E, F). These patches reflect a changing Trx interactome caused by an antibiotic stimulus. The first patch of the three patches, consisting of W29, E31, W32, G34, M38, I42, E45, D62, Q63, and L95 for tetracycline-induced changes and D11, W29, E31, W32, G34, M38, and Q63 for streptomycin-induced changes, contains hydrophobic and negatively charged residues. This patch of residues is similar to the quinary interaction surface of Trx (Figure 2.8 G) recognized by previous comparison of in-cell Trx with cell lysate Trx spectra\textsuperscript{24}, further confirming the influence of RNA on the quinary structure of Trx.

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Figure 2.8. Binding of an antibiotic to ribosomes affects residues that match the Trx quinary interaction surface. (A) Distribution of singular values, SV, of each data set index (binding mode) for Trx residues in the presence of tetracycline. (B) Contribution of each amino acid in response to adding tetracycline for the first (blue) and second (red) binding modes. (C) Distribution of SV of each data set index (binding mode) for Trx residues in the presence of streptomycin. (D) Contribution of each amino acid in response to adding streptomycin for the first (blue) and second (red) binding modes. (E) Residues involved in quinary interactions (red) due to the presence of tetracycline are mapped onto the molecular surface of Trx (Protein Data Bank entry 1X0B). (F) Residues involved in quinary interactions (red) due to the presence of streptomycin. (G) Quinary interaction surface (red) of Trx in the absence of antibiotics. (H) Electrostatic surface map of Trx showing regions of positive, 4 kT/e (blue), and negative, −4 kT/e (red), potential, where $k$ is the Boltzmann constant, $T$ is the temperature, and $e$ is the electron charge. In panels A and C, linear fits of SVs are shown by dotted lines. In panels B and D, the horizontal line represents the threshold used to highlight the amino acids with the strongest effect on the quinary structure. The threshold value was set to 1.5 times the largest contribution from the second binding mode, which represents the average noise of the NMR spectra.

The second and third patches consist of residues G98, K101,
and E102 and residues V92 and G93 for tetracycline-induced changes and residues E45, K97, G98, and K101 and residues R74, G75 A88, T90, K91, V92, and G93 for streptomycin-induced changes, respectively. Both the second and third patches contain positively charges and hydrophobic residues, functional groups that mediate RNA binding (Figure 2.8 H). Several other stretches of continuous amino acids contribute to the first binding mode of both tetracycline and streptomycin (Figure 2.8 B, D). These amino acids consists primarily of buried hydrophobic residues and likely reflect tertiary structural rearrangements of Trx. The effect is more pronounced for streptomycin.

2.4 Discussion:

The results of RT in-cell NMR were consistent with the proposed model that ribosomes and Trx compete for binding along mRNA. Antibiotics that weaken the interaction between ribosome and mRNA result in extensive mRNA-Trx quinary interactions that can be seen in the broadening of the spectra of in-cell Trx (Figure 2.7). Changes to the quinary structure of Trx as a result of the ribosomal antibiotics are almost certainly not unique. A combination of chemical cross-linking and mass spectrometry resulted in the positive identification of >800 proteins that can potentially bind to mRNA or ribosomes in various eukaryotic cells. Many of these are drug targets, such as the RNA binding proteins involved in intermediary metabolism. The ubiquity of RNA-mediated changes in quinary protein interactions may therefore provide a physical basis for ribosome inhibition and other regulatory pathways.

Developed in the course of this study, RT in-cell NMR spectroscopy maintains cells in a metabolically active state for >24 h in the NMR tube. This is a critical step for monitoring intracellular responses to stimuli, in particular those induced by drugs, which
can take hours to mature into detectable signals. The advent of this technology will allow temporal changes in target protein structural interactions to be monitored in real time and will help reveal the inner workings of the cell with unprecedented accuracy and resolution.
CHAPTER 3: Active Metabolism Unmasks Functional Protein-Protein Interactions in Real Time In-cell NMR

3.1 Introduction:
Characterizing functional protein-protein interactions, PPIs, within the cellular environment is important to understand the impact PPIs has on the biological activity of a cell\textsuperscript{102-104}. PPIs can be found in most important cellular processes, leading PPIs to be a target for drug studies. The activity of PPIs is governed by the metabolic state of a cell and may only be visible during particular metabolic states, for this reason PPIs should be studied within living cells that allow for regulation of the interaction surface by the metabolome and its byproducts\textsuperscript{105, 106}. In-cell NMR spectroscopy allows for the spectra of a target protein labeled with NMR active nuclei, $^{15}$N and $^{13}$C, within living cells to be studied, allowing for the study of PPIs to be conducted with amino acid resolution\textsuperscript{46, 48, 107-110}.

In-cell NMR studies have long been plagued by the inability to maintain metabolic activity as well as cell viability\textsuperscript{56, 66, 111}. Protein leakage resulting from cell death is common during lengthy experiments and measures are taken to insure the resulting spectra are from the target protein within intact cells. Traditional in-cell NMR is limited in the depth that PPIs can be studied, this is because in-cell NMR employs cells that are in an intermediate energy state which are metabolically inactive and are limited in the ability to produce the full interaction network, interactome, needed to be able to study PPIs in living cells\textsuperscript{42, 46, 112}. Employing a bioreactor during PPIs in-cell NMR studies allows for the exchange of growth medium and the removal of waste, establishing a favorable environment promoting cell growth\textsuperscript{56, 64, 66, 74, 111}. 
Currently our bioreactor can maintain a metabolic energy state for up to >24 h with a ~40% loss of ATP, ADP, NAD⁺, and NAD(H) concentrations, while maintaining cell viability plus limited cell growth. Transcription, translation, replication and other ATP-dependent processes all require a high energy state in the cell. Improvements to the bioreactor allow for high levels of ATP to be maintained and protein expression to be achieved. Maintaining a high energy state allows for ATP-dependent PPIs to be identified through the combination of structural interactions, STINT¹¹³, and real time in-cell NMR,RT in-cell NMR⁶⁶.

The study of functional PPIs in the cell was conducted using a system where high energy interactions play a central role, for this reason the mycobacterial proteasome system¹¹⁴ was utilized. Prokaryotic ubiquitin-like protein, Pup, marks proteins for degradation by the mycobacterial proteasomal ATPase, Mpa, and the 20S proteasome. Pupylation affects up to 5% of the M. tuberculosis, Mtb, proteome¹¹⁵-¹¹⁷. The persistent infection of macrophages by Mtb¹¹⁸-¹²⁰ lead to the Pup-proteasome system being identified as a drug target.

The Pup-Mpa interaction was re-examined⁹⁰,¹²¹ in E.coli using an improved bioreactor that promotes a high energy metabolic state for an extended time, >24 h, within the cell. Pup-Mpa interactions were conducted in E.coli as opposed to Mtb in an effort to isolate the Pup-Mpa interaction from Mtb proteasome factors that may form unfavorable interactions with the Pup-Mpa complex. The extent of the observed Pup-Mpa interaction while in an active metabolic state exceeded previously reported interactions reported by traditional in-cell NMR⁹⁰,¹²¹.
3.2 Material and Method

Cell Growth

Fifty milliliters of LB broth was supplemented with 150 μg/mL of carbenicillin and 50 μg/mL of kanamycin and was inoculated with a single colony of *E. coli* strain HI-Control BL21 (DE3) transformed with pASK-Pup and pRSF-Mpa and incubated overnight at 37 °C in a rotating shaker. Proteins under a T7 promoter may experience leaky expression when grown in LB medium. HI-Control BL21 (DE3) were used over BL21 (DE3) as HI-Control BL21 (DE3) tightly regulates expression from the T7 promoter preventing leaking expression of Mpa. The overnight culture was transferred to a fresh 500 mL of LB containing 150 μg/mL of carbenicillin and 50 μg/mL of kanamycin and grown at 37 °C in a rotating shaker until an OD$_{600}$ of 0.5-0.6 was reached. Cell were collected by centrifugation at 200g for 20 min and prepared for casting in alginate.

