Biophysical Characterization of a De Novo Elastin

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BIOPHYSICAL CHARACTERIZATION OF A *DE NOVO* ELASTIN

by

KELLY NICOLE GREENLAND

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ABSTRACT

BIOPHYSICAL CHARACTERIZATION OF A DE NOVO ELASTIN

by

Kelly Nicole Greenland

Adviser: Dr. Ronald L. Koder

Natural human elastin is found in tissue such as the lungs, arteries, and skin. This protein is formed at birth with no mechanism present to repair or supplement the initial quantity formed. As a result, the functionality and durability of elastin’s elasticity is critically important. To date, the mechanics of this ability to stretch and recoil is not fully understood. This study utilizes de novo protein design to create a small library of simplistic versions of elastin-like proteins, demonstrate the elastin-like proteins, maintain elastin’s functionality, and inquire into its structure using solution nuclear magnetic resonance (NMR).

Elastin is formed from cross-linked tropoelastin. Therefore, the first generation of designed proteins consisted of one protein that utilized homogony of interspecies tropoelastin by using three common domains, two hydrophobic and one cross-linking domains. Basic modifications were made to open the hydrophobic region and also to make the protein easier to purify and characterize.

The designed protein maintained its functionality, self-aggregating as the temperature increased. Uniquely, the protein remained self-aggregated as the temperature returned below the critical transition temperature. Self-aggregation was additionally induced by increasing salt
concentrations and by modifying the pH. The protein appeared to have little secondary structure when studied with solution NMR.

These results fueled a second generation of designed elastin-like proteins. This generation contained variations designed to study the cross-linking domain, one specific hydrophobic domain, and the effect of the length of the elastin-like protein. The cross-linking domain in one variation has been significantly modified while the flanking hydrophobic domains have remained unchanged. This characterization of this protein will answer questions regarding the specificity of the homologous nature of the cross-linking domain of tropoelastin across species. A second protein has additional hydrophobic domains flanking the originally designed elastin-like protein. The characterization of this protein will answer questions regarding the functionality of longer or more hydrophobic elastin-like proteins. The final variation designed is one hydrophobic domain and the new cross-linking domain repeating several times. The characterization of this protein will answer questions regarding the specific hydrophobic domain and its functionality.
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CHAPTER 1: INTRODUCTION

This dissertation is aimed at gleaning new information regarding elastin by studying small model elastin-like proteins that contained strong homology with the monomeric protein tropoelastin which forms elastin when cross-linked with additional copies of tropoelastin. This work is broken down into several sections: background, which pertains to techniques utilized in the study of the model proteins, literature review, which briefly covers what other studies have been done with tropoelastin model elastin-like proteins, the new research that was done, and future directions to lead subsequent investigations.
2.1: PROTEIN BACKGROUND

2.1.1: AMINO ACIDS

Proteins are comprised of twenty amino acids grouped into general categories of polar or non-polar, charged or uncharged, hydrophobic or hydrophilic, or if they possess special properties as seen in Figure 1. Polar amino acids can further be separated into positively charged, negatively charged or non-charged species, as depicted below. The special properties that can typically be utilized to characterize amino acids include aromatic side chains, side chains that can form disulfide bridges, or side chains that have unique folding properties.\(^3\)

Amino acids are traditionally read from the amine NH\(_3^+\) terminus to the carboxylic acid COO\(^-\) terminus. They are attached to each other via a peptide bond when the hydroxide on the carboxylic acid terminus is removed upon reaction with the amine terminus of another amino acid, forming water and bonding the two termini together. The two amino acids are then linked, forming the beginning of a protein backbone.\(^4\)
The twenty amino acids separated into common traits. A. Amino acids with charged side chains. B. Amino acids with polar but uncharged side chains. C. Special case amino acids. D. Amino acids with hydrophobic side chains.

FIGURE 1: AMINO ACIDS

The twenty amino acids separated into common traits. A. Amino acids with charged side chains. B. Amino acids with polar but uncharged side chains. C. Special case amino acids. D. Amino acids with hydrophobic side chains.
2.1.2: PRIMARY STRUCTURE

As mentioned earlier, a protein is a bonded chain of amino acids. Thus the primary structure is the sequential order of amino acids. As an example, a protein that will be discussed later, \( 20'_{21-23} 24'_{21-23} 24' \), contains 182 amino acids, with the primary structure of the protein displayed in Figure 2. Each amino acid has been represented by either a one-letter symbol designation instead of the full name for each amino acid or the three letter designation seen in Figure 1. The one-letter designation for each amino acid is listed in Figure 3.

\[
\text{WVPGVGGVPVGVPVGVPGVGPVGVPVPVGGVGPVGGVGPVGGV}
\]
\[
\text{EAQAAAAKAAYGVPAAAAAAKAAAQAQFG}
\]
\[
\text{APGVGVAPGVGVAPGVGVAPGVGVAPGVGVAPGVGVAPGVGV}
\]
\[
\text{EAQAAAAKAAYGVPAAAAAAKAAAQAQFG}
\]
\[
\text{APGVGVAPGVGVAPGVGVAPGVGVAPGVGVAPGVGVAPGVGVW}
\]

FIGURE 2: PRIMARY SEQUENCE OF 20'_{21-23} 24'_{21-23} 24'

Protein 20'_{21-23} 24'_{21-23} 24' is hydrophobic domain 20', the first line of the sequence, followed by cross-linking domain 21_{23}, which is the second and fourth line of the sequence. This connects to the hydrophobic domain 24' which is the third and fifth line of the sequence. Hence, the name of the protein is each domain as it appears in the sequence.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>1-Letter Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>A</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>I</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
</tr>
</tbody>
</table>

FIGURE 3: ONE LETTER AMINO ACID CODE
Protein secondary structure is the initial folding of areas of the protein into lower energy conformations. The most common structures, alpha helices and beta sheets, are displayed in Figure 4. A complete understanding of folding is still an unsolved problem in biophysics, but progress has been made\(^5\). Proteins are believed to fold due to driving forces including hydrophobic interactions, hydrogen bonds, ion pairs, and space-limiting Van Der Waals interactions.\(^6\)

**FIGURE 4: PROTEIN SECONDARY STRUCTURE**\(^7\)
On the left is an example of an alpha helix and on the right is an example of a beta sheet, two of the most common examples of secondary structure within a protein.
2.1.4: TERTIARY AND HIGHER ORDER STRUCTURE

Tertiary structure of a protein is formed when the entire protein completely folds into the lowest conformational energy state. This structure may have one or more secondary structures with or without unfolded areas. Quaternary structures are possible when multiple proteins, or copies of the same protein, come together into a unique structure that further reduces the energy state of each protein. A common example of this is hemoglobin, which has two copies of chain A and two copies of chain B associated into a heteroquartamer, as is seen in Figure 5.

One could argue that elastin’s quaternary structure is cross-linked monomeric tropoelastin. However, this also raises technical issues since lysyl oxidase is required to modify the cross-linking lysine residues before the tropoelastin can cross-link to form elastin.

Figure 5: Hemoglobin
Hetero-tetramer hemoglobin imaged with two A subunits in red and two B subunits in blue.
2.2: CHARACTERIZATION BACKGROUND

Proteins possess a plethora of properties that allow them to be studied. Several non-invasive methods of characterization which do not require high concentrations of proteins utilize ultraviolet and visible wavelengths of light to identify properties of the protein. These techniques were utilized to characterize proteins within this study and merit a brief explanation.

2.2.1: ULTRAVIOLET-VISIBLE SPECTROSCOPY

Ultra-violet (UV-Vis) spectroscopy uses light in the 200-1000 nm wavelength region to excite ground state electrons to available excited states. The transition is called absorption, as the photon is “absorbed” by the electron in the ground state and used to transition to the excited state. The energy released when the electron transitions back to the ground state is typically thermal energy; there is no photon emitted. The spectrophotometer, the machine used in UV-Vis spectroscopy, measures the intensity of the photons passed through a solution sample and compares it to the initial intensity of the photons. The spectrophotometer then takes the negative logarithm of final intensity divided by the initial intensity; the resulting value is absorbance.

\[ A = -\log \left( \frac{I_f}{I_i} \right) \]  

(1)

Beer’s Law states that the absorbance, A, is linearly related to the concentration of the absorbing species, listed as C in Equation 2.

\[ A = \varepsilon CL \]  

(2)

This allows for the determination of the concentration of a sample of protein that contains tryptophan, since tryptophan absorbs photons strongly at 280 nm. Additionally, the length of
sample that the photon passed through is needed, $L$, is typically 0.4 or 1.0 cm, and depends on the sample container. The final variable is known as the extinction coefficient, which is a measurement of the strength that a species absorbs photons. This value is known for each amino acid and the total can be calculated from a protein’s primary structure. This is often accomplished by inputting the sequence into online calculators such as ExPASy ProtParam tool.\(^9\)

The caveat of UV-VIS spectroscopy is that the absorbance value needs to be kept at or below 1.0 because above 1.0 the linear relationship begins to break down. As a result, the highly concentrated samples needed for solution NMR cannot be measured completely. As such, the concentration can be determined by one of two methods, though both methods can introduce error; the degree of error dependent on the accuracy of the volume measurement.

The first method is to measure the concentration of a dilute sample and then concentrate it. Then, using the ratio of initial dilute volume to final concentrated volume, it is possible to determine the final concentration. This method can have significant errors if, for example, the initial volume is 15.0 mL as measured by a 25 mL pipette, which has an accuracy of 1 mL, then the sample is concentrated down to 1 mL, as measured by a 1 mL pipette, which has the smallest division of 2 uL. The uncertainty of the initial volume is half the final volume. A thorough uncertainty derivation is difficult to determine because the absorbance varies, as it is dependent upon the equipment’s lamp, the temperature, and the scale utilized.

A second method for determining the concentration of a highly concentrated sample is to take 10 uL of sample and add it to 990 uL of buffer. This process typically dilutes the sample to a level that will be within the linear region of the spectrophotometer. Next, the dilute sample’s determined concentration value is multiplied by 100 in order to determine the concentration of
the high concentration sample. The ratio does not need to fall in a range of 10 uL to 1 mL if the sample will yield an absorbance value less than 0.1, as the error rate of the absorbance is higher at lower levels. This method also yields potential error if a low absorbance value is recorded for the diluted sample. Additionally, inaccurate measurements may be an issue, but typically the volumes used for this method are measured with glass syringes that have 0.25 uL smallest divisions and 10 uL divisions for the 25 uL volume syringe and 1 mL volume syringe, respectively.
2.2.2: SCATTERING

An additional application of UV-VIS spectroscopy is the ability to detect light scattering. As scattering characteristics are dependent upon the size of the particle in solution, it is necessary to have an idea of the size of the particle. The scattering assay used in this project began with molten-globular protein and as the conditions were modified, it self-aggregated into larger and larger particles. Fundamentally, a 500 amino acid folded protein has an average diameter of 2-3 nm. The largest protein studied with this experiment had 254 amino acids, and even if the protein is molten globular instead of in a unique fold, all proteins can be approximated as 2-3 nm in diameter at the largest. This is 100 times less than the lowest wavelength used in scattering. Consequently, in the regime wherein the proteins have not begun to self-aggregate, Rayleigh scattering approximations are valid. However, because the Rayleigh approximation states particles are at least ten times smaller than the wavelength of the light, as the proteins begin to self-aggregate, the particles become much larger and are no longer valid.

One assay completed had 10 μL of solution removed every 5°C and the samples were placed on glass slides, covered, and sealed. When imaged by a confocal microscope, it was possible to see particles as small as 2 um. Samples taken at temperatures above the critical coacervation temperature had structures that exceeded 50 um. All visible particles were orders of magnitude larger than the wavelength of the light used in the UV-VIS spectrophotometer, so while Rayleigh was no longer the correct scattering regime, it was clear that the light was still scattered.
As previously stated, when the proteins have not begun to self-aggregate, any scattering present falls under Rayleigh scattering. In Rayleigh scattering, the intensity of light scattered is proportional to the inverse wavelength to the fourth power, as seen in Equation 3.

\[ I \propto I_o \left( \frac{1}{\lambda^4} \right) \tag{3} \]

This relationship demonstrates a dependence upon the wavelength of the light that samples the solution. The shorter wavelengths on the blue end of the spectra are therefore scattered more than the longer wavelengths on the red of the spectra. This phenomenon can be seen in scattering assay data presented in Figure 6, particularly in the 50°C data curve.