Cell Casting

Previously cells were cast in agarose threads allowing the agarose to supply a supporting matrix for the cells within the bioreactor. As improvements to the bioreactor technology enriched cell health and promoted cell growth, agarose proved to be brittle and resulted in a significant portion of the NMR sample to be washed out of the bioreactor and a loss of the NMR signal. Alginate was chosen to replace agarose as the supporting matrix as it posed similar properties to agarose permitting cell growth. Alginate/cell mixture was cast into beads to maximize the surface area permitting the exchange of medium. Alginate/cell beads were cast with an atomizer that allowed control of bead size and was reproducible. Pelleted cells (~500 μL) were mixed 1:1 (v/v) with a 2% alginate solution in hybrid grown medium salts, HGM salts, 50 mM HEPES, pH 7.5, 2 mM CaCl$_2$, 0.8 mM MgSO$_4$, 5.3 mM KCl, 100 mM NaCl, and 3 mM NaH$_2$PO$_4$. 
The alginate/cell mixture was loaded into a 3 mL syringe fitted with a Luer-lok tip connected to a 40 mm length of tygon tubing (I.D. 0.79 mm) with a blunt 21 gauge needle fixed on the end and placed at a 45 ° angle under an airflow. The alginate/cell mixture was injected into the atomizer by a syringe pump (New Era Pump System NE-300) at a rate of 300 μL/min. Cells were cast with an airflow rate of 5.5 liter per minute. The atomizer was centered over 25 mL of 150 mM CaCl₂. As the alginate/cell mixture was passed through the atomizer and collected in the CaCl₂, Ca²⁺ polymerized the alginate encapsulating the cells within the beads. The 150 mM CaCl₂ solution was decanted off the beads and replaced with 25 mL of complete HGM which consisted of HGM salts supplemented with 4 g/L glucose, 1 mg/mL NH₄Cl, 1 mg/mL ISOGRO (Sigma Aldrich) and 1 mg/mL thiamine. The overexpression of small proteins such as Pup (7 KDa) was achieved after cells were cast into alginate beads and placed in the bioreactor to prevent leakage of protein from the cell.

**Bioreactor Setup**

Improvements to the bioreactor mentioned in chapter 2 allowed for increased reproducibility in the experimental setup and increased efficacy of the delivery of growth medium to the cells. As in the earlier bioreactor a standard screw-cap NMR tube with a PTFE/silicone septum was employed to create a seal around the inlet and outlet tubing. Flow of medium was established in the inlet tubing by a gravity siphon allowing bubbles to be eliminated from the tubing that may restrict the flow of medium. The flow of medium created by height difference between the inlet and outlet reservoirs often resulted in changes in flow rate as the experiment progressed, a peristatic pump (Pharmacia LKB) was placed along the outlet tubing to allow for increased control the flow of medium. Flow of medium was maintained at 80 μL/min.
Developments to the horizontal drip irrigation increased the reproducibility of the pores allow for easy replacement of the horizontal drip system should it fail or be damaged. Creating a horizontal drip irrigation stem improved the rigidity of the system and dammed the beads from flowing out of the NMR active region of the NMR tube. The horizontal drip irrigation stem was fabricated from 3 lengths of plastic microhematocrit capillary tubes (Globe Scientific) connected by 3D-printed (Dremel Model 3D45) 10 mm joints bonded together with plastic bond (Loctite). Fixed to the end of the capillary tubes with plastic bond was a 13 mm length of ultrahigh molecular weight, UHMW, polyethylene porous rod (Scientific Commodities) that was reduced to an O.D. of 1.55 mm on a metal lathe with an 8 mm long channel bored through the center with a diameter of 0.85 mm. The opposing end of the capillary tubing was passed through the PTFE/silicone septum and a 50 mm length of Tygon tubing fitted with a Luer-Lok syringe connector was attached. Additionally a 50 mm length of PTFE tubing fitted with a tubing connector (GE Healthcare) was passed through the PTFE/silicone septum to allow for waste medium to be removed. The addition of the Luer-Lok and tubing connector allows for the detachment of the horizontal drip irrigation stem and outlet tubing from the bioreactor should the septum fail or damage to the stem occurs.

Ensuring the medium was maintain at 37 °C reduced temperature shock, promoting cell health. To counter temperature loss in the inlet tubing the fresh medium reservoir was placed into a 42 °C water bath (Branson model 2800). The 2 m length of tygon tubing (I.D. 0.79 mm) that connected the fresh medium reservoir to the NMR tube was fit with a Luer-Loc syringe connector at both ends allowing one end to be connected to the horizontal drip irrigation stem and the other to provide addition weight
allowing the inlet tubing to rest at the bottom of the inlet reservoir. The inlet tubing was insulated by a 1.21 m length of ½ in foam pipe insulation (Everbilt) filled with polyester fibers (Poly-fil) to further prevent temperature loss. The alginate/cell beads were transferred to the bioreactor with a 2 mL pipette (Falcon) and the horizontal drip irrigation stem was inserted. The bioreactor was allowed to flow on the benchtop for 30 min before experimentation to ensure a proper flow of medium.

Expression of \([U-^{15}N]\) Pup

The high concentration of CaCl\(_2\) used in the encapsulation of cells negatively affects the ability of *E.coli* to retain Pup within the cells. 150 mM CaCl\(_2\), commonly used to enhance the competency of cells during tranformation\(^{125}\), changes the permeability of the cell membrane\(^{126}\) allowing small proteins to leak from the cell. For this reason the overexpression of \([U-^{15}N]\) Pup was achieved after cells were encapsulated in alginate within the bioreactor as opposed to what is traditionally done when conducting STINT NMR\(^{90, 121}\). The overexpression of \([U-^{15}N]\) Pup was achieved on the benchtop prior to placing the bioreactor in the NMR spectrometer. The NMR tube containing the alginate/cell beads was placed in a 37 °C water bath and \([U-^{15}N]\) HGM which consisted of HGM salts supplemented with 4 g/L glucose, 1 mg/mL \(^{15}\)N-NH\(_4\)Cl\(_2\), 1 mg/mL \(^{15}\)N-ISOGRO (Sigma Aldrich) and 1 mg/mL thiamine containing 150 μg/mL of carbenicillin and 50 μg/mL of kanamycin was flowed through the bioreactor. Overexpression of Pup was induced with 2 mg/mL of anhydrotetracycline in dimethylformamide to a final concentration 0.2 μg/mL and allowed to proceed for 18 h.

Expression of Mpa

Succeeding the overexpression of Pup the bioreactor was moved to the NMR spectrometer. Overexpression of Mpa was achieved following the replacement of \([U-
\(^{15}\text{N}\) HGM with \(^{2}\text{D}-\text{HGM}\), HGM prepared in 90% H\(_2\)O/10% D\(_2\)O containing 150 μg/mL of carbenicillin and 50 μg/mL of kanamycin, \(^{15}\text{N}\)-NH\(_4\)Cl\(_2\) with NH\(_4\)Cl\(_2\), and \(^{15}\text{N}\)-ISOGRO with 1 g/L of Bacto tryptone (BD Biosciences) and 1 g/L Bactro yeast extract (BD Biosciences). Before the overexpression the bioreactor was allowed to equilibrate for 30 min. Overexpression of Mpa was induced by adding 1 mM isopropyl-\(\beta\)-D-1-thiogalactopyranoside.

**NMR Spectroscopy**

All NMR spectra were recorded at 298 K using a 600 MHz Avance III NMR spectrometer equipped with a QCI-P cryoprobe (Bruker). \(^1\text{H}-^{15}\text{N}\) SOFAST-HMQC spectra were acquired with 256 scans. The spectral widths in the \(^1\text{H}\) and \(^{15}\text{N}\) dimensions were 14 and 31 ppm, respectively and were digitized by 1024 and 64 points in the \(^1\text{H}\) and \(^{15}\text{N}\) dimensions respectively with a recycling time of 100 ms. Prior to the over expression of MPA, a \([U^{15}\text{N}]\) Pup spectrum was recorded to establish a reference spectrum. 1 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside was added to the fresh medium reservoir and \(^1\text{H}-^{15}\text{N}\) SOFAST-HMQC spectra were recorded 5 times in succession over 5 hours.

Proton- decoupled \(^{31}\text{P}\) spectra were recorded for *E. coli* cells cast in alginate beads with and without the bioreactor. Spectra were recorded at 3.5 h intervals with a total acquisition time of 25 h for both *E. coli* cells. The \(^{31}\text{P}\) peak intensity at −11.5 ppm that contains contributions from the \(\alpha\)-phosphate of both ATP and ADP and diphosphate of NAD+ and NAD(H) was integrated with three different integration ranges to estimate a standard error of the mean. All spectra were processed with Topspin version 3.2 (Bruker) and analyzed by using CARA software.
Data analysis

Data analysis follow was conducted following similar guidelines in section 2.2. Cross peaks in the $^1$H-15N SOFAST-HMQC spectra of free Pup and the Pup-Mpa interaction at different time intervals were determined. Peak intensities were normalized and the changes in intensity that arose from the Pup interaction with Mpa were calculated as $\Delta I$, $\Delta I = \frac{(I/I_{ref})_{bound}}{(I/I_{ref})_{free}}$, where $(I/I_{ref})_{free}$ represents the normalized intensity of individual cross peaks in the spectra of free Pup and $(I/I_{ref})_{bound}$ represents the normalized intensity of individual cross peaks in the spectra of Pup-Mpa complexes. $I_{ref}$ is a glutamine peak at 7.52 ppm and 122.2 ppm in the proton and nitrogen dimensions, respectively, that does not display changes in chemical shift as the complex is formed. Data was compiled into a Matrix M using Excel (Microsoft) and exported as a ASCII text file that was read by MATLAB (Mathworks). Random matrices were generated to observe the effect of random noise on SVD analysis, this was achieved by generating a random matrix of values between -1 and 1 using the $A=-1+2*\text{rand}(64.5)$ command in MATLAB.

Matrix M was analysis by SVD, executed by the $[U, S, V] = \text{svd}[M]$ command in MATLAB. A scree plot of singular values, from matrix S, permitted visualization of the individual binding modes. Scree plots were fitted by linear regression (Microsoft Excel) to define the coefficient of determination, $r^2$. Experimental noise is described by the magnitudes of the second- and higher-order binding modes. The threshold for determining the amino acids involved in the Pup-Mpa complex was set to the maximum contribution of the second binding mode.
**Mpa Calibration curve**

The creation of a calibration curve of Mpa concentration allows for the quantification of Mpa expression levels within the bioreactor. The overexpression and subsequent purification of Mpa began with a fifty milliliters of Luria broth (LB) supplemented with 50 μg/mL of kanamycin, was inoculated with a single colony of pRSF-Mpa and grown overnight at 37 °C in a shaking incubator. The overnight culture was transferred to 500 mL of fresh LB with 50 μg/mL of kanamycin and grown to an OD$_{600}$ of 0.7-1.0. 1 mM isopropyl β-D-1-thiogalactopyranoside was added to induce overexpression of Mpa and was completed over 12 hrs. Following overexpression the culture was centrifuged at 200g for 20 min at room temperature and resuspended 20 mL of lysis buffer, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0, before sonicating with a Model 250 Digital Sonifier (Branson). The lysate was centrifuged at 30,000g for 45 min at RT and the supernatant was collected. MPA was purified by Ni-NTA affinity chromatography (Qiagen). A Bradford colorimetric assay was used to determine the concentration of the stock Mpa solution. A calibration curve of Mpa concentration was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel. Coomassie blue G-250 was used to stain the gel. Precision Plus Protein (BioRad) was used as a molecular size standard. Band intensity was quantified using ImageJ software$^{127}$.

**In-cell Mpa concentrations**

Fifty milliliters of Luria broth (LB) supplemented with 50 μg/mL of kanamycin was inoculated with a single colony of pRSF-Mpa and grown overnight at 37 °C. The overnight culture was transferred to 500 mL of LB with 50 μg/ml of kanamycin and grown to an OD$_{600}$ of 0.7-1.0. The culture was centrifuged at 200g for 20 min at and
cast in alginate beads. Cells were placed into bioreactor and setup in benchtop with a 37 °C water bath to replicate conditions within the NMR spectrometer. Overexpression of Mpa was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. Samples of alginate beads were removed from the bioreactor every 2 hours for 12 hours and a final 24 hour sample and normalized to 33 mg for analysis. Alginate bead aliquots were suspended in 500 μL of lysis buffer and were sonicated with a Model 250 Digital Sonifier (Branson). The lysate was centrifuged at 30,000g for 45 min and the supernatant was collected. Mpa was purified from the supernatant by Ni-NTA affinity chromatography (Qiagen). Purified Mpa was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel. Coomassie blue G-250 was used to stain the gel. Precision Plus Protein (BioRad) was used as a molecular size standard. Band intensity was quantified using ImageJ software and compared to the standard curve generated using purified Mpa to calculate in-cell Mpa concentrations. Assumptions were made to allow for in-cell concentration to be determined, assuming the volume of E.coli to be 2.5 μm³ while having 2.5 x 10¹⁰ cells in each 33 mg sample, the total in-cell volume of E.coli was calculated. It was found that the Mpa concentration in-cell are 10 fold greater than that of the Mpa concentration found within the NMR tube.

*Binding curve*

An approximate binding curve for the Pup-Mpa interaction was generated. Mpa binding curves were constructed from Mpa expression concentrations in the bioreactor and the intensity of isoleucine 18 located at 7.934 ppm and 122.046 ppm ^1^H and ^1^5^N dimensions respectively. Isoleucine 18 was selected for the binding analysis because SVD analysis determined that isoleucine 18 is a participating residue in the formation of
the Pup-Mpa complex. Due to the minimal spectral overlap with other peaks in the Pup spectra isoleucine 18 was selected. An isotherm was fit to a single binging site equation $Y = B_{\text{max}} \times X / (K_d + X)$, using Prism 6 (Graphpad), where $Y$ is the peak intensity of the isoleucine 18 peak in the Pup spectra, $B_{\text{max}}$ is the maximum value of $Y$, $X$ is the concentration of Mpa in μM, and $K_d$ is the equilibrium dissociation binding constant, in μM.

3.3 Results

Maintaining cells in a metabolically active state is vital for the success of any in-cell NMR experiment. Traditional in-cell NMR experiments often are conducted in a non-idealized environment as a large number of cells are suspended in NMR buffer for several hours while the NMR spectra are being collected. As acquisition proceeds, the cellular environment is constantly changing as waste is being produced, and the pH and redox state of the cell is changing. The changing cellular environment leads to changes in the NMR spectra as the protein adapts to the new environment. Changes in the cellular environment negativity impact the quality of the in-cell NMR spectra as lengthy experiments often lead to cell death which allows leakage of target protein from the cell resulting in NMR spectra that are a combination of in-cell and free protein.

Improvements to bioreactor

Maintaining cells in a high energy metabolic state required improvements to the bioreactor (Figure 3.1) that increased experimental reproducibility and maximized the exchange of medium. The original orifices used in the previous bioreactor limited exposure of cells to fresh medium and relied on diffusion to distribute fresh medium. Replacing orifices with an ultrahigh molecular weight, UHMW, microporous polyethylene
diffuser, maximized the fresh medium delivered to the cells. With a range of 50-100 \( \mu m \) pores, the microporous diffuser provided an even distribution of medium throughout the NMR tube. Additional medium was kept at 42 \(^\circ\)C to account for the temperature loss during medium transfer so that cells were maintained at 37 \(^\circ\)C and not exposed to additional temperature stress.