Once the proteins began to self-aggregate and were on the same scale as the wavelength of light, the scattering entered the Tyndall regime. This scattering is what gives the appearance of milk to be slightly blue, as well as motorcycle exhaust. While Tyndall scattering does not have a rigorous mathematical formulism, if the particle is assumed to be spherical, it can be represented by Mie scattering, an analytical solution to Maxwell’s equation using an infinite series expansion.

It is evident from the assay that as the particles become larger the wavelength dependence changes. As the particles become very large the wavelength dependence appears to be minimally impacted. This phenomenon can be seen in Figure 6, with both the 90°C and 100 °C data. Occasionally, the particles became so large that the scattered light was not detected and the scan had to be repeated when the large particle was no longer in the path. That was believed to be the initial case for the 100°C data, but after several repetitions it was concluded that the data accurately represented the scattering of the large size particles.
Scattering assay of 25 uM 20’-21-23-24’-21-23-24’ in 50 mM Tris, 1 mM CaCl$_2$, pH 6.0. Temperature was increased from 5°C to 100°C at 1°C per minute with constant stirring. The 5c shows slight scattering, while 50c shows more significant Rayleigh scattering. The 90c and 100c data shows scattering from particles at least on the same size scale as the wavelength of the light.

FIGURE 6: SCATTERING ASSAY
Scattering assay of 25 uM 20’-21-23-24’-21-23-24’ in 50 mM Tris, 1 mM CaCl$_2$, pH 6.0. Temperature was increased from 5°C to 100°C at 1°C per minute with constant stirring. The 5c shows slight scattering, while 50c shows more significant Rayleigh scattering. The 90c and 100c data shows scattering from particles at least on the same size scale as the wavelength of the light.
While circular dichroism (CD) was not completed on the proteins in this work at the time of publication, it is a characterization tool which will be utilized. CD is a non-destructive method of protein characterization that needs only a low concentration of protein. CD utilizes a small volume of protein in solution in a narrow cuvette that is inserted into a CD Spectroscopy apparatus. This machine utilizes the same mechanism as absorption spectroscopy utilized in UV-VIS spectroscopy. CD spectroscopy differs from UV-VIS spectroscopy in that the light used to excite the electrons of the protein is polarized.

Light is an electromagnetic wave; the electric field is perpendicular to the magnetic field. Non-polarized light has the components, while still perpendicular, at all different angles so that they average to zero. Linearly polarized light can be thought of as light that has gone through a picket fence. The “fence” would block any wave whose electrical component is not vertical, resulting in all light that has a completely vertical component. In our scenario the picket fence represents a polarizer. In reality, a linear polarizer tends to have a composition wherein long strands are connected excite along the strands but not between the strands. In this case, the waves are transmitted through the long strands but any waves that are not along the direction of the strands will be disrupted.

Circularly polarized light is similar to linearly polarized light except the axis of polarization rotates either clockwise or counterclockwise. The creation of circularly polarized light is most easily created in a two-step process wherein which non-polarized light first passes through a linear polarizer then through a second quarter-wave polarizer. The quarter-wave
polarizer is made of a media that allows light to pass through in one direction quickly while passing more slowly perpendicularly. This results in a net circular polarization.

The circularly polarized light is utilized in assays because left-handed circularly polarized light passes through absorbing material at a different rate than right-handed circularly polarized light. This difference can be detected with the equipment. The difference relates to the chirality of the protein, which relates to the fold of the protein and not the actual structure; *E. coli* expresses the proteins with amino acids of the same natural chirality.

![FIGURE 7: EXAMPLE SIGNALS OF VARIOUS PROTEIN CD CONFORMATIONS](image-url)

The Solid line is alpha helical protein, long dashed line is anti-parallel beta sheet and short dashed line is irregular structure.
Figure 7 shows the two most common protein secondary structures and their CD signals. With the possible exception of the termini, which may be small, non-folded regions, the alpha helical signal was created by using a protein that is completely helical. The beta sheet signal was likewise created by using a protein that is completely folded into the beta sheet conformation with only minimal areas not folded, such as the turns needed to stack the sheets and the termini which may be unfolded. The irregular structure is from a protein which has either no or minimal secondary structure. From these three signals and libraries of similar signals, it is possible to determine information from a protein whose secondary structure is unknown. Algorithms have been created that utilize these large libraries of signals to de-convolute the unknown signal and give the relative percentage of different structures.\textsuperscript{11}
2.3: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY BACKGROUND

Nuclear magnetic resonance (NMR) spectroscopy can be utilized to characterize proteins in a non-destructive fashion, similar to UV-Vis spectroscopy. Crystallography can also determine three-dimensional structure. However, only NMR can be utilized to investigate kinetics and dynamics of proteins.\textsuperscript{12}

Protein NMR utilizes a characteristic of certain nuclei whose spin angular momentum is \(\frac{1}{2}\). Spin angular momentum is a quantum mechanics phenomenon intrinsic to all nuclei. Nuclei that have an odd number of total neutrons have a spin angular momentum of at least \(\frac{1}{2}\); the most commonly studied nuclei being \(^1\text{H}\), \(^{13}\text{C}\) and \(^{15}\text{N}\). Additionally, \(^2\text{H}\) is used, which has a spin angular momentum of 1. Proteins with all or some of these nuclei are placed in a strong magnetic field that orients the nuclei along the field. Then electromagnetic radiation at resonant radio frequencies is pulsed to perturb the aligned nuclei. The nuclei absorb and re-emit the radiation that can be detected, amplified, and mathematically converted into useful information about the nuclei. This phenomenon is called chemical shift. The chemical shift is dependent primarily upon the local environment and can be utilized to determine structure and kinetics of the nuclei. Chemical shift peaks are reported in terms of parts per million (ppm), which is the difference in magnetic field strength.

However, NMR has some drawbacks which include: NMR sample concentrations are significantly larger than UV-Vis samples, NMR often requires a large time commitment on the equipment, and the equipment is expensive.
One dimensional NMR is the first experiment run on a new protein. The spectrum is used as a baseline to compare subsequent samples and conditions. Very small proteins, with less than 20 amino acids, have the potential to be nearly fully defined with 1D experiments. However, as seen in Figure 8, ubiquitin, a protein with 62 amino acids, the chemical shifts overlap to the point where it is impossible to differentiate 62 peaks.

In 1D NMR the $^1\text{H}$ nuclei are excited with electromagnetic energy corresponding to the resonance frequency. Then the signal from the $^1\text{H}$ nuclei is detected, amplified and converted from the time domain using a fourier transform. This experiment required the shortest length of time on the spectrometer, and the number of repetitions depended on sample concentration.

FIGURE 8: UBIQUITIN 1D NMR
One-dimensional solution NMR spectrum of the 62 amino acid protein ubiquitin.
2.3.2: TWO DIMENSIONAL SOLUTION NMR

Two dimensional NMR was used extensively in this research as a method to probe ideal conditions for further studies. The most frequently utilized experiment was $^{15}$N Hetero Single Quantum Coherence spectroscopy ($^{15}$N-HSQC). This experiment excites the $^1$H and then transfers the energy to the $^{15}$N, which in turn transfers energy back to the $^1$H, which is then detected and transformed to chemical shift. Multiple scans are completed, modifying the $^{15}$N, indirect, dimension while the entire $^1$H, direct, dimension is run with each scan. The time devoted in this experiment is therefore dependent upon the concentration of the protein and the number of scans in the indirect dimension.

A NHSQC is capable of relaying folding information regarding a protein as well as aggregation. An unfolded protein will have most peaks all together around 8.0 ppm, while a folded protein will have peaks well dispersed from below 7.0 ppm to 9.0 ppm and even higher. Figure 9 demonstrates a protein completely unfolded, partially folded and fully folded, and the corresponding NHSQC spectra for each condition.

![Figure 9: Apolmyoglobin NHSQC NMR](image)

Apolmyoglobin at various pH resulting in unfolded, partially folded and fully folded protein.
Three dimensional NMR was used in this study to investigate the structure of proteins. The first experiment generally run is an HNCO. This experiment utilizes $^1$H, $^{15}$N and $^{13}$C isotopes. The experiment scans over the full range of $^1$H at selected values of $^{15}$N and $^{13}$C to create slices similar to those in 2D NMR.

Figure 10 is an example of the data set that is collected in an HNCO experiment. On the left is a 3D representation while on the right are two slices; the bottom is at a constant $^{15}$N and the top is at a constant $^{13}$C value.

**FIGURE 10: 3D REPRESENTATION OF HNCO SPECTRA**
The rationale in first running an HNCO as the first 3D NMR experiment can be seen in Figure 10, on the top right. When all the $^{13}$C planes are compressed down onto one $^1$H-$^{15}$N spectra, it should be identical to the NHSQC that is collected via 2D NMR. Therefore, by running this experiment first, it is possible to check parameters and conditions again to maximize resolution, sensitivity, and signal separation before proceeding to the additional suite of 3D NMR experiments.

Typically, 3D NMR experiments geared towards structure determinations are run in pairs. Once a good HNCO spectra has been collected, its pair, the HN(CA)CO, is often run. The reason pairs of experiments tend to be run is the way in which the magnetic energy is transferred from one isotope to another. As seen in the top left of Figure 11, the HNCO experiment starts by the excitation of $^1$H, which then transfers to $^{15}$N. Then the energy is transferred only to the $^{13}$C labeled CO to the left, which is the preceding amino acid along the backbone, often referred to as the i-1 residue. The HN(CA)CO transfers the energy from the $^{15}$N to both the i-1 residue and the $i^{th}$ residue. This is accomplished by transferring through the CA atom, but as it is not the desired signal, the experiment is not tuned to detect the energy from this atom.

As seen in Figure 11, the HN(CO)CA and HNCA are also run as a pair where the CA atom is the desired signal from both the i-1 residue and the $i^{th}$ residue. This is also the case for the HN(CO)CACB and HNCACB experiments. For these experiments, both the CA and the CB signals are desired whereas the CO is not. The rationale for not simply exciting the CA and CB is due to sensitivity issues resulting from magnetic flux, relative population of isotope, and other factors. The sensitivity of $^{13}$C is 64000 times less sensitive than $^1$H. It is for this reason that $^1$H is first excited and then the energy transferred to the other isotopes. Subsequently, the energy is
usually transferred back to be detected. However, this method can take a long time, which allows for relaxation and the decay of signal. Because of this, many researchers choose to run a CBCA(CO)NH instead of an HN(CO)CACB, wherein the CB is directly excited and the energy is transferred to the $^1$H for detection.

**FIGURE 11: 3D NMR EXPERIMENTS**

Pairs of 3D NMR experiments run for backbone assignment of proteins. The top line of experiments is used to identify the i-1 and i$^{th}$ CO. The second line of experiments is used to identify the i-1 and i$^{th}$ CA. The bottom line of experiments is used to identify the i-1 and i$^{th}$ CA and CB atoms.

With these spectra collected, they can be analyzed to connect i$^{th}$ atoms from plane to the i-1 atom. This pattern can be extended to form a chain of atoms that match and begin to form a chain of the atoms in the backbone. Ideally, this chain can be extended forward and backward to completely identify each atom in the backbone. An example of this process can be seen in Figure 12. The far left column is an i-1 atom highlighted in red for the CB on the top row, CA on the
middle row and CO on the bottom row. This is then matched with the \(i\)th atom of another scan. This process can then be continued to connect the backbone. One difficulty in this process is that the amino acid proline does not have an \(i\)th atom peak due to its unique side chain structure. As a result, this breaks the backbone chain into pieces at each proline appearance.