Figure 3.1. Bioreactor setup. (A) (Left) A peristaltic pump is used to draw fresh medium through the bioreactor at a rate of 80 \( \mu L/\text{min} \). Samples are maintained at 37 \(^\circ\)C in the bioreactor and a water bath is used to keep the medium at 42 \(^\circ\)C to compensate for temperature loss during transfer to the NMR tube. (Right) A magnified image of the diffuser tip. (B) Disassembled bioreactor components are shown: A drip irrigation stem and capped NMR tube. (C) The assembled bioreactor.

Improved bioreactor

Maintaining a high concentration of cells within the NMR active region of the NMR tube was achieved by encapsulating \textit{E.coli} cells in 1\% alginate through the use of
an atomizer (Figure 3.2 A). The atomizer created highly reproducible uniform
dispersion of alginate beads with an average diameter of 0.85 μm\textsuperscript{124}. Replacing agarose
matrix with alginate still provides the appropriate density to withstand the upward drag
created by the flow of medium, but unlike the agarose, alginate expands during cell
growth maintaining the structural integrity of the bead. Cast alginate beads have an
average diameter of 0.83 ± 0.3 mm, after 25 h the diameter of the alginate/cell beads
expanded ~6% to 0.90 ± 0.4 mm a 25% increase in volume. Growth of cells within the
alginate beads led to cells being ejected as cells grow. Despite the loss of cells from the
beads, the overall cell density within the bead stays constant allowing for the signal
strength to be unaffected by this change. Ejected cells that may negatively affect the
NMR spectra were washed out of the bioreactor by the flow of medium so that they will
not contribute to the spectra.
Figure 3.2. Schematic of the cell casting apparatus and time course of the energy charge of cells in the bioreactor. (A) An atomizer was used to cast *E. coli* in 1% alginate. 150 mM CaCl₂ was used to polymerize alginate into beads upon contact. (B) and (C) Continuous medium exchange maintains high levels of phosphate-containing metabolites. (B) In-cell $^{31}$P spectra of phosphate-containing metabolites in *E. coli* without (red) and with the bioreactor (black). (C) Signal intensity of intracellular $^{31}$P-containing metabolites over time.

A previous study$^{53}$ categorized *E. coli* cells into three different metabolic states based on the adenylate energy state. The high energy state is characterized by a high concentration of ATP, vigorous cell growth and a high metabolic state. For cells in a stationary phase of a growth cycle or during times of malnourishment, the concentration of ATP decreases and cells enter an intermediate energy state while retaining viability and the ability to form colonies. As the ATP concentration decreases further metabolism...
becomes inactive and cell death may occur. Improvements to bioreactor technology allows for cells used during in-cell NMR studies to achieve a high metabolic state with a high concentration of ATP allowing the study of functional PPIs. Studies were conducted on *E. coli* because these three distinct energy states can be clearly differentiated.

Assessing the viability of *E. coli* within the improved bioreactor with and without the flow of fresh medium (Figure 3.2 B, C) allowed for the metabolic state of the cells to be determined. As cells enter different stages of growth, changes in intensity in the $^3$P spectra reflect fluctuations in intracellular concentration of phosphate containing metabolites, ATP, ADP, NAD+, and NAD(H), as the experiment progressed. Observing the level of ATP within the cell informs about the metabolic state of the cells. Using the bioreactor to provide fresh medium, cells sustained a constant viability over a 24 h period unlike cells without continuous exchange of medium, which saw a drop in phosphate containing metabolites by 10% after 8h, 30% after 12h, and 40% after 24h. Maintaining a high level of phosphate containing metabolites is consistent with a high energy state and growing, metabolically active cells.

**Protein overexpression**

To best illustrate the capability of the improved bioreactor to achieve a high metabolic state, protein expression in *E. coli* was achieved and monitored in real time. Because agarose was replaced with alginate as the supporting matrix within the bioreactor, growth medium was designed to contain a minimal amount of phosphate. This was due to the fact that phosphate chelation of calcium from the alginate beads break down the encapsulating beads and releases cells into the medium. The hybrid
growth medium, HGM, used to express protein in cell cast in the alginate matrix is similar to M9\textsuperscript{131} but is buffered with HEPES instead of phosphate to prevent the decomposition of the alginate beads. Supplementing the HGM with \([U^{\text{15}}\text{N}]\) ISOGRO (Sigma Aldrich) powder during the uniform expression of \textsuperscript{15}N-labeled proteins by providing the cells with \textsuperscript{15}N-labeled metabolic precursors reducing the cellular energy required for protein expression as \textit{de novo} metabolites are not required. For overexpression of unlabeled proteins, ISOGRO is replaced with yeast extract and tryptone.

Overexpression of Mpa within the bioreactor confirmed that the improvements lead to the ability to maintain cells in a high energy metabolic state with a high concentration of intracellular ATP, enough to support ATP dependent processes such as protein overexpression. Overexpression of unlabeled Mpa was conducted within the bioreactor over a 24 h period at 37 °C on the benchtop. Expression levels were monitored through the removal of beads from the bioreactor every two hours and electrophoresis on a 10 % denaturing polyacrylamide gel (FIGURE 3.3 A). The cellular concentration of Mpa saw a steady increase over a 12 h period followed by a decrease over the final 12 hours. The decrease in cellular concentration of Mpa was expected as intracellular proteases degrade overexpressed proteins over time.
Figure 3.3 Overexpression of Mpa and Pup in the bioreactor. (A) SDS-PAGE of Mpa overexpression. Lanes 1-7 are Mpa expression samples collected every 2 hours for 12 hours, lane 8 is Mpa expression after 24 hours. (B, C, D, E) $^{15}$N-edited SOFAST-HMQC spectra of Pup overexpression in the bioreactor during in-cell NMR after 0 (B), 6 (C), 12 (D), and 24 (E) hours.
Overexpression of $[U^{15}\text{N}]$ Pup by the bioreactor was monitored inside the NMR spectrometer by using SOFAST-HMQC NMR (Figure 3.3 B,C,D,E). Assignments of the in-cell NMR spectra of Pup were identified from the cross-peaks of purified Pup. Similar to the expression of Mpa, the Pup spectrum reached maximum intensity after 12 hours. After 24 hours spectral peaks for all 64 amino acids were still present but lower in intensity. The ability to observe the overexpression of proteins expands the versatility of in-cell NMR and allows for functional PPIs to be studied.

Functional protein-protein interactions

Illustrating the expanded ability of the bioreactor, functional protein-protein interactions were observed. The interaction between Pup and Mpa was revisited with the aid of real time using RT-STINT NMR spectroscopy and the bioreactor. Previous characterization of the Pup-Mpa $^{90,132}$ interaction obtained both by *in vitro* studies and by STINT in-cell NMR serve as a comparative standard for the ability of RT-STINT NMR and the bioreactor to discern the interaction within the cellular environment. STINT-NMR allows for the changes in the NMR spectra of a $[U^{15}\text{N}]$ target protein to be monitored as the concentration of the interacting unlabeled protein increases over time $^{109}$. To properly preform a RT-STINT NMR study, both the target and interactor protein were expressed in the bioreactor.