FIGURE 12: 3D NMR PROCESSING\(^{17}\)
Chaining corresponding chemical shift peaks to assign the backbone of a protein. The far left column represents one set of peaks corresponding to the \(i-1\) and \(i-2\) amino acids of the backbone, the second column represents the \(i-1\) amino acid, and the third column has the \(i-1\) and the \(i\)th amino acid. The far right column has the \(i\)th amino acid as well. The top row is the CB chemical shifts, the center row is the CA shifts and the bottom row is the CO chemical shifts.
3.1: MINIATURE TROPOELASTIN

Tropoelastin contains two primary domains: a hydrophobic non-polar region and a hydrophilic cross-linking region. The hydrophobic non-polar region primarily contains the amino acids valine, proline, alanine and glycine. These amino acids typically appear in motifs that repeat four, five or six times. Common motifs include: Val-Pro-Gly-Gly, Val-Pro-Gly-Val-Gly, and Ala-Pro-Gly-Val-Gly-Val. In contrast, the hydrophilic region contains many alanine and lysine residues which are responsible for the cross-linking of tropoelastin to form elastin. It is believed that while the hydrophilic regions are responsible for cross-linking, the hydrophobic domains are responsible for the mechanical properties of elasticity within tropoelastin and elastin.\(^{18}\)

Tropoelastin is found in many other species where one would expect elastin’s functions to facilitate respiration and blood flow, such as in chickens, rats and cattle. The sequences of tropoelastin and the genes that encode this protein within humans and the above mentioned animals are very similar and possess a high-level of homology.\(^{19}\)

Small proteins designed from repeats of the motifs listed above have been designated ELPs, elastin-like proteins. These small proteins can be used to functionalize desired proteins for purification by fusion to the end of desired protein. This process has been designated ELPylation.\(^{20}\)
Coacervation is the reversible self-aggregation initiated by temperature changes. This inverse phase transition of tropoelastin is termed the transition temperature, and it occurs in a narrow window of temperature variation, typically 2-3°C. The self-aggregation can be monitored by measuring scattering and turbidity of the protein solution using UV-VIS spectrophotometry. Below the transition temperature the proteins are soluble and do not scatter photons. Above the transition temperature, the protein self-aggregates and becomes insoluble, scattering photons and increasing turbidity.\textsuperscript{21,22} Self-aggregation and turbidity is completely reversed when the temperature returns to below the transition temperature.

The transition temperature is determined by several factors including buffer, protein concentration, salt concentration, the size of the protein, the ionization influence of side chains of each amino acid, and the arrangement of polar interactions.\textsuperscript{23,24,25,26} The transition temperature will decrease as salt concentration increases or as the number of repeats of the motif increases.
3.3: CROSSLINKING

3.3.1: LYSYL OXIDASE

According to Eyre et al lysyl oxidase is the only chemical needed to cross-link collagen and elastin. It is believed that amino acids near the lysine that are cross-linked have developed as active sites to promote cross-linking. Lysyl oxidase functions by converting the amine side chains of lysine and hydroxylisine into aldehydes. Then the aldehydes condense to covalently cross-link. The crystal structure of lysyl oxidase was determined in 2006 by Duff et al and is shown below in Figure 13.

FIGURE 13: LYSYL OXIDASE
In 2007, Lim et al demonstrated use of an organophosphorous cross-linker, \( \beta\)-[tris(hydroxymethyl)phosphino]propionic acid (THPP). Unlike lysyl oxidase, THPP does not turn the amine group of the lysine into an aldehyde and condense to covalently cross-link. Instead, the amine group of the lysine binds to a hydroxide on the THPP, releasing water. Because THPP has three hydroxide binding opportunities, a THPP has the ability to cross-link three different lysine amino acids. This can be seen in Figure 14.

![THPP Cross-Link](image)

**FIGURE 14: THPP CROSS-LINK\(^{30}\)**

Gluteraldehyde is an additional cross-linking chemical. Its cross-linking mechanism is the same as lysyl oxidase, converting the amine of lysine to aldehyde. However, the selectivity of gluteraldehyde is low, so it will convert all available amines to aldehyde. It is therefore used in sterilization and is considered toxic because of its ability to convert the amine terminus of any protein to aldehyde.
3.4: APPLICATIONS

Given the properties of elastin-like proteins, it has been hypothesized that it should be possible to build switchable nanostructures designed to open or close by fluctuating the temperature. Additionally, the self-aggregation could make capsules to introduce nano-devices in a biocompatible environment.

In vitro and in vivo studies of the repeating hydrophobic motif, Val-Pro-Ala-Val-Gly, have failed to demonstrate any cytotoxicity, general inflammation response or cellular respiratory depression, a result indicative of strong biocompatibility. Their biocompatibility and chemical similarity to human elastin makes them immunologically invisible and ideal for biomedical applications such as drug delivery and cellular matrices.

3.4.1: DRUG DELIVERY

Elastin-like proteins self-aggregate as the temperature is raised above the transition temperature. This feature can help focus pharmaceuticals that have the elastin-like protein tag to areas of hyperthermia. By focusing the pharmaceuticals to desired locations, side effects including cytotoxic material entering undesired locations, as is typical in chemotherapy, can be significantly reduced. There are many potential applications including encapsulation to allow for sustained release over extended lengths of time. Additionally, aggregated elastin-like proteins have a longer half-life and could be a potential depository for long-term drug delivery.

Cell proliferation has also been seen to be inhibited when elastin-like protein tagged protein aggregates within or nearby. This has the potential to be a thermal-dependent target for solid tumors. Additionally, when the Tat peptide is tagged with the elastin-like protein, it has been shown to inhibit cell adhesion, invasion, spreading, and migration of ovarian cancer cells.
3.4.2 CELLULAR MATRICES

Elastin-like proteins have been genetically designed to mimic the ocular surface extracellular matrix. This has been useful for in vitro culture of ocular surface cells.\textsuperscript{40} Additionally, elastin-like proteins have been engineered as a hydrogel matrix to grow human adult stem cells.\textsuperscript{41} Polymers containing elastin-like proteins have also been developed as a type of adhesive to hold mammalian cells to culture dishes. One layer of elastin-like protein is hydrophobically bound to the culture dish and a second layer is bound to the mammalian cells and as the temperature is raised, the elastin-like proteins self-aggregate between the two layers holding the cells to the dish. When the temperature is lowered, the cells are released.\textsuperscript{42}

3.4.3 PROTEIN PURIFICATION

Currently recombinant proteins are typically purified using affinity chromatography, wherein a tag is added to the desired protein that will utilize its affinity to separate the tagged desired protein from various other proteins that may have been concurrently expressed. The elastin-like protein domain provides a novel tag that utilizes temperature fluctuations rather than utilizing affinity. This technique, inverse transition cycling,\textsuperscript{43} was first utilized in 1996 to purify elastin-like proteins expressed from e-coli cells.\textsuperscript{44} Following this, in 1999, Meyer purified desired proteins that had been tagged with elastin-like proteins. This was accomplished by lycing cells at low salt and temperature, employing centrifugation to separate out cell debris, then raising the temperature of the supernatant above the transition temperature, causing the elastin-like proteins to self-aggregate and precipitate. The solution was centrifuged and the precipitate was then resuspended in low-salt buffer at a temperature below the transition temperature. This led to a nearly complete removal of host proteins and recovery of tagged desired proteins.\textsuperscript{45}
Cleavage of the elastin-like protein tag is the final step in the inverse transition cycling purification method listed above. This can be accomplished by treatment with specific proteases like thrombin or factor X. Alternatively, an intein can be added to form a triple fusion protein, which would induce self-cleavage upon a specific temperature or pH fluctuation. This addition would ease scalability, as only a second round of temperature variation would need to be added, or a variation in acidity. Then an additional centrifugation step would separate the intein bound to the elastin-like protein tag.
4.1 BACKGROUND

Elastin is found in arteries, lungs, ligaments, arteries and skin. It is a protein comprised of multiple tropoelastin molecules that cross-link together. Each tropoelastin contains thirty-six small domains that are either primarily lysine rich or hydrophobic. The lysine rich domains are responsible for the cross-linking required to form elastin. The hydrophobic regions are believed to be responsible for the phenomenon coacervation, wherein the tropoelastin aggregates. The process of coacervation is dependent upon several factors including pH, ionic strength of the solution, and temperature.
Tropoelastin is approximately 72 kDa with 724 amino acids, which is too large for traditional NMR experiments. Additionally, at such a large size, it would be difficult to see small changes resulting from mutations or small changes in environment. This can be combatted by representing tropoelastin with a “minielastin” protein, which is composed of hydrophobic regions and cross-linking regions modeled after tropoelastin’s domains.
4.2: MATERIALS AND METHODS

4.2.1: PROTEIN DESIGN AND SYNTHESIS

The minielastin proteins that we have created were modeled off the tropoelastin domains 20, 21-23, and 24. Modifications can be seen in the protein alignment in Figure 16. These model proteins were not designed from first concepts. Instead, the proteins were composed of selected hydrophobic and cross-linking domains and subsequently received small modifications. The most obvious modification was the addition of two tryptophan amino acids, one at either end of each model protein. This was done to assist in quantifying the concentration of protein samples using tryptophan’s strong 280 nm absorption. Additionally, many leucine and isoleucine amino acids were replaced in the hydrophobic regions with alanine or glycine. The rationale behind this decision was to reduce the bulk of side chains that could prevent a denser hydrophobic packing scheme. For some of the model proteins the standard cross-linking domain, 21-23, was modified significantly. This modified cross-linking domain is represented by 21-23’ in the naming scheme. The original cross-linking domain was made much shorter; the glutamine was replaced with asparagine and the tyrosine was replaced with a phenylalanine. The replacement of the glutamine with asparagine modification changed a polar uncharged amino acid to a polar charged amino acid, and also replaced a longer side chain with a shorter one. The replacement of tyrosine by phenylalanine swapped one aromatic amino acid with another, but the phenylalanine is more hydrophobic than the tyrosine, a fact that may lend itself well as a transition from the cross-linking domain to the hydrophobic domain following it.
Four protein sequences were designed; the first sequence studied was 20’–21-23–24’–21-23–24’. The non-modified counterpart 20_21-23_24_21-23_24 is a sequence that has been extensively studied and allowed for systematic comparisons of the resulting changes made.48,49,50

The second sequence designed was 20’–21-23’–24’–21-23’–24’. This sequence took the original modifications one step further in modifying the cross-linking domain. The modified cross-linking domain, as seen in Figure 16, varies significantly from the original cross-linking domain. The significant variations between 21-23 and 21-23’ were the attempt to simplify the cross-linking domain to allow for a more thorough study of the hydrophobic domains via solution NMR.

The third sequence designed was 20’–20’–21-23–24’–21-23–24’–24’. This sequence was designed to test the hypothesis that a longer sequence would have a greater association energy. Adding two additional hydrophobic domains to the protein, one at the beginning and one at the end, created this sequence. For consistency, the initial domain added was identical to the subsequent domain and the final domain added was identical to the previous domain.
The fourth sequence designed was 24’_21-23’_24’_21-23’_24’_21-23’_24’. This sequence was created primarily to study the 24’ domain. By removing the 20’ domain, the only hydrophobic effects were necessarily the result of the 24’ domain. Additionally, the simplified cross-linking domain was utilized to minimize the effects of the larger cross-linking domain. A review of the sequences can be seen in Figure 17.

The genes were synthesized by GENEWIZ (South Plainfield, New Jersey). Genes contained an N-terminus TEV cut site and were inserted into an ampicillin-resistant pET32a (+) vector by Novagen. This construct was then cloned into *E. coli* (*Escherichia coli*) strains NICO21 for production or DH5A for storage.
Displayed are the protein sequences of the four elastin-like proteins that were designed. The red font indicates the 20’ domain, the blue font indicates the 24’ domain and the black font was used for both cross-linking domains. Additional information provided includes molecular weight and total number of amino acids in the sequence.

**FIGURE 17: ELASTIN LIKE PROTEIN SEQUENCES**
4.2.2: PROTEIN PURIFICATION

The genes were transformed into DH5A and NICO21 competent *E.coli* cells using the method outlined in Appendix 3: Lab Protocols. Briefly, 2 uL of the DNA received was added to 500 uL of *E. coli* competent cells that had been stored at -80°C. The cells were incubated on ice for 30 minutes. The cells were then placed in a 42°C water bath for 45 seconds for heat shock. The cells were then incubated for 5 minutes on ice. Then 1 mL of luria broth, LB, was added and the cells were incubated at 37°C for 30 minutes. Finally, 100 uL was spread onto an ampicillin plate and incubated overnight at 37°C.