$[U^{15}\text{N}]$ Pup was overexpressed for 12 hours in the bioreactor on the benchtop before the bioreactor was transferred to the NMR. Prior the expression of unlabeled Mpa, an $^{15}\text{N}$ edited SOFAST-HMQC in-cell spectrum of Pup was collected to establish the base spectrum for each sample. Spectra were collected in hour intervals with
minimal cell growth occurring over this time. Cross peaks in the spectra of Pup associated with the interaction with Mpa broadened as the intracellular concentration of Mpa increased over time (Figure 3.4 A). Cross peak intensities were compiled into matrix, M, and subjected to analysis using SVD to distinguish the residues involved in the binding interaction.\(^9\)

![Figure 3.4 RT-STINT NMR of the Pup-Mpa interaction.](image)

**Figure 3.4 RT-STINT NMR of the Pup-Mpa interaction.** (A) Overlay of $^{15}$N-edited SOFAST-HMQC spectra of Pup before (red) and after (blue) 12 h of Mpa overexpression. (B) Apparent binding isotherm of Pup to Mpa in *E. coli*.

Affinity of the Pup-Mpa interaction was estimated using RT-STINT NMR as a test of the reliability of the technique (Figure 3.4 B). To do so the benchtop expression of Mpa was quantified. Exact quantification could not be achieved as the in-cell spectra of Pup displayed significant overlap within peaks involved in the Pup-Mpa interaction. To best estimate the affinity of the Pup-Mpa interaction differential broadening of the I18 peak of the in-cell Pup spectra was correlated with the concentration of Mpa found in
the benchtop overexpression. Because it is not possible to distinguish the cells that only expressed either Pup or Mpa and did not participate in the Pup-Mpa interaction, the analysis of binding is an approximation at best. The resulting curve was fit with an apparent $K_d$ of 28 ± 3 μM, which is in rough agreement with the previous estimated value of 3-4 μM$^{132}$.

SVD analysis of the Pup-Mpa interaction allows for the amino acids involved in interaction to be identified. Scree plots of the singular values generated by SVD analysis represent the contribution from each binding mode, fitting Scree plots with a linear regression helped determine the presents for real signal vs spectra noise. A poor linear fit $r^2 = 0.61$ in the scree plot from the SVD analysis of the Pup-Mpa interaction revealed the first binding mode constituted a real signal that was distinguished from the noise (Figure 3.5A). Removal of the first binding mode from the scree plot of Pup-Mpa interaction results in a good linear fit $r^2 = 0.99$, confirming the additional bind modes constitute noise.
Figure 3.5 Functional Pup-Mpa interaction in metabolically active cells. (A) and (B) Singular values and changes in cross peak intensity for each amino acid upon binding to Mpa for the first (blue) and second (red) binding modes. The threshold line was set to the maximum contribution of the second binding mode, which represents the average noise of the NMR spectra. Continuous and dashed lines in (A) represent linear fits of SVs with and without first binding mode, respectively. (C) Residues comprising the first binding mode (red) between Pup (blue) and Mpa (gray) are mapped onto a Pup–Mpa complex (PDB 3M9D). (D) The mechanism of Pup threading into the Mpa cavity can be observed in-cell using the improved bioreactor.

SVD analysis of the Pup-Mpa interaction revealed the interaction surface within the helical section of Pup, residues 20-52, confirming what was previous reported both in vitro and by using STINT in-cell NMR. SVD analysis also identified a second surface of interaction previously unobserved that encompasses the N-terminus of Pup.
(Figure 3.5 B,C). N-terminal interactions implicate the presence of an ATP-dependent process that is critical for pupylated substrates to enter the proteasome for degradation. ATP-dependent interactions were only able to be visualized due to the improvements in bioreactor technology that made maintaining a high metabolic state possible.

Striebel et al. demonstrated such a result in the absence of ATP, they showed that without ATP the pupylated substrates bound to the Mpa coiled coil domain in the helical region of Pup but no degradation of the pupylated substrate was observed. Mpa does not possess the ability to unfold Pup in the absence of the Pup N-terminal interactions with the interior channel of Mpa. The ATP-dependent interaction of the Pup N-terminus allows the pupylated substrate to be threaded through the Mpa channel (Figure 3.5 D).

3.4 Discussion:
Previous studies that employed STINT-NMR in metabolically inactive cells to characterize the interaction between Pup and Mpa, were able to visualize the interaction between the C-terminus of Pup and Mpa coiled coil but failed to see the ATP-driven interaction between the N-terminus of Pup with the inner channel of Mpa. The lack of visibility of the N-terminal interaction in the previous studies was due to insufficiently high levels of ATP within the cells during traditional in-cell NMR. To best visualize such high energy interactions using in-cell NMR, cell health must be maintained for the duration of the NMR experiment. The improvements in bioreactor technology made maintaining a high energy state in *E. coli* possible allowing for the study of functional protein-protein interactions to be monitored in real time.
The metabolic state has an effect on protein-protein interactions in the cell\textsuperscript{106}. Metabolic enzymes and complexes in the cell are often regulated by allosteric inhibitors and enhancers that are present in the cell at a steady state concentration\textsuperscript{138} rendering the creation a cellular environment mimic increasingly difficult. A potential way to bypass the need for a cellular mimic by conduct studies within living cells. Allowing for a contestant metabolic state to me maintained for the duration of the in-cell study. By monitoring the level of ATP within bacteria during an in-cell study we can glean information about the stability of the metabolic state of cells. Bacteria, which do not carry an internal ATP buffering system, allow for the probing of the metabolic state as the ATP concentration is exclusively the result of synthesis and degradation\textsuperscript{139, 140}. Real time in-cell NMR with a bioreactor allows for the metabolic state of a cell to remain at a high level so that the structure and function of protein-protein interactions and be examined. The improved bioreactor shown here is able to maintain a high metabolic state within \textit{E. coli} expanding the capabilities of in-cell NMR to study functional protein-protein interactions.
CHAPTER 4: Metabolic studies by using bioreactor-based NMR spectroscopy

4.1 Introduction

The metabolic state of a cell can provide information important for drug discovery, disease diagnostics and changes in biochemical pathways that result from mutations, aging, diet, exercise, or lifestyle\textsuperscript{141, 142}. Studying the metabolic states of a cell of requires the quantification of small low molecular weight (<1 kDa) molecules such as amino acids, carbohydrates, cofactors, fatty acids and nucleotides in the cellular environment\textsuperscript{143}. Metabolomics looks to provide a description of the physiological state of an organism as it responds to changes in environments.

In-cell NMR offers a non-invasive technique permitting metabolomics studies to be carried out within living cells\textsuperscript{144}. In-cell NMR offers the opportunity to observe metabolites that are often missed by traditional metabolomics studies that rely on mass spectrometry as they are either too difficult to ionize or require derivatization\textsuperscript{145}. Samples intended for in-cell NMR metabolomics studies need minimal to no sample preparation reducing the analytical variability between samples\textsuperscript{145}. In-cell NMR is not without its disadvantages as poor sensitivity limits detection to metabolites with concentrations larger than 100 nM\textsuperscript{146}, also limited spectral resolution prohibits metabolites with similar chemical shifts from be visualized, and lastly instruments are costly\textsuperscript{147}. 
Metabolites are highly susceptible to changes in pH, temperature, concentration, and ionic conditions making NMR spectral assignments difficult when the environment is changing during a NMR experiment\textsuperscript{147}. The addition of a bioreactor to metabolomics studies offer a way to minimize the stresses that may alter the metabolic state of a cell\textsuperscript{56}. By employing a bioreactor during in-cell NMR studies, a cell can maintain a high energy state that allows for ATP dependent metabolic pathways to be studied\textsuperscript{148}. In-cell NMR allows for an \textit{in vivo} examination of biological pathways not feasible in traditional \textit{in vitro} studies as many pathways are activated or inhibited by the presence of other enzymes and metabolites and would be too complex to accurately reconstruct \textit{in vitro}\textsuperscript{144}.

Metabolomics studies employ magnetically sensitive isotopes such as $^{13}\text{C}$, $^{15}\text{N}$, and $^{31}\text{P}$ that allow metabolites to be monitored as they progress through a metabolic pathway\textsuperscript{146, 149}. Using $^{31}\text{P}$ allows changes in the energy state of a cell to be determined as $^{31}\text{P}$ is incorporated into phosphate containing metabolites\textsuperscript{148}, and with the use of $^{13}\text{C}$-labeled glucose, downstream metabolites of glycolysis, the TCA cycle, and pentose phosphate pathways can be quantified\textsuperscript{146}. Previous studies utilizing bioreactor technology to studying metabolism suffered from poor spectral resolution due to the use of 10 mm NMR tubes\textsuperscript{57, 70, 150}. Oppositely, high resolution metabolic studies fail to maintain the cellular environment during the collection of the NMR spectra\textsuperscript{151-153}.

1D $^1\text{H}$ NMR spectra have long been used as an analytical tool for identification of cellular metabolites\textsuperscript{154, 155}. And while 1D $^1\text{H}$ NMR provides valuable information, increased spectral overlap of peaks makes identification and quantification of metabolites difficult. $^{13}\text{C}$ edited 2D NMR spectroscopy correlates covalently bonded $^{13}\text{C}$
and $^1$H atoms and allows for spectral overlap in traditional 1D $^1$H NMR to be resolved. While 2D NMR provides enhanced resolution over 1D NMR, the long acquisition times prohibit the use of 2D NMR in real time metabolic studies. Decreasing the acquisition time while retaining enhanced resolution can be achieved by collecting only the first FID in a 2D $^1$H-$^{13}$C HSQC experiment. During acquisition of a 2D $^1$H-$^{13}$C HSQC spectrum the indirect dimension is resolved through the collection of successive FIDs during which the evolution time after the INEPT-like polarization transfer is incremented $^{156}$. Limiting the acquisition to the first FID that is collected before the evolution time is incremented results in a 1D spectrum that is a projection of the total signal. $^1$H-$^{13}$C HSQC limits the detectable signal to only metabolites enriched in $^1$H-$^{13}$C bonds. Due to the 2 % natural abundance of $^{13}$C, metabolites that do not incorporate the $^{13}$C label are filtered out of the final spectra. The result is a 1D isotope edited spectra with enhanced resolution and fast acquisition times, required for real time metabolic studies.

We show in these studies that the use of real-time in-cell NMR combined with isotope edited 1D experiments allows for metabolic pathways to be monitored as $^{13}$C-glucose is metabolized. Here we observe the effect of the diaphanous homolog 1 (Diaph1), a member of the Rho family of GTPases that promotes the elongation of actin filaments within cells under times of stress, on eukaryotic cardiomyocyte, H9C2 cell, metabolism.

4.2 Material and Methods:
Preparation of H9C2 cells

H9C2 cells containing either Diaph1 or Diaph1 knockdown (Diaph-KD) were grown by following a previously reported procedure\textsuperscript{24}. Eight 150 cm\textsuperscript{2} culture dishes (Corning) were each seeded with \textasciitilde{}5 x 10\textsuperscript{6} of H9C2 cells (ATCC) and allowed to grow to 80\% confluence in complete low-glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10\% fetal bovine serum (FBS,Gibco). To reach 80\% confluence H9C2 cells were incubated for 3 days in a 5\% CO\textsubscript{2} incubator at 37 °C. Once confluence was reached cells were collected by exposing the cells to 0.25\% trypsin/EDTA (Sigma-Aldrich) for 15 min in a 5\% CO\textsubscript{2} incubator at 37 °C. After the incubation trypsin was neutralized with a 5-fold dilution of DMEM. To collect the H9C2 cells, the medium was centrifuged at 200g for 10 min at 25 °C and the resulting pellet was washed with phosphate-buffered saline. Eight 150 cm\textsuperscript{2} culture dishes at 80\% confluence resulted in \textasciitilde{}3 x 10\textsuperscript{7} H9C2 cells for in-cell NMR study. 1 μg/mL of puromycin was used to select for cells with the Diaph1-KD. Puromycin selects for cells that contain the transformed vector, which expresses a short hairpin RNA (shRNA) cassette complementary to the mRNA for Diaph1. The shRNA causes RNA interference (RNAi) with the mRNA for Diaph1 forming an RNA-induced silencing complex (RISC) that facilitates the degradation the Diaph1 mRNA\textsuperscript{157,158}. The result is cells still contain the gene for the expression of Diaph1 but expression is silenced. H9C2 cells containing Diaph1 and Diaph1-KD were provided by collaborators at NYU Langone medical center.

Casting

Alginate/cell beads were cast with an atomizer\textsuperscript{124} that allowed reproducible control of bead size. Pelleted cells (%500 μL) were mixed 1:1 (v/v) with a 2\% alginate
solution in Heart buffer salts, HB salts, 50 mM HEPES, pH 7.2, 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, and 3 mM NaH₂PO₄. The alginate/cell mixture was loaded into a 3 mL syringe fitted with a Luer-lok tip connected to a 40 mm length of tygon tubing (I.D. 0.79 mm) with a blunt 21 gauge needle fixed on the end and placed at a 45 ° angle under an airflow. The alginate/cell mixture was injected into the atomizer by a syringe pump (New Era Pump System NE-300) at a rate of 300 μL/min. Cells were cast with an airflow rate of 5.5 liter per minute. The atomizer was centered over 25 mL of 150 mM CaCl₂, as the alginate/cell mixture was passed through the atomizer and collected in the CaCl₂, Ca²⁺ polymerized the alginate encapsulating the cells within the beads. The 150 mM CaCl₂ solution was decanted off the beads and replaced with 25 mL of complete HB supplemented with 5 mM glucose, 0.4 mM palmitate, 0.4 mM BSA, and 70 mU/L of insulin before the beads were placed into the bioreactor.

Bioreactor
The bioreactor used to conduct metabolic studies was identical to the bioreactor described in chapter 3 with the addition of a sample loop. A sample injector (Rheodyne) was placed 120 cm down the inlet line from the fresh medium reservoir. The sample injector was fit was a 5 mL injection loop constructed out of polyethylene tubing (Clay Adams) with an I.D. of 1.19 mm. The addition of the sample injector allows for the rapid introduction of an analyte to the cells within the bioreactor expanding the capability of the bioreactor to conduct pulse-chase analysis. HB was supplemented with 5 mM ¹³C glucose and loaded into the injection loop, allowing for metabolomics studies to be conducted as the cells digest the isotopically labeled glucose. HB containing ¹²C glucose was flowed through the bioreactor at a rate of 100 μL/min and allowed to
equilibrate providing time for the cell/alginate beads to settle into their final position within the bioreactor. To establish a metabolic steady state prior to the injection of the HB containing $^{13}$C glucose, HB containing $^{12}$C glucose was flowed through the bioreactor for 1.5 hours, during this time $^{31}$P spectra of the ATP signal were collected to determine the viability of cells in the bioreactor.

NMR experiments

All NMR spectra were recorded at 300 K using a 600 MHz Avance III NMR spectrometer equipped with a QCI-P cryoprobe (Bruker). $^{13}$C-isotope edited $^1$H spectra were acquired with 32 scans. The spectral widths in the $^1$H and $^{13}$C dimensions were 16 and 80 ppm respectively and were digitized by 2048 and 1 points in the $^1$H and $^{13}$C dimensions. The time of acquisition for each $^{13}$C-isotope edited $^1$H spectra experiment was 48 seconds. For each experiment an $^{13}$C-isotope edited $^1$H spectra was collected prior to the injection of the $^{13}$C labeled medium to establish a baseline reading of the metabolites present in the sample. Once injected 120 consecutive $^{13}$C-isotope edited $^1$H spectra were collected over a 1.5 hr period allowing the digestion of the $^{13}$C-glucose in the medium to be monitored as it progressed through different metabolic pathways.