Proteins were expressed as fusions with a thioredoxindomain and a hexa-histadine tag. Initially, *E. coli* were grown for six hours until turbid in 5 mL of sterile LB. This growth was transferred to 100 mL of either sterile LB or sterile M9 (minimal media), if the protein was to be labeled for NMR studies. Then 100 uL of stock 100 mg/ml ampicillin was added to ensure growth of only the ampicillin-resistant e-coli. We then added 200 uL of 500 X sterile trace metal solution, 2.5 mL of sterile 40% glucose and 100 uL of 1000 x sterile vitamin solution to the M9. The 100 mL growths were shaken at approximately 250rpm overnight at 37°C. Half, 50 mL, of the overnight growth was added to either 1 L of sterile TPP (tryptone phosphate LB) or sterile M9 (minimal media) in the case of NMR samples. Next, 1 mL of 100 mg/ml ampicillin stock was added to the growth, 25 mL of sterile 40% glucose, 2 mL of sterile trace metal solution and 1 mL of sterile vitamin solution. The growths then shook at 37°C until OD600 was between 0.6 and 1.0. Protein production was then induced with 0.5 mM ITPG (isopropyl-β-thiogalactopyranoside). *E. coli* growths in TPP were then shaken for five hours at room
temperature, while M9 growths were shaken at room temperature at approximately 250 revolutions per minute for seven hours.

Cells were collected by centrifuging the 1 L in three containers at 4°C at 10,000 rpm for 10 minutes. Cells were resuspended in a low imidazole-working buffer, known as wash buffer, with 1 ug/mL DNasel and a pipette tip of Pefabloc. Cells were resuspended by gentle rotation for 30 minutes at 4°C. Cells were broken open using a Mini Cell Pressure Cell French press by Glen Mills, Inc., which applied over 1000psi three times to the cells. Material collected was separated by centrifugation at 15,000 rpm at 4°C for 30 minutes.

Following manufacturer’s directions, the supernatant was then purified on a Ni-nitrilotriacetic acid column (Qiagen, Inc.). This utilized imidazole-binding affinity. Consequently, an elution buffer was poured over the column with a higher imidazole concentration, which replaced the locations the hexa-histadine tags had bound.

The fusion protein collected was then dialyzed overnight into 50 mM Tris-HCl, 1 mM DTT (dithiothreitol) at pH 8.0. His6-tagged TEV protease (Invitrogen, Inc.) cleaved the fusion protein for at least six hours at room temperature with gentle nutation. The mixture was then dialyzed back into low imidazole-working buffer and filtered by running it back over a Ni-nitrilotriacetic acid column. The protein was then dialyzed into lyophilization buffer and lyophilized. Protein was resuspended in minimal amounts of 50 mM phosphate buffer, pH 7.4, and run on a reverse-phase HPLC (high pressure liquid chromatography) C18 prep column (Higgins Analytical) with a water/acetonitrile gradient 30%-70% in 40 minutes with 0.2% TFA (trifluoroacetic acid). Protein was collected, run through a rotovap to remove acetonitrile, snap frozen and lyophilized. All stages of purification were monitored by SDS-PAGE gel.
Coacervation studies were conducted using an HP 8452A diode array photospectrometer running Olis Spectral Works equipped with a peltier temperature controller (Quantum Northwest TC125). Protein concentrations were determined using extinction coefficient calculations at an absorbance of 280 nm, with the predicted coefficient 13980 M⁻¹ cm⁻¹. Lyophilized protein was dissolved in either coacervation buffer, 50mM Tris, 1.5M NaCl, 1mM CaCl₂ pH7.5, or a listed variation utilized to test the effect of the change.

Samples were placed into a 1.5mL quartz cuvette and placed in the sample holder and equilibrated at initial temperature for at least five minutes. The temperature was then increased at 1°C/min with stirring. Scattering was monitored at 440nm. Upon completion of the temperature titration, samples were cooled to 10°C and centrifuged at 13,200rpm to separate coacervated protein that did not return to solution. This material was washed with deionized water and stored for solid-state NMR.

Optical microscopy samples were obtained during coacervation studies by removing 50μL of solution every five degrees and immediately placing the cover slide and sealing the slide with nail polish. Images were collected on a Zeiss LSM710.
Protein was initially grown in *E. coli* following the procedure listed above in Appendix 3.3.9: TPP Media Growing Instructions. Briefly, *E. coli* strain NiCO21 contained the Pet32a(+) vector that had the plasmid and DNA was grown in LB and then TPP against ampicillin. The *E. coli* was induced to produce the protein and after four hours was spun down. The pellet was resuspended in wash buffer and lyced open. The lyced solution was spun down and separated into solid and supernatant. The solid was then resuspended in high concentration urea to denature any protein remaining. The supernatant had 3 M salt added to the solution and brought to room temperature. This temperature and salt increase coacervated the 20'-21-23-24'. The supernatant was then spun down at 13,200 rpm for 10 minutes at room temperature. This supernatant was decanted. The solid was resuspended in low concentration imidazole buffer and chilled. A SDS-PAGE gel was run on the initial lyced debris in urea, the lyced supernatant with 3 M salt, the supernatant of the room temperature spin, and the solid resultant from the room temperature spin.
4.2.5: SOLUTION NMR

4.2.5.1: 1D SOLUTION NMR

One dimensional solution NMR was conducted at Louisville University using a Varian 800 MHz. Data was collected at 25°C. Protein concentration was approximately 300uM in 10% D2O in a 25mM phosphate, 25mM KCl buffer at pH 7.0. Water gating was used to suppress the water signal.

4.2.5.2: 2D SOLUTION NMR

Two dimensional solution NMR data was collected at both Louisville University on a Varian 700 with cryoprobe and Varian 800 with cryoprobe and at City College of NY (CCNY) on a Varian 700 with cryoprobe. Data collected at Louisville came from a sample with protein concentration of approximately 300 uM, in 10% D2O at 25°C in 25 mM phosphate, 25 mM KCl buffer at pH 7.0 and 5.9. The experiments completed were NHSQC, TOCSY-HSQC and CHSQC.

Data collected at CCNY came from samples with approximate protein concentrations of 800 uM unless specified with the data. The samples contained 10% D2O. Buffers and temperatures were varied and are listed with each data set. The experiments completed were NHSQC.

4.2.5.3: 3D SOLUTION NMR

Three dimensional solution NMR data was collected at Louisville University on a Varian 700 with cryoprobe and Varian 800 with cryoprobe. The protein concentration was approximately 300 uM in 10% D2O at 25°C in 25 mM phosphate, 25 mM KCl buffer at pH 7.0
and 5.9. The initial experiments completed were HNCA, CBCANH, CBCACONH, HNCO, and HNCOCA.
4.3: RESULTS AND DISCUSSION

4.3.1 COACERVATION STUDIES

To determine whether the designed protein functioned in the same fashion as native tropoelastin, coacervation assays were completed to demonstrate that the protein self-aggregated when the temperature was increased above the critical temperature, if the salt concentration was substantially increased, or if the pH was varied. As seen in Figure 18, coacervation did occur when heated above a critical temperature of coacervation and the particles that formed were large enough to be seen by the naked eye.

FIGURE 18: TEMPERATURE COACERVATION OF 20'_{21-23}_{24'}_{21-23}_{24'} 20'_{21-23}_{24'}_{21-23}_{24'} was heated 1°C/min to 80°C and gently stirred.
Once it was confirmed the protein coacervated, characterization of self-aggregation was necessary. Coacervation was quantified using the scattering at 440 nm in the UV-Vis spectrophotometer. The critical temperature of the coacervation did follow trends demonstrated by previous research, wherein adding salt reduced the heat required to self-aggregate. This result can be seen in Figure 19 when the same concentration of protein was assayed multiple times with varying amounts of salt. As the temperature was increased, self-aggregation occurred at lower temperatures for higher concentrations of salt.

The coacervation did not appear to be dependent upon the rate of stirring. However, as evidenced by the black 0 M salt trial in Figure 19, when the stirring rate was not sufficient, the particles became heavy enough to fall out of suspension and rest on the bottom of the container, yielding a decrease in scattering and a break in the coacervation curve.

**FIGURE 19: SALT DEPENDENT COACERVATION OF 20'21-23_24'21-23_24'**

The coacervation study utilized 50 mM Tris Buffer with 1 mM CaCl₂, with 25 uM samples varying NaCl concentration. All samples had temperature increased at 1°C/min with gentle stirring.
Additionally, reducing the pH of the protein lowered the critical temperature of coacervation, as can be seen in Figure 20. While scattering of the same concentration of protein with the same salt and buffer constituents appeared to approach the same asymptote, the lower pH sample began to coacervate sooner with more scattering detected at lower temperatures. Contrasting this is the higher pH sample that had nearly no scattering detected until five degrees from maximal scattering.

One feature of the coacervation scattering assays is the baseline. While it can be seen in Figure 20, it is more prominent in the 1.5 M NaCl trial in Figure 19. Even at the lowest temperatures of the assay, some scattering is detected and prevents a common baseline as a result. This initial scattering is worth noting and therefore not removed as background.

FIGURE 20: PH DEPENDENT COACERVATION OF 20'_21-23_24'_21-23_24'
The coacervation study used 50mM Tris Buffer, 1.5M NaCl, and 1mM CaCl2 as a constant buffer, varying the pH between trials with both trials increasing the temperature one degree per minute with gentle stirring.
Coacervation critical temperature variations have been demonstrated by temperature modulation, pH modulation and salt concentration. It was therefore hypothesized that by solely increasing the salt concentration at a fixed temperature the critical temperature could be shifted to the point of coacervation. Figure 21 demonstrates the successful implementation of this hypothesis. A salt solution was titrated into the protein solution at 48°C until the protein coacervated. Salt solution continued to be added in order to observe the effects upon scattering after coacervation, as well. Approximately 2 M salt concentration with a pH 6 at 48°C is the peak of coacervation. Beyond this concentration of salt the scattering signal began to decrease, possibly because the ionic strength of the solution was interfering with additional self-aggregation, or even protein folding.

![Coacervation varying NaCl 48C, 25uM, pH 6.0](image)

**FIGURE 21: SALT COACERVATION ASSAY**

25 uM protein in 50 mM Tris, 1mM CaCl₂ at pH 6.0 was warmed to 48°C and then aliquots of NaCl were added, 100 mM at a time and stirred. Scattering was observed at 440 nm.
During a temperature coacervation assay, 50 uL samples were removed from the overall sample and sealed on microscope slides. The samples were then imaged using a confocal microscope using 500 times zoom. Samples below the critical temperature had very little structure visible at all, as is seen in Figure 22A where the sample was removed at 5°C. In contrast, samples above the critical temperature had large formations, as is seen in Figure 22B. The large formations visible above critical temperature appear to be particle-like and not fibers, while tropoelastin cross-links to form elastin in the shape of fibers.

The microscope’s resolution limit was a determining factor in this assay, as it was very difficult to visualize any particles smaller than 10 um. As mentioned earlier, the size of the protein can be approximated to be roughly 5-10 nm. Therefore, the smallest self-aggregation would be 10-20 nm, well below the threshold of the microscope. Scattering of the smallest self-aggregated proteins can be visible with Rayleigh scattering while the particles detected with the confocal microscope would be well beyond the size limit of Rayleigh scattering, following Mie scattering theory instead.

FIGURE 22: IMAGED COACERVATION 5°C AND 85°C SAMPLE
Confocal microscopy images of 25 uM 20’-21-23_24’-21-23_24’ samples in 50 uM Tris, 1 mM CaCl₂ at pH 6.0 with 1 M NaCl. A) Very little coacervation was detected at 5°C, with most protein not visible within the microscope range. The scale is 20 um. B) Coacervation was easily visible within the range of the microscope, with larger structures of various sizes forming.
One of the concerns was the non-reversibility of the construct upon coacervation. It is known that an increase in temperature can induce non-reversible protein aggregation. To demonstrate the functionality of 20’-21-23-24’-21-23-24’, the protein was cross-linked with 0.02% gluteraldehyde for eighteen hours and imaged using a confocal microscope. Long strands of cross-linked protein, resembling elastin, were visible, as is seen in Figure 23.

FIGURE 23: CROSSLINKED 20’-21-23-24’-21-23-24’
Cold 40 uM protein had 0.02% gluteraldehyde added. Sample was then placed in a hot water bath with gentle agitation for 18 hours. Subsequently 50 uL was sealed on microscope slide and imaged with confocal microscope.
Solution NMR was conducted to ascertain structural information on the designed protein. The first experiment completed on the protein was 1D NMR to obtain a baseline for all future experiments. This can be seen in Figure 24. The aliphatic protons can be seen in the 0-2 ppm region, the alpha protons in the 3-4 ppm region, and the amide protons 7-8.5 ppm with two very small peaks at 10 ppm, possibly representing the two tryptophan amino acids.

FIGURE 24: 1D NMR SPECTRA OF 20’-21-23-24’-21-23-24’
The spectrum was collected on a Varian 700 with protein in 25 mM Phosphate, 25 mM Potassium Chloride buffer, at pH 7.0 at 25°C.
Initial NMR experiments were undertaken prior to HPLC purification of the protein. It was hypothesized that the HPLC step of purification would modify the spectra obtained from NMR. As a result, pre and post HPLC data was connected to determine any potential differences that may have resulted from the removal of the low concentration of contaminants that remained following standard purification. The primary observation made from Figure 25 is slight chemical shift following purification. However, this could be the result of the spectrometer being utilized on different days with potential temperature fluctuations. Aside from the slight up-field chemical shift, many peaks appear much more distinct. However, the concentration following HPLC was significantly higher, and as a result, peaks were able to be distinguished much more easily from background. Therefore, it appears differences from purification are minimal.

FIGURE 25: NHSQC OF 20'_21-23_24'_21-23_24'
Protein was in 50 mM Phosphate buffer at pH 5.9 at 25°C. The black sample is prior to HPLC purification and approximately 300 uM, and the red overlay is the protein after HPLC purification and approximately 800 uM.
After investigating the effects of small concentrations of contaminations, the effects of pH was investigated. The initial pH ran was 7.0, close to that of physiological conditions, which were believed to be ideal for tropoelastin. It was compared with pH 5.9, which was believed to yield higher quality NMR spectra. As seen in the coacervation studies, a decrease in pH does allow for self-aggregation at lower temperatures, but with the temperature of the sample maintained around 25°C, coacervation effects were kept to a minimum.

While the red pH 5.9 spectra comes from a much more concentrated sample, it actually appears to have fewer peaks in some regions of the NHSQC. In particular, the down-field $^{15}$N region, especially around $^1$H 7.75 ppm appears to have more overlapping peaks. This could be indicative of the protein being more molten globular and less folded. There are some areas where it appears that there are more signals with the lower pH, but this may be the result of a higher concentration and better signal to noise.

**FIGURE 26: NHSQC OF 20'21-23_24'21-23_24'**
The protein was in 25 mM Phosphate, 25 mM Potassium Chloride buffer at 25°C. The black 300 μM sample is at pH 7.0, the red 800 μM sample is at pH 5.9.
Many of the coacervation assays in the literature were completed with 1 mM CaCl$_2$. To determine whether this addition interacts with the protein, an NMR experiment was performed prior to calcium chloride being added then an excess was added and the sample were-evaluated. As can be seen in did not affect the NMR spectra.

Figure 27 there was a slight down-field shift after the addition of the calcium chloride, but no other appreciable differences were detected. This result indicates that while the calcium chloride was used in the coacervation buffer, it did not affect the NMR spectra.
FIGURE 27: NHSQC OF 20'_{21-23}_{24}'_{21-23}_{24}'
Protein was in 50 mM Phosphate Buffer pH 5.9 at 10°C. The black sample does not contain CaCl$_2$. The red sample contains 1 mM CaCl$_2$. Approximate protein concentration was 750 uM.
Another hypothesis tested was the possibility that the protein underwent conformational changes as it approached the critical temperature of coacervation. To test this hypothesis a temperature titration NHSQC was run. It is well known that as temperature increases there is a corresponding up-field \(^1\)H shift. This phenomenon helps to minimize peak overlap by all peaks shifting to the right as the temperature increases. However, there were approximately five peaks whose \(^1\)^15\(^N\) peaks did not follow the trend of shifting up-field. Additionally, not all up-field shifts appeared to happen at the same rate. This is believed to be the result of hydrogen bonds interacting differently in their surroundings as presented by Cordier and Grzesiek in 2002.\(^{52}\)

![FIGURE 28: NHSQC OF 20’_21-23_24’_21-23_24’](image)

Sample was in 50 mM Phosphate Buffer, pH 5.9. A temperature titration was completed with black at 15°C, violet at 20°C, sea green at 25°C, peach at 30°C, orange at 35°C and red at 40°C. After each dataset was collected, the instrument temperature was increased, then given five minutes additional time to equilibrate. Approximate protein concentration was 880 uM.
Peak intensity as a function of temperature had the potential to produce results that would have indicated the concentration of sampled protein had decreased because the protein aggregated. However, as seen in Figure 29, variations between different peaks are so wide that there is no discernable trend. This is counter-intuitive because it was visible to the naked eye that as the temperature increased, the protein coacervated, in turn, reducing the concentration in solution. One possible explanation for the lack of decreasing trend could be the relative concentration differences. The NMR sample was very concentrated, which while producing very clear spectra, may have made it insensitive to small fluctuations in the concentration.

FIGURE 29: NHSQC PEAK INTENSITY OF 20’_21-23_24’_21-23_24’
The peak intensity was plotted as a function of temperature. Protein was in 50 mM Phosphate Buffer, pH 5.9. Approximate protein concentration was 880 uM. The NHSQC spectra on the right has the various peaks circled to indicate where on the spectra the intensity peaks originate.
Three dimensional NMR experiments HNCO, HN(CA)CO, CBCANH, and CBCACONH were conducted on 20’-21-23-24’-21-23-24’ in an attempt to completely assign the backbone of the protein. However, this proved impossible due to the molten globular conformation the protein adopted. The HNCO shows approximately 60 peaks. However, the protein contains 182 amino acids, and with only 21 prolines, there should be approximately 161 peaks without overlapping. The fact that roughly 100 peaks are missing is indicative of the molten globular conformation. When investigated more closely, it can be recognized that one repeat in 20’, one repeat of 24’ and one cross-linking domain can be approximated by the 60 peaks visible on the HNCO. This section of the backbone was assigned, but it is not possible to determine which segment of the 20’ or 24’ domains or which cross-linking domain is being seen.
In determining if the elastin-like protein functioned as a typical elastin-like protein, it was used to purify thioredoxin. In many laboratories a thioredoxin His$_6$ tag is appended to the desired protein. In purifying 20'-21-23-24'-21-23-24' a thioredoxin tag was appended with a TEV cleavage site. This created a nickel affinity that aided in purification. However, the construct could also be looked at in a different light, wherein the elastin-like protein was added to the thioredoxin tag. Now the unique self-binding affinity of the elastin-like protein at high temperatures can be utilized to separate it from other soluble proteins. Figure 30 demonstrates the entire process of thioredoxin purification prior to the removal of the elastin-like protein tag. The most telling information appears in lanes 2 and 13. Lane 2 is a sampling of all proteins that were within the lysed _E. coli_ supernatant. Lane 13 is all solid precipitant from Lane 2 that subsequently went back into suspension. The key features are the increase in concentration of the sample and the disappearance of the band immediately above 12 kDa. Purification protocols using elastin-like proteins for separation of target proteins often use coacervation multiple times, whereas the results in Error! Reference source not found. are after only one coacervation and suspension.
FIGURE 30: THIOREDOXIN PURIFICATION
A. Lane 1 is the molecular weight marker, lanes 2-4 are the lyced supernatant before adding salt and raising temperature, lane 2 is full concentration, lane 3 diluted in half, lane 4 is diluted to 25%. Lanes 5-7 are the lyced debris resuspended in 8 M urea wash buffer. Lane 5 is full concentration (but much of the sample did not transfer to the gel due to aggregation in the pipette. Lane 6 is 50% and lane 7 is 25% concentration. Lanes 8-10 is the supernatant after centrifugation of the high temperature salt solution. Lane 8 is 25% concentration, lane 9 is 50% and lane 10 is full concentration. Lanes 11-13 are the high temperature solid resuspended in cold wash buffer. Lane 11 is 25% lane 12 is 50% and lane 13 is full concentration.
B. Lanes 1, 2, and 13 are shown side-by-side to demonstrate the before and after purification.
4.4: CONCLUSIONS

By coacervating as the temperature increases, this work demonstrates that the designed elastin-like protein 20’_21-23_24’_21-23_24’ behaves similarly to native tropoelastin. Additionally, the protein’s self-aggregation is also dependent upon the ionic strength of the salt concentration, the pH, the protein concentration, and the choice of buffer-- which is expected due to native tropoelastin’s similar coacervation profiles.

Preliminary cross-linking work indicates that the de novo elastin does cross-link, further indicating the functionality of the protein. Images showed fiber-like structures beyond the basic particle formation seen during coacervation.

Nuclear Magnetic Resonance spectroscopy indicates that the ideal pH for the designed protein is near physiological conditions, provided the sample concentration is high. Additionally, the calcium chloride utilized in the coacervation buffer is not necessary in the NMR samples because there was no negligible difference between samples with and without the calcium chloride. Salt titrations were not carried out due to the tendency for high ionic strength to yield poor NMR results. However, temperature titrations were completed in order to look for conformational changes as the protein approached coacervation. A few small changes were observed, but there did not appear to be significant changes in folding.

Furthermore, three-dimensional NMR was used to assign resonances which correspond to different parts of the protein, specifically one GVGGV repeat of 20’, one cross-linking domain, and one GVGVA repeat of 24’. The molten globular nature of the de novo protein constrains the other chemical shifts to be either overlapping with the identified or missing due to relaxation or exchange.
CHAPTER 5: FUTURE DIRECTIONS

5.1: MODIFICATIONS OF MODEL PROTEINS

5.1.1: 20’_21-23’_24’_21-23’_24’

Initial modifications of 20’_21-23’_24’_21-23’_24’ have been completed and synthesized. These new designs must be studied to determine the effects of the changes implemented. From these studies, it will be possible to design a possible third generation of small elastin-like proteins to test the conclusions drawn from the initial modifications.

Future studies on the modified protein motif should begin with experiments on 20’_21-23’_24’_21-23’_24’. In comparing this protein with the original 20’_21-23’_24’_21-23’_24’, it is possible to explicitly determine the effects of varying the cross-linking domain. The studies to be completed include temperature, salt, pH and concentration coacervation assays, CD and NMR.

If the coacervation data shows a decrease in coacervation temperature, those results would be indicative of an increase in ease of self-aggregation. This could mean the designed cross-linking domain 21-23’ is more solvent-exposed or active than the original 21-23 domain. Additionally, the coacervation assay will indicate if the self-binding affinity decreased.

The original protein had such a high self-binding affinity that coacervation was permanent, a phenomenon highly uncommon in elastin-like proteins. The second half of the coacervation assay of the new protein yielded a basic indication of the level of self-binding affinity. As the temperature of the sample was decreased, the original protein showed no indications of disassociation. The new protein’s coacervation assay could show a decrease in scattering as the temperature is decreased. This behavior would be consistent with other elastin-like proteins that go back into solution below the critical temperature of coacervation.
Additionally, NHSQC 2D solution NMR on a sample with identical conditions to those the original protein was in when data was collected, which is listed with each figure, should be collected. The NHSQC spectra collected can be used as a basis to determine folding of the protein. The original protein contains 182 amino acids; the NHSQC of the original protein contained approximately 60 unique chemical shift peaks. The protein contains 21 proline amino acids that do not exhibit a peak on a NHSQC; if the protein were completely folded, there should be 161 peaks. The fact that over 100 amino acid peaks were not visible on the NHSQC is indicative of the chemical shifts overlapping or loss due to exchange. If the new protein is folded more, it will have additional peaks on the NSHQC compared to the original. If enough peaks are present, it may be possible to determine the structure of the designed protein with 3D NMR experiments.

5.1.2: 20’ _20’ _21-23 _24’ _21-23 _24’ _24’

The second protein that should be studied is 20’ _20’ _21-23 _24’ _21-23 _24’ _24’. This design attaches an addition hydrophobic region to each end of the original de novo protein. This protein should be characterized in the same fashion as the original protein, utilizing coacervation assays, circular dichroism and solution NMR. If the coacervation studies indicate a lowering of the critical temperature for self-aggregation, then that would indicate that a larger elastin-like protein is more stable than a smaller one. The fact that the number of cross-linking domains remained constant should help support the hypothesis that the number of hydrophobic domains is the driving force behind the lowering of the critical temperature rather than the ability for additional cross-linking.
This larger protein may introduce additional difficulties in NMR, as the larger protein, in part, will have more relaxation effects because the tumbling rate will be slower. This will cause line broadening. This can be compensated for to an extent by additional scans, which adds time to the experiment; and the longer the protein is in the equipment, the more opportunity for degradation, which will also decrease the quality of the data. These difficulties may make CD a more useful source for the secondary structure of the protein.