Prior to each trial in the metabolomics study a proton- decoupled $^{31}$P spectrum was collected. Doing so allowed the metabolic state of the cell to be assessed prior to the study through the presence of an ATP signal. The $^{31}$P peak intensity at $-11.5$ ppm contains ATP and ADP as well as diphosphate of NAD+ and NAD(H) all vital
metabolites and can be used as a good assessment of cell vitality. All spectra were processed with Topspin version 3.2 (Bruker).

Data analysis

For the metabolic studies the $^1$H-$^{13}$C HSQC spectra were first analyzed in Topspin version 3.2 (Bruker) allowing for the glucose in each peak to be normalized. To achieve normalization, each spectrum was overlaid with a $^{13}$C-glucose standard, the HEPES peak at 2.90 ppm was scaled to match the signal intensity of the experimental spectrum and the scaling factor was recorded. Each spectra exported from Topspin as an ASCII file. ASCII files for each trial were compiled into a matrix using Excel (Microsoft) and a data within a ppm range of 4.71 to 0.013 ppm were analyzed.

Peak intensities were calculated as $I = \frac{((I/I_{ref}) \cdot S.F._{HEPES}) \cdot S.F._{ATP}}{(I/I_{ref})}$, where $(I/I_{ref})$ represents the normalized peak intensity after background subtraction, S.F._{HEPES} represents the individual scale factor for HEPES, S.F._{ATP} represents the different between ATP spectra of the cells that contain Diaph1 and Diaph1-KD at $-11.5$ ppm, allowing for the two different runs to be compared.

For each trial the first 20 experiments were excluded from analysis as they contained no $^{13}$C signal and represent the void volume of the bioreactor. The change in intensity of each metabolite peak was analyzed, rate of reaction were determined from the regression models that provided the best fit. Because the intensity of the lactate peak reached plateau an exponential regression model was used to fit the data. The one phase association model from Graphpad Prism was used to fit lactate intensities, the equation being $Y = Y_0 + (\text{Plateau}-Y_0) \cdot (1-\exp(-K\cdot x))$, where $Y_0$ is the initial intensity of peaks at time zero, Plateau is the maximum intensity of the peak, $K$ is the rate constant.
Linear regression was used to determine the reaction rates for alanine and glutamate because the intensity of the peaks never reached a plateau and continuously increased in intensity.

4.3 Results
To achieve the best results cells must be free of any outside stresses that may affect the rates of metabolism as the cells respond to metabolic tracer. To alleviate stress, implementing a bioreactor during metabolic studies allows for the metabolic state of cells to be maintained for an extended period of time allowing for studies on the metabolome to take place.

Utilizing NMR active isotopes in metabolomics studies allows for metabolic pathways to be monitored as the isotope is incorporated into different metabolites as the isotope progresses through a metabolic pathway. Doing so requires a steady state of metabolites to first be established. Steady states were reached by continuous flow of medium through the bioreactor that supplied the cells with a known concentration of nutrients to support cellular function. Changing to a $^{13}$C-labeled medium allowed for metabolism to be monitored as the $^{13}$C was incorporated into metabolites.
To facilitate the change from an unlabeled $^{12}$C-medium to a labeled $^{13}$C-medium an injection valve was added along the inlet tubing (Figure 4.1). The injection valve was placed 120 cm from the fresh medium reservoir and contained a 5 mL loop that was filled with $^{13}$C-medium. Upon injection 12 min elapsed before the $^{13}$C-medium was seen by NMR as the void volume on the inlet tubing was replaced. Over the next 11.2 min the $^{13}$C-medium saturated the bioreactor during which time metabolites start to incorporate the $^{13}$C label as glycolysis of the $^{13}$C-glucose begins. A steady state of isotope label metabolites was reached over the following 18.4 min, during this time the isotopic label was observed progressing through the metabolic pathways. The $^{13}$C-medium was depleted from the NMR tube over a 21 min period whereupon the $^{13}$C signal returned to a level prior to the introduction of the $^{13}$C-medium. (Figure 4.2)
Figure 4.2. Introduction of carbon within bioreactor. The amount of $^{13}$C-glucose in the bioreactor was monitored by comparing a 5 mM standard of $^{13}$C-glucose with each experiment. The glucose peak at 3.60 ppm was used to determine the amount of glucose in the NMR tube.

Prior to and following each injection of $^{13}$C-medium, a $^{31}$P NMR spectrum was collected to assess the viability of cells within the bioreactor. Previous studies have shown that the ATP signal rapidly decreases as the cellular environment changes leading to cell death$^{66,148}$. Maintaining a strong ATP signal correlates to a high metabolic state within the cells. The strong signal intensity at $-11.5$ ppm, which contains contributions from the $\alpha$-phosphate of both ATP and ADP and diphosphate of NAD+ and NAD(H), allows for the determination that cells are in stable metabolic state for the duration of the metabolic study. Cells was determined to be stable metabolic state as the signal intensity of the $^{31}$P spectra remained constant. (Figure 4.3)
Figure 4.3. Energy state of the cell remain unchanged during metabolic studies. $^{31}$P spectra of cardiomyocytes show that ATP levels remain constant before and after metabolic studies. Blue) before study. Red) after study

One of the disadvantages of conducting metabolomics studies by NMR is the increased complexity of proton NMR spectra as all metabolites can be observed and overlapping of spectral peaks leads to the masking of metabolites of interest as they may have a similar chemical shifts. In order to simplify the 1D spectra to allow for only the metabolites of interest to be observed, $^{13}$C-isotope edited $^1$H spectra were collected. The collecting of data was limited to a single FID in the carbon dimension, doing so resulted in 1D spectra that result only from metabolites that contain the $^{13}$C label thus simplifying the resulting spectra to only peaks of interest. One disadvantage of collecting 2D spectra is that data collection varies from several minutes to hours,
Because glycolysis is achieved over a ~10 min time scale and TCA over a ~ 2 hr \(^{149}\), each spectrum must be collected on a time scale that will allow multiple points to be collected as the amount of metabolites change. By reducing the collection of a traditional \(^1\)H-\(^{13}\)C HSQC spectrum to a single FID in the carbon dimension the total acquisition time of each experiment is reduced to 48 seconds allowing for metabolic rates to be approximated. Prior to each injection of \(^{13}\)C-medium a \(^{13}\)C-isotope edited \(^1\)H spectrum was collected to establish the background intensity of metabolites. After each injection consecutive \(^{13}\)C-isotope edited \(^1\)H spectra were collected over a 1.5 hour period allowing the \(^{13}\)C-metabolites to be monitored while they progressed through glycolysis and the TCA cycle.

\[\text{Figure 4.4.} \quad ^{13}\text{C}-\text{isotope edited} \quad ^1\text{H spectra of metabolites in cardiomyocytes contain a Diaph1 knockdown.}\]

The first FID in a \(^1\)H-\(^{13}\)C HSQC spectra allows for a one dimensional spectrum to be collected. Peaks in the resulting spectra consist of only metabolites that incorporated the \(^{13}\)C label.
From the 1D spectra of cardiomyocytes it was determined that three metabolites; lactate, alanine, and glutamate can be used to analyze the change in metabolism in-cell as a result of the presence or absence of Diaph1 (Figure 4.4). These metabolites were selected for metabolic analysis as each metabolite generated an isolated peak within the 1D spectra that resulted solely from the metabolite and not from overlapping metabolites. As the $^{13}$C label is incorporated into the metabolic pathways the changes in peak intensity over time are monitored. Monitoring the changes in lactate, alanine, and glutamate we can observe the changes in metabolism in response to the presence and absence of Diaph1.