5.1.3: 24’-21-23-24’-21-23-24’-21-23-24’

The third protein that has been designed focuses on the 24’ hydrophobic domain. This protein will also need to be tested against the same experiments run on the original 20’-21-23-24’-21-23-24’ protein. Coacervation studies varying concentration, pH, salt and temperature will all provide insights into this protein. Comparing the critical temperature of coacervation as well as the salt concentration will allow for hypotheses regarding the functionality of the cross-linking and elasticity of the resulting mini-elastin. Circular dichroism and NHSQC will help to determine the secondary structure of the protein to determine if the repeating motif allowed for a unique folded conformation, or if the protein is molten-globular or unfolded.
5.2: CROSS-LINKING STUDIES

Very initial cross-linking data was collected, demonstrating that the model proteins were functionally viable for cross-linking. Systematic studies of cross-linking are a next logical step. One variable that can be tested is the type of cross-linking chemical. Natural elastin uses lysyl oxidase for cross-linking, which would be the obvious choice to investigate; however, using gluteraldehyde, data has also been collected to successfully cross-link. While gluteraldehyde would most likely have more immunological interactions, a comparison between the two cross-linking agents would be worthwhile.

A second variable to investigate is cross-linking conditions. Initial data was collected with 0.02% gluteraldehyde added to protein solution and the solution was warmed and gently agitated for 18 hours. The amount of cross-linking agent is a variable to consider, as well as the temperature of the solution, the length of time allowed for cross-linking, and the amount of agitation. Additionally, varying the protein concentration is a condition that merits investigation. This would be different than varying the concentration of the cross-linking agent because the self-aggregation of the protein is dependent upon protein concentration and the self-aggregation is required for cross-linking.

Assays of the cross-linking also needed to be investigated. Use of the confocal microscope gave a qualitative result of successful cross-linking, but there was little quantitative data derived. Data that could be collected in the future would be average density, thickness, and length of cross-linked strands. Additionally, cross-linking forms large particles, so the scattering assays used for coacervation could yield results on speed of cross-linking. Another method to test cross-linking is via the elasticity studies described in the next section.
5.3: ELASTICITY STUDIES

Collaborators will utilize Atomic Force Microscopy (AFM) to study the elasticity of the proteins. The AFM uses a very fine tip and deflection of the tip is recorded and translated into position signal. Traditionally, as a tip scans across a surface, it is able to record grooves and peaks; and with systematic scans along a sample, it can recreate the surface topology. In this application of the AFM, the non-cross-linked coacervated protein will “stick” to the tip of the AFM and the machine will slowly pull back the tip; the resistance will be indicative of the stretch of the protein. When the resistance stops or significantly decreases, the protein has stretched to the point where it snapped or its bond with the tip or base surface broke. This experiment will be repeated for many hours with the results represented in a histogram that will ideally have one narrow peak indicative of the elasticity of the protein.

Upon completion of the experiment with non-cross-linked coacervated protein, it will be repeated with cross-linked protein. The elasticity of the cross-linked protein should be substantially larger. This experiment will be very valuable in determining optimal cross-linking conditions as well as providing insights into the variations of the different protein constructs that have been designed to probe the functionality of the hydrophobic domains and the cross-linking domains.
**5.4: SOLID-STATE NMR**

Solid-state NMR (SSNMR) can be used to study the self-aggregated cross-linked protein as opposed to solution NMR, which studies the protein prior to self-aggregation. The SSNMR samples are not in solution; rather they are spun down and dried. This method can allow for studying the solids that can actually make the solution NMR signals less ideal. The primary difficulty with SSNMR in this case is the quantity of protein needed for the experiment.

Samples that are run in SSNMR should be prepared in identical fashion, so a great deal of 20’_21-23_24’_21-23_24’ should be purified initially and prepared in multiple fashions to find the ideal conditions for future proteins to be prepared for SSNMR. Once solid-state samples have been prepared, they can be run to help clarify the structure of the protein utilizing the chemical shifts.

**5.5 DIFFERENTIAL SCANNING CALORIMETRY**

Another method to study the self-aggregated and cross-linked proteins is with Differential Scanning Calorimetry (DSC). In this experiment, 50 uL of sample would be placed in a cold pan and held at 5°C until equilibrated. It is then heated and the difference of energy needed to heat the sample compared to the reference sample would be recorded. The parameters used in preparing the 20’_21-23_24’_21-23_24’ sample for DSC would need to be tested and varied initially so that once the optimal conditions are found, they can be repeated for additional proteins to be tested via DSC. Some parameters to investigate would be pH, salt concentration, protein concentration, and cross-linking parameters.
Circular dichroism is scheduled to be completed on 20’_21-23_24’_21-23_24’ in the immediate future. There are a few parameters that can be modified in order to explore the secondary structure of the protein. A temperature titration using CD would yield very interesting results showing whether the protein folded with the increase of temperature as it approached the coacervation critical temperature. Varying the buffer, pH and salt concentration and running additional temperature titrations has the potential to yield further interesting results.

Circular dichroism on 20’_21-23’_24’_21-23’_24’ would be completed after the results of 20’_21-23’_24’_21-23’_24’ have been analyzed. The conditions for the new CD temperature titrations would need to be identical to those of the initial titration. This parameter would allow for clear comparison between the two to determine whether or not the modification of the 21-23 cross-linking domain improved the secondary structure. Additionally, by waiting until the analysis of the data for 20’_21-23’_24’_21-23_24’, it would be possible to determine the ideal parameters to run the subsequent experiments, thereby reducing the number of CD titrations on the future generations of proteins.

The next protein to have CD temperature titrations would be 20’_20’_21-23’_24’_21-23_24’_24’. This protein would also utilize the same conditions for the temperature titrations as the original protein for ease of comparison. If the secondary structure improves with the modifications made to this protein, it would indicate that the adding of additional hydrophobic domains improved the ability to fold, since that was the only modification.

The final protein on which to run CD temperature titrations would be 24’_21-23’_24’_21-23_24’_21-23_24’. This experiment would also utilize the same conditions as the initial protein
to allow for comparison. If the secondary structure is less molten-globular than the initial protein, it will warrant further investigations by creating additional proteins. The secondary structure may have improved because this protein has three cross-linking domains as opposed to two, which the previous proteins have. It is also possible that the protein structure improved because only the 24’ hydrophobic domain was utilized while the 20’ hydrophobic domain was omitted. This could be studied by creating two additional proteins: 24’_21-23_24’_21-23_24’ and 20’_21-23_20’_21-23_20’. The first new protein is identical to the original protein with the exception that the 20’ hydrophobic domain has been replaced by the 24’ hydrophobic domain. The second construct is identical to the final in the first series of revisions except each of the 24’ hydrophobic domains has been replaced by the 20’ hydrophobic domain. The secondary structure of these proteins would help to further deduce whether improvements were due to additional cross-linking domains or if the improvements were the result of the 24’ hydrophobic domain.
APPENDIX 1: LIST OF PUBLICATIONS

1.1: PUBLICATIONS IN PREPARATION


CDM13 Catalyst Structural Studies – James Murray

CDM13 Enzymatic Catalysis– Ross Anderson

CDM13 Water Relaxation – Anna Peacock

FHFH NMR Structure – Lei Zhang

1.2: PUBLICATIONS
2.1: NITRIC OXIDE ENZYMATIC ACTIVITY

Protein nitric oxide (NO) affinity can be characterized where enzymatic rates can be investigated. Two controls can be used-- horse heart myoglobin (Mb) and flavohemoglobin (fHb).

The NO enzymatic rate investigation utilizes a combination of Gardner\textsuperscript{53} and Hargrove\textsuperscript{54} procedures. Commercial NO gas arrives as NOx and must be passed through sodium hydroxide to remove unwanted forms, leaving a more pure NO.\textsuperscript{55} The NO is blown over the 0.1 M potassium phosphate pH 7.4 buffer in a gas tight container while being stirred for at least ten minutes. The saturated buffer can be stored at 4°C for approximately one month,\textsuperscript{56} but for optimal results due to leaks, this should be made fresh or re-saturated for each trial. The concentration of the saturated solution is temperature dependent upon initial saturation. At 25°C, the concentration is approximately 3 mM.\textsuperscript{56, 57}

A World Precision Instruments NO probe is used to determine the NO concentration in a 1-3 mL sample, provided the concentration is within the linear range of the detector. The linear range is determined by calibrating the probe with increasing injections of known amounts of NO and creating a response curve.\textsuperscript{58} The linear region of the response curve is the functional region for the probe. Current experiments are being conducted with a probe that has a linear response from below 5 uM to 30 uM and a second probe which has a linear response from 20uM to more than 80 uM, as seen in Figure 31.
Calibration of the NO and oxygen probes follows the guidelines set forth within ISO-NOP manual by World Precision Instruments. The oxygen probe is calibrated by first determining the amperage output from the probe in 0.1 M phosphate buffered saline (PBS) solution with atmospheric oxygen present (21%). Then the PBS is saturated with 100% oxygen for more than five minutes and the amperage output is recorded. Finally the PBS is saturated with 100% nitrogen for more than five minutes and the amperage output is recorded for 0% oxygen. The points are then plotted and the equation of the line is used to convert the amperage output to percent oxygen in the sample.
Calibration of the NO probe is completed in 0.1 M potassium phosphate buffer with a pH of 7.4. With 0% NO in the 1 mL sample, baseline amperage is recorded. Then a small aliquot of NO is added, often 5 uM. Once the amperage plateaus, the value is recorded. Double the amount of NO is then added, usually 10 uM. Once the amperage plateaus, the value is recorded and a third aliquot of NO is added, usually 20 uM. The amperage is recorded once it plateaus. The additions are added via a gas-tight Hamiltonian 10 uL syringe as quickly as possible, as the NO within the sample is being converted into other forms of NOx and the amperage will begin to decrease quickly after plateauing. With the four values of amperage at various NO concentrations, a response curve can be constructed to convert the amperage to molar concentration of gas present in the sample. A sample curve and fit is presented in Figure 32.

![Figure 32: Nitric Oxide Probe Calibration](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
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<td>a (intercept)</td>
<td>1.1103</td>
<td>0.0916</td>
</tr>
<tr>
<td>b (gradient)</td>
<td>1.0028</td>
<td>0.0172</td>
</tr>
</tbody>
</table>

**FIGURE 32: NITRIC OXIDE PROBE CALIBRATION**
Calibration points for NO are at 0, 1.25, 2.5, 5, 10 and 20 uM concentration. The gradient and intercept are used to calculate the conversion between nA to uM of NO.
A key feature of the probes is that they are temperature dependent and also dependent upon the stirring rate. Prior to calibration, to ensure consistency throughout experiments, the reaction chamber is placed in a water bath that is maintained at 25°C and the stirring rate is set at 600 rpm. Neither setting is adjusted for the remainder of the experiment.

NO consumption experiments are completed in a 1 mL volume. The volume contains 3 mM glucose-6-phosphate (G6P), 60 uM nicotinamide adenine dinucleotide phosphate (NADP+), 1 uM ferrodoxin-NADP reductase (FdR), 0.7 units of glucose-6-phosphate dehydrogenase (G6PD), 5-20 uM protein, and 2.5-25 uM NO, with the remainder of the solution is potassium phosphate buffer, with a pH of 7.4. The above concentrations are variations of the set-up presented by Hargrove’s group. It has been concluded that the NADP+ needs to be prepared fresh daily, as use of older supplies gives inconsistent results (figures omitted). All solutions should be kept on ice or refrigerated when not in use. Proteins tested so far include Mb, fHb and de-novo designed HHHH. The HHHH and fHb require heme added to the protein to create a complex. However, heme is stored in dimethyl sulfoxide (DMSO) which is an organic solvent that destroys the membrane and probe. Thus, upon creating a complex of heme and protein, the DMSO must be dialyzed out. The HHHH complex could be put over a desalting column; however the fHb does not travel through the column unaffected. It is hypothesized that the protein breaks into two domains; the flavin traveling more slowly through the column. As a result, dialysis is the optimal method for fHb, and the HHHH complex is treated in the same fashion.

Maintaining constant protein concentration and varying the amount of NO added completes consumption experiments. This varies the amount of buffer, but all other solutions
remain constant. The G6P, NADP+, FdR, protein, and buffer are added to the reaction chamber and degassed until the oxygen concentration is approximately 4% as determined from the oxygen sensor. The rationale for 4% oxygen is that the reaction of FdR and NADP+ requires a small amount of oxygen, but NO reacts with oxygen. Thus, it is essential to minimize the amount present to allow the NO to be consumed by the protein rather than the oxygen. The aliquot of NO is added, the amperage spikes to indicate the presence of NO, and then quickly decays as the protein reacts with it. The initial decay slope is the initial velocity, which is used in enzymatic calculations including the Michaelis-Menten equation and Lineweaver-Burke Plot for the $K_m$, the Michaelis constant, and $K_{cat}$ the catalysis rate constant. Figure 33 is an example response curve of 20uM NO being added to 10uM Mb. This is seen with the red curve, and 10uM NO is added to buffer as seen by the black curve.

![Figure 33: Nitric Oxide Enzymatic Activity](image)

The black curve is the background decay rate of NO in buffer, to determine the rate that NO is converted to NO$_x$, where 10 uM of NO is injected to the sample that does not contain the studied protein. The red curve is indicative of 20 uM of NO added to 10 uM Mb. The initial slope is significantly sharper than the background black curve.
Important lessons learned from this set-up include NO’s ability to corrode copper. As a result, all tubing, needles and fittings need to be stainless steel, plastic or rubber. Rubber will become brittle more quickly in the presence of NO, and as a result it is recommended to replace stoppers weekly. Additionally, cleaning the reaction chamber should be done with care to reduce the probability of the chamber becoming brittle and scratching and absorbing proteins.

Quick cleaning of the chamber between runs can be accomplished using a long blunted needle to withdraw the maximal amount of liquid and rinsing several times with de-ionized water. Thorough cleaning is needed at the end of the day or when switching between protein samples. To thoroughly clean the reaction chamber, first withdraw the maximal amount of liquid using a blunted needle. Next, add approximately 1 mL of 1:256 diluted in water Coverage Plus NPD cleaner. Allow solution to stir with the stir bar for at least two minutes, then using the blunted needle, withdraw as much liquid as possible. Rinse the reaction chamber more than three times to ensure the cleaner is removed. Avoid using ethanol as much as possible, as this will make the plastic even more brittle.

Additionally, the sodium hydroxide bubbler that the NO is passed through will lose efficacy over time. It is recommended to replace the 1 M sodium hydroxide solution bi-weekly to ensure purity of NO. To replace the solution, the first and most important step is to vent the gas train with nitrogen before removing the bubbler from the gas line. If unfamiliar with the NO regulator that has the option to vent with nitrogen read the manual, but a brief and incomprehensive summary is to switch the top gas valve to off so NO will not leave the regulator or permit nitrogen to back in to the NO tank. Next, turn on the nitrogen tank then switch the valve on the nitrogen tank to go into the NO regulator. Then turn the NO regulator valve to allow nitrogen to vent through it, in effect purging all lines with nitrogen and removing all NO. Once
nitrogen has run through the system for at least ten minutes, the nitrogen can be stopped and the bubbler can be removed from the gas train. Pour out old solution while still in the hood, taking care not to inhale the vapors or allow the liquid to contact skin, as in addition to being 1 M sodium hydroxide, it also contains various forms of NO\textsubscript{x}. Once the bubbler is empty, rinse it with ethanol and de-ionized water. Refill the bubbler with 40 mL of 1 M sodium hydroxide and replace it in the gas train. Prime the gas line with nitrogen for an additional ten minutes before switching over to NO by reversing the process of allowing the nitrogen to flow through the regulator. It is critically important to close the valve to the nitrogen line before opening the NO, as it will be damaging for the lines if NO enters into the lines connecting the nitrogen tank, especially when it is time to replace the nitrogen tank.

If at any time brown fumes are visible outside the hood, NO is leaking from the container. Quickly try to stop the leak by turning off the tank. Then evacuate and ventilate the room. Contact emergency personnel if warranted. Signs of exposure to NO may include feeling faint, burning sensations from inhalation, and rapid heartbeat. The rapid heartbeat may last many hours after exposure.

To run the experiment, two pieces of software are used in Koder Lab. The first piece of software is for an MCQ Instruments three-channel gas blender. Open the software “Gas Mixer Manager”. In order to use the software, you must click on the “Connect Device” button. Two channels have been set up for use: nitrogen and oxygen. Nitrogen percentage is determined by changing the percentage of oxygen from 0-100%; thus nitrogen will be 100-0%.

The second piece of software is for the WPI gas sensors. To run the hardware, open the software “LabScribe2”. This software can record up to four channels. To vary the display, change units, or change the scale, open the Edit drop-down menu and select Preferences. The
most common problem experienced when using this software is the unit scale mis-matched to the
manual scale on the hardware. Also, the software can sample ten points a second, a feature that
can crash older computers. It has been found that averaging the ten points per second yields an
accurate representation without risk of slowing or crashing computers.
Oxy-lifetime binding has been attempted using kinetic traces on the UV-VIS spectrophotometer. The hypothesis tested was that with the temperature decreased sufficiently, the lifetime of the sample could be recorded using the single second interval traces within the Olis kinetic software. Below is the procedure utilized for this experiment.

First turn lamp on the spectrophotometer. It should warm up for at least thirty minutes to allow for optimal repeatability. While waiting, acquire an ice bucket and fill with ice. Place borate buffer and protein in the ice. Do not place heme or diacetyl-deuteroporphyrin IX (DADPIX) in ice, as the dimethyl sulfoxide (DMSO) has a high freezing point and the mixture will re-freeze. Next, acquire the low temperature sleeve and slide on top of peltier controlled stage, aligning windows to allow light beam to pass through. Do not touch the windows, as they are made of very sensitive optical glass from which oils are incredibly difficult to clean. On the large chiller below the table, press the knob in once then rotate it to adjust the maintaining temperature to \(-3^\circ C\). Press the knob in once more to allow it to begin changing the setting. It is prudent to add some antifreeze to the chiller, as most lab users only take the chillers to room temperature. Next, adjust the peltier temperature control above the table to \(-15^\circ C\) in 2\(^\circ\) increments. To turn on the peltier, press the run/stop button to have the red light flashing for the unit to be on and begin changing temperatures. If the instrument signals “Low Flow”, then the unit was unable to regulate the temperature at the rate desired. In that case, turn it off and then on again and slowly work down to \(-15^\circ C\). It will help if the chiller below the table is well below room temperature.

Prepare a blank sample to minimize background signals from the UV-VIS spectrophotometer. Acquire a 3 mL cuvette, fill with chilled borate buffer, and place in UV-VIS
spectrophotometer. This step assumes there are two identical cuvettes and thirty minutes have
not elapsed since preparation of the samples. If there is only one 3 mL cuvette, it will be
necessary to run the blank, clean it, and prepare the sample as described in the next step.

To prepare sample, first acquire a 3 mL cuvette, then fill with 0.9 mL 100% ethylene
glycol and 2.1 mL complex. Add a very small clean stir bar that is usually kept by the sink.
Avoiding the creation of bubbles, gently flip upside-down and right side up until thoroughly
mixed. Try to minimize fingerprints while still holding the cuvette securely, as fingerprints need
to be removed with Kimwipes. Ensure the cap of the cuvette is the type that has puncture-able
rubber in the center. Place the sample in the sample holder on the stage. The low temperature
peltier controller above the table has a stir bar controller which should be set to the maximum of
10 in order to facilitate maximal stirring, but it will not be sufficient due to the size of the stir bar
and the viscosity of the solution.

As the temperature of the sample is lowered, condensation will form around it, making
the UV-VIS readings inaccurate. To deter this reaction, dry air is blown around the sample
chamber. To do this, remove the tube from the air connection at the wall and gently turn on the
air. When it is just barely flowing, reconnect the tube. The pressure coming out is enough to
blow the connections, so pay careful attention to the glass fittings that are rubber banded together
by the nitrogen train. If the Dri-Rite is pink instead of blue, it will need to be replaced with fresh
that will absorb any liquid in the air.

Next, turn on the nitrogen gas train. Begin by opening a valve after the gas train to
prevent excess pressure from building up within the glass bubblers. Next, open the valves at the
nitrogen tank; ensure the pressure is below the rating of the glass bubblers. The end of the train
should be in de-ionized water and should begin to bubble, indicating the gas is flowing. Pour 4
ml buffer into a falcon tube and degas it by inserting a clean bubbling needle into the buffer. This needs to be kept cold. The buffer can be prepared ahead of time for Sodium Dithionite. This must be degassed for at least fifteen minutes.

Once the system has reached -15°C, blank the system using the prepared blank and the stir bar spinning. This will eliminate small noises it may cause later in the sample. Next, insert the sample with the stir bar into the cold stage and let it reach -15°C. While it is reaching temperature, degas it by running the primary nitrogen line in to it and a secondary line out of it and into the buffer. Since the sample has protein in it, do not allow the nitrogen to bubble the sample; instead, have it flow on top of the sample. Once the nitrogen has flowed over the sample, at least fifteen minutes, take a spectrum of the sample, which is the oxidized spectrum.

Once the sample is reduced, oxygenated buffer will be added to the sample to determine binding. To create the oxygenated buffer, a serum bottle of buffer will be needed. Bubble oxygen directly into the buffer for at least ten minutes to ensure complete oxygenation. When the oxygenated buffer is added, it will be need to be done immediately after removing the oxygen bubbler, as the serum septum does slowly leak. Additionally, the buffer will need to be transferred with a 10 or 25 mL gastight syringe. Again, this is done after reducing the sample, but since the preparation takes more than ten minutes, it is prepared before reducing the sample.

To reduce the sample, remove the Sodium Dithionite from the decanting jar and promptly seal the jar by sliding it shut. Taking a clean micro-spatula, scoop a tip-full into the cold, degassed buffer and seal it. Shake the falcon tube to ensure all the powder has been dissolved.

Using a 10 or 25 uL gastight syringe, add 1 uL of the sodium dithionite buffer at a time to the sample. Because the sample is currently at -15°C, the best method of adding the buffer is to
drip the drop above the sample and then lower the needle into the liquid. The liquid will freeze in the needle otherwise.

To ensure the sodium dithionite is homogeneously mixed within the sample, remove the cuvette and gently flip it upside down and right side up and return it back to the holder. Do this after firmly cleaning the path lengths with a Kimwipe, as condensation will form when removing it from the dry, cold environment.

Take a spectrum of the sample on the UV-VIS spectrophotometer to determine if the sample is reduced. A reduced spectrum will have a characteristic shift of the soret peak with the formation of smaller Q bands as seen in Figure 34. Continue to add additions of sodium dithionite until the spectrum shift appears to be complete. The amount of sodium dithionite buffer will vary with each preparation, as it is dependent upon the degassing and the amount of solid added to the volume of buffer. In Figure 34, the sample needed 10 uL in order to be completely reduced. The spectrum is displayed only from 350 to 750 nm, as sodium dithionite has a large peak in the 300-340 nm range.

Once reduced, change the set up of the spectrophotometer to take kinetic runs. Select “Repeated Scans,” then “Repeated scans as a function of: “Time.” Change the top number to 40 and the bottom to 120. These settings will take a scan every three seconds for two minutes.
FIGURE 34: REDUCTION OF HFHF
Approximately 60 uM HFHF protein with diacetyldueteroporphyrin (DADPIX) is reduced with sodium dithionite. Indications of reduction can be seen in the shift of the soret peak from 424 to 448 nm and the growing in of the Q bands at 550 and 590 nm.

Now that the sample is reduced, oxygen will be bubbled through the sample. Retrieve the septum with oxygenated buffer and 10 mL syringe. Fill the syringe with oxygen gas. A very long needle is essential because it needs to reach the bottom of the cuvette.

Insert the 10 mL needle to the bottom of the cuvette, insert an outlet needle, trying to keep a finger over the outlet to prevent oxygen leaking in, and select start on the kinetic trace. Quickly bubble 5 mL of the oxygen gas through the sample while avoiding bubbling solution out the top outlet needle. It is possible to slightly peak in by taking off the insulator’s cap, not the cuvette’s cap, as removing the cuvette cap will oxidize the sample and require restarting the process.
Ideal data will have clear oxidized and reduced peaks with an additional steady peak that the complex appears to stay in for an extended period of time, indicative of third state, wherein which the reduced iron formation binds with oxygen.