To test the hypothesis that the presence of Diaph1 in cardiomyocytes changes the cell’s metabolism, the metabolic rates of lactate, alanine and glutamate were determined in-cell. We believe that the presence of Diaph1 in cardiomyocytes restricts the movement of mitochondria within the cytosol affecting the cell’s ability to utilize the TCA cycle and other metabolic pathways that occur within the interior of the mitochondria. Without the ability to move glycolysis products into the TCA cycle, pyruvate is ushered into other metabolic pathways, which will lead to an increase in the cellular concentration of metabolites such as lactate and alanine, for this reason we use lactate and alanine as reporters for glycolysis. In the absence of Diaph1 the cell will have full access to the TCA cycle, glutamate can act as a reporter of the TCA cycle as it is commonly produced through the conversion of α-ketoglutarate, a TCA intermediate. We believe that the restriction of the mitochondria’s movement in the cytosol can be visualized by changes in glutamate biosynthesis in cardiomyocytes containing the Diaph1 protein.
Figure 4.5. In-cell metabolic rates in the presence and absence of Diaph1. Metabolic rates were determined for lactate, alanine and glutamate with and without Diaph1.

In the presence of the Diaph1 protein we observed that the cellular concentration of lactate was twice that seen in the Diaph1-KD cells. Although the cellular concentration of lactate was larger in the Diaph1 cells the metabolic rate was slower \( k = 488 \ \mu M/s \) when compared to cells that contained the Diaph1-KD, \( k = 1.2 \ mM/s \) (Figure 4.5 A, B). The same effect can be seen when looking at alanine. In the presence of the Diaph1 protein the metabolic rate \( k = 1.95 \ \mu M/s \) opposed to \( k = 7.52 \ \mu M/s \) in cells.
without the Diaph1 protein, confirming that the inability to move glycolysis products into the TCA cycle results in an increase that can be seen in metabolites generated by the glycolysis end product (Figure 4.5 C, D). The hypothesis was further confirmed upon analysis of the glutamate peak which saw a metabolic rate of $k = 4.60 \, \mu M/s$ in cardiomyocytes that did not contain the Diaph1 protein as opposed to a metabolic rate of $k = 7.74 \, \mu M/s$ in the presence of the Diaph1 protein (Figure 4.5 E, F). Additionally we observed a delay in glutamate response in the presents of Diaph1. In the presents of Diaph1 cells took six times as long to generate a glutamate signal oppose to cells with the Diaph1-KD. Cells containing Diaph1 did not produce a glutamate signal till 41.6 min after introduction of the $^{13}$C-glucose while cells with the Diaph1-KD saw a glutamate peak after only 6.88 min. We attribute the delay in glutamate production seen in the presents of Diaph1 to the decrease in mitochondrial motion that results from the increased molecular crowding.

The decrease in the metabolic rate of lactate and alanine along with the display in the glutamate peak intensity in the presence of Diaph1 suggests that the presence of Diaph1 restricts the cell’s ability to access the TCA cycle, as a result metabolites that are associated with glycolysis accumulate within the cell as they are prohibited from any furthers metabolism.

4.4 Discussion
Integration of bioreactor technology with isotopic tracing of metabolites allows for metabolic pathways to me monitored. Monitoring the ATP signal in $^{31}$P spectra showed that minimal changes in intensity, indicating that a steady state of metabolism was maintained for the duration of the metabolic study. Isotope edited 1D $^1$H spectra showed
that lactate, alanine and glutamate best illustrate the changes in metabolism of the cell in different cellular environments. Changes in the 1D spectra revealed that the metabolites that incorporated isotopic label to yield the strongest intensity peaks were lactate, alanine, and glutamate. Bioreactor technology has increased the analytical capability of in-cell NMR showing that metabolic rates can be observed within the cellular environment to help understand the effects proteins have on the cell.

The presence of Diaph1 in the cell regulates the production of actin, increased concentrations of filamentous actin in the cellular environment results changes in the extent of molecular crowding and limits the movement of mitochondria in the cell.\textsuperscript{164, 165} Observing the TCA cycle, which occurs within the mitochondria, the central hub for energy and biosynthesis in the cell,\textsuperscript{166} allowed for the effect of increased molecular crowding to be assessed. It was observed that restricting mitochondrial motion with Diaph1 lead to a decrease in the cellular production of glutamate. While the cellular production of lactate and alanine was unaffected by Diaph1 the rate of metabolism was less than half observed in cells without Diaph1 which reflects the limited metabolism that results from the restriction of the movement of mitochondria. We observed that when the movement of mitochondria was restricted the metabolism rates of lactate and alanine were also reduced leading to the assumption that metabolism as a whole is reduced in the presence of Diaph1.
Chapter 5: Conclusion

Quinary interactions of a protein are affected by changes in the cellular environment\textsuperscript{55, 167}. Maintaining a stable cellular environment minimizes the effect cellular stress has on the structure of a protein\textsuperscript{56}. Using bioreactor technology allows for a stable cellular environment to be maintained during in-cell NMR studies. By continuously supplying cells with fresh growth medium, stress that arise from changes in pH, redox state and waste buildup are minimized\textsuperscript{56}. Implementation of horizontal drip irrigation with bioreactor technology allowed for delivery of fresh medium throughout the entire cell suspension facilitating the exchange of nutrients while minimizing cell death during an in-cell NMR study. Monitoring the $^{31}\text{P}$ spectra of cells within the bioreactor demonstrated that a high metabolic state can be maintained for more than 24 hours.

Improvements to the bioreactor expanded the capability of in-cell NMR to include detection of protein-protein interactions by facilitating the ability to overexpress protein during a NMR experiment. To improve the consistency of the bioreactor, the horizontal drip irrigation with orifices was replaced by ultrahigh molecular weight porous polyethylene, UHMW, and a peristatic pump to regulate the flow of medium. Changing the encapsulating matrix from agarose to alginate maintained a cell density required for in-cell NMR while providing the flexibility needed to support an active metabolic state. The high metabolic state was confirmed through the overexpression of $[\text{U}^{15}\text{N}]$ Pup and Mpa within the bioreactor.

Using bioreactor technology changes in the quinary interactions of Trx were detected in response to antibiotic stress. By coupling real time in-cell NMR with SVD
analysis it was concluded that the observed spectral broadening of Trx was a result of changes in quinary structure between Trx and mRNA. Tetracycline and streptomycin resulted in strong changes in the quinary structure of Trx as both inhibit the binding of the 30S ribosome subunit to mRNA. Decreases in the concentration of ribosome along mRNA may facilitate the increase in quinary interactions of Trx with mRNA in the cell.

We show here that employing bioreactor technology for STINT-NMR allows protein interactions that are dependent upon a high metabolic state to be observed within the cell. Previous studies employing STINT-NMR to observe the interaction between Pup and Mpa failed to see the interaction involving the N-terminus\textsuperscript{90}. The threading of the N-terminus of Pup through the inner channel of Mpa is an ATP dependent process that previous studies failed to observe as cells were not maintained in an active metabolic state. By using bioreactor technology we showed that in an active metabolic state, protein expression can be observed within the bioreactor allowing for STINT-NMR to be utilized. Real-time in-cell NMR allows for the interaction of the Pup N-terminus to be characterized within the cellular environment.

Lastly we showed that with the addition of the injection loop to the bioreactor, metabolic tracing can be conducted by NMR. Isotopic edited 1D experiments decreased the complexity of traditional 1D NMR allowing only metabolites that contain the $^{13}$C label to be observed allowing for the rapid sampling rate required for determining metabolic rates. Metabolism was traced as cardiomyocytes incorporated a $^{13}$C label in the presence of the Diaph1 protein. Changes in the metabolic rates of lactate, alanine and glutamate were affected by the presence of Diaph1 in the cell, as Diaph1 limited the ability of the cell to access the TCA cycle.
Bioreactor technology has greatly enhanced the field of in-cell NMR by reducing the stress exerted on the cell during long NMR experiments. In-cell NMR allows protein interactions to be observed within the cellular environment, but as the cells respond to stress the changing cellular environment can mask the interactions of interest.

Bioreactor technology eliminates any changes in the in-cell NMR spectra that arise from the changing cellular environment so the protein interactions can be observed.
References:


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