![Graph showing spectral data for FHFH DADPIX with 5 mL O2.](image)

**FIGURE 35: OXY-LIFETIME**
FHFH protein with a concentration of 63 uM with diacetyl-dueteroporphyrin (DADPIX). The anerobic sample was held at -15°C, reduced with sodium dithionite and 5 mL of oxygen gas was bubbled through. Spectra were collected every 12 seconds for nine minutes. This data is inconclusive regarding an oxy-ferrous binding state.
3.1 ANTIBIOTIC STOCK SOLUTIONS

3.1.1: CARBENCILLIN STOCK SOLUTION (50 MG/ML)

Dissolve 2 g in 40 ml de-ionized water. Sterile filter the solution and aliquot 1 ml into green 1.5 ml eppendorf tubes. Store in -20°C.

3.1.2: CHLORAMPHENICOL STOCK SOLUTION (35 MG/ML)

Dissolve 1.4 g in 40 ml 100% ethanol. Sterile filter the solution and aliquot 1 ml into red 1.5 ml eppendorf tubes. Store in -20°C.

3.1.3: AMPICILLIN STOCK SOLUTION (100MG/ML)

Dissolve 4 g in 40 mL de-ionized water. Sterile filter the solution and aliquot 1 ml into green 1.5 ml eppendorf tubes. Store in -20°C.

3.1.4: KANAMYCIN STOCK SOLUTION (50MG/ML)

Dissolve 2 g in 40 ml De-ionized water. Sterile filter the solution and aliquot 1 ml into blue 1.5 ml eppendorf tubes. Store in -20°C.
3.2 PROTEIN/SDS-PAGE GELS

3.2.1: PROTEIN GEL STAIN: 600 ML

Combine 1.2 g coomassie blue, 300 mL methanol, 60 mL acetic acid, and 240 mL de-ionized water. This can be reused for three to six months.

3.2.2: PROTEIN DE-STAIN: 1 L

Combine 400 mL methanol, 100 mL acetic acid and 500 mL de-ionized water.

3.2.3: BLUE SILVER FIXING SOLUTION

Solution can be made at any volume using the following ratios: 60% water, 30% methanol and 10% acetic acid.

3.2.4: BLUE SILVER STAIN

Start with 100 mL de-ionized water. Add 100 mL phosphoric acid and mix. Add 100 g ammonium sulfate, mix until dissolved. Add 1.2 g coomassie R250 mix until mainly in solution. Bring the volume up to 800 mL with de-ionized water and mix. Add 200 mL methanol.

This should be stored in a dark bottle. To stain, soak the gel in fixing solution for 1 hour, then soak in the stain solution for at least 2 hours and destain using water. This is more sensitive than the regular coomassie method.
3.2.5: MES/SDS RUNNING BUFFER (20X) (1 LITER)

Mix together 195.2 g of MES (1 M), 121.2 g TRIS Base (1 M), 20 g SDS (69.3 mM) and 6g of EDTA (20.5 mM). Bring the final volume up to 1 liter with de-ionized water. This will take time to dissolve; heating the water will decrease wait time.

3.2.6: LOADING BUFFER (TO MAKE A 4X STOCK)

In order to make a 4 x concentrated stock, combine 62.5 mM Tris-HCl; pH 6.8 (2.5 mL 1 M Tris-HCl; pH 6.8), 2% SDS (0.8 g SDS, sodium lauryl sulfate), 10% Glycerol (4.0 mL 10% Glycerol), and 0.02 % Bromophenol Blue, 8.0 mg. Add de-ionized water to bring volume to 8 mL. Aliquot 800 uL into 1.5 mL epis and freeze. Add 200 uL bME (beta mercaptoethanol) before use.
3.3 GROWTH MEDIA

3.3.1: LB (LURIA-BERTANI) SOLUTION

Combine the following into 500 mL de-ionized water: 10 grams bactotryptone or bacto peptone, 5 grams of yeast extract, and 10 grams of NaCl. Adjust to 7.5 pH with NaOH. Adjust final volume to 1 L with de-ionized water. Pour out in 100 ml flasks and autoclave.

3.3.2: LB (LURIA-BERTANI) AGAR (FOR PLATES)

Make 500 mL in 1 L flask. Add 5 g bactotryptone, 2.5 g yeast extract, 5 g NaCl and 7.5 g Bacto Agar (this is just agar). Adjust to pH 7.5 with NaOH. Autoclave the solution with a stirring bar inside the container. Two stacks of plates will be created with 500 mL volume.

Once out of autoclave, slowly cool to baby-bottle warmth, stirring to prevent solidification so it does not hurt to touch. Only once to baby-bottle warmth is it time to add antibiotics if making antibiotic plates. Pour solution to form a half-moon in the plate, then rotate plate to cover full surface.

3.3.3: 40% GLUCOSE

Slowly add 400 g of glucose or sucrose to 600 ml of de-ionized water while stirring until dissolved. Add de-ionized water to make a final volume of 1 L. Autoclave the solution.

3.3.4: TPP SOLUTION (PER LITER)

Add 20.0 g bactotryptone, 15.0 g yeast extract, 8.0 g NaCl, 4.0 g Na₂HPO₄, and 2.0 g KH₂PO₄. Adjust to pH 7.5 with NaOH. Autoclave the solution. After autoclaving, (for growth)
Add 1% glucose (25ml 40% sterile stock solution) and whatever antibiotic you are selecting against.

3.3.5: M9 OR MINIMAL MEDIA (PER LITER)

Add 12.0 g disodium phosphate $\text{Na}_2\text{HPO}_4$, 6.0 g monopotassium phosphate $\text{KH}_2\text{PO}_4$, 0.5 g NaCl, and 1.0 g $^{15}\text{N} \text{NH}_4\text{Cl}$ use heavy isotope for NMR samples. Adjust pH to 7.4 and autoclave. Wait until the solution approaches room temperature or just before use and add sterile solutions of: (per liter) 25 mL 40% glucose, 2 mL trace metal solution (500X) and 1 mL vitamin solution (1000X).

3.3.6: METAL STOCK SOLUTION (100 ML)

Combine the following:

- 8 ml concentrated HCl
- 5 g FeCl$_2$·4H$_2$O
- 184 mg CaCl$_2$·2H$_2$O
- 64 mg H$_3$BO$_3$
- 40 mg MnCl$_2$·4H$_2$O
- 18 mg CoCl$_2$·6H$_2$O
- 4 mg CuCl$_2$·2H$_2$O
- 340 mg ZnCl$_2$
- 605 mg Na$_2$MoO$_4$·2H$_2$O
Bring to a volume of 100 ml with de-ionized water. Initially, the solution will be green and will need to be stirred for several hours before everything dissolves. Store the metal stock solution at room temperature.

*The metal salt is not as important as the metal itself. If no iron chloride is available, use iron sulfate, for one example.

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3.3.7: VITAMIN SOLUTION (1000X) (1L)

Combine the following:

1.1 mg biotin
1.1 mg folic acid
110 mg PABA para-aminobenzoic acid
110 mg riboflavin
220 mg pantothenic acid
220 mg pyridoxine HCl
220 mg thiamine HCl
220 mg niacinamide

Add 500 mL de-ionized water and 500 mL high-purity ethanol and filter to sterilize.

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3.3.8: TRACE METAL SOLUTION 500X (500 ML)

Add 10 ml Metal Stock Solution (above) to 26.8 g MgCl2·6H2O then add de-ionized water to 500 ml. Filter the solution to sterilize and store at room temperature.

Note that the Metal Stock Solution is used only to prepare the "O" solution and is not added to the minimal media.
Expect yield to drop about 50% for the same expression systems in LB or TPP. Also, doubling times increase to 40-50 minutes.

3.3.9: TPP MEDIA GROWING INSTRUCTIONS

From fresh plate, pick one colony and grow in 5 mL LB and appropriate antibiotic for at least six hours at 37°C. It is recommended to do this process during the day. Add the 5 mL to 100 mL LB and grow overnight with antibiotic. Next, add 50 mL from the 100 mL LB to 1 L TPP in 6 L Erlenmeyer flask that has the antibiotic that is being selecting against and glucose (25 mL of 40% glucose, equivalent to 1% w/v). Add 1% metal stock and vitamin stock. Allow *E.coli* to grow until OD600 is approximately 1 but not higher. The waste is bio-hazardous, so add bleach to liquid to neutralize. Sterile pipettes such as 10 mL and 25 mL are not bio-hazardous unless they came in contact with *E.coli*. Induce the 1 L of growth to produce protein with 200 mg of IPTG (0.5 mM). Induce for 3-6 hours, depending on protein. Spin down at 10,000 rpm for ten minutes in Sorvall SLA-3000 and collect bacterial pellets. To optimize expression of new protein, collect 1 mL samples every hour and run all samples on a SDS-PAGE to compare expression levels. Freeze each sample to prevent growth while waiting to collect subsequent samples. For proteins that are difficult to express, try expression at lower temperatures such as 30/25°C. It is likely to need to induce for 4-5 hours at lower temperatures,.
3.3.10: MINIMAL MEDIA GROWING INSTRUCTIONS:

Preheat the 100 mL and 1 L M9 media for minimal lag/delay. Add the glucose, divalent metals, and antibiotics to minimal media as close as possible to use.

With the appropriate antibiotic, grow 5 mL LB cultures of bacterial strain at 37°C until cloudy, at least 6 hours, during the day. Use sterile technique and transfer 2 mL of the 5 mL to 100 mL of minimal media. Add antibiotic and grow overnight. Using sterile technique, transfer 50 mL of the 100 mL growth to 1 L M9, add glucose, antibiotic, metals, vitamins (25 mL of 40% glucose equivalent to 1% w/v). The final volumes of metal stock and vitamin stock should be 1%. If using C\textsuperscript{13} glucose, add 2.0 g C\textsuperscript{13} glucose.

Grow to OD600 greater than 0.6 and less than 1. This will take longer than TPP; approximately twice as long. Induce for an additional two hours, six to eight hours total. Again, 10 mL and 25 mL sterile pipettes are not bio-hazardous unless they came in contact with \textit{E.coli}. Liquid waste is bio-hazardous, so add bleach to neutralize.
3.4: FRENCH PRESS INSTRUCTIONS

3.4.1: DNASEI (2 MG/ML)(200X)

Place 20 mg DNase I in 10 mL 20% glycerol and 75 mM NaCl. Aliquot the solution into 1 mL eppendorfs and freeze it at -20°C. To digest DNA during bacterial lysis, add to pellet for a final concentration of 10 µg/mL in lysis or wash buffer, including 5 mM MgCl₂.

3.4.2: NATIVE LYSIS BUFFER 10X (PER LITER) FROM QIAGEN

Combine 500 mM NaH₂PO₄, 69.0 g NaH₂PO₄·H₂O (MW 137.99) and 3 M NaCl 175.4 g NaCl (MW 58.44) and Add 0.02 g Azide NaN₃ to 1 L of de-ionized water. Adjust to pH 8.0 with NaOH.

Wash buffer can be substituted for this buffer.

3.4.3: PELLET PREPARATION

Combine and resuspend the bacterial pellets in 20-30 mL of wash buffer. Add the DNase stock solution to reach 1X concentration. Add minimal amount of Pefabloc-- roughly a micospatula tip. Rotate in cold room for thirty minutes. This step ensures homogenous suspension. While this is rotating, prepare the French Press.

3.4.4: PRESS PREPARATION AND USE

The French Press often causes splashes of liquid. It is therefore strongly recommended that proper personal protective equipment is utilized, including lab coat, safety glasses, and gloves.
First, retrieve the French Press cell from cold room. Place the cell in the press from the rear of the press. Secure the cell in place by tightening the finger screws on the top plate.

Remove the outlet valve and replace the 1/8″ nylon ball with a new one. To remove the ball from the valve, it may be easiest to heat the end of a metal paperclip red hot then insert into the nylon ball. Wait for the ball and clip to cool completely, then spin the clip left and right to loosen the ball from the valve. Wash the French Press cell with 20 mL of ethyl alcohol, and two times 20 mL de-ionized water.

When using the cell, first ensure all valves are closed. Next, open the inlet valve on the back of the cell. Pull the cell plunger up to draw in liquid. The plunger shaft has volume measurements listed on one side that can be used as reference for amount of liquid pulled into the cell. Once 20-30 mL of liquid has been pulled in, close the inlet valve. Turn the press on to medium and allow it to compress the cell and bring the pressure over 1000 psi. For washing the cell, the press can be left on medium. Open the outlet valve ¼ turn at a time until flow starts. The valve can continue to be opened further as long as the pressure remains above 1000 psi. If any air is in the cell or was pulled in with the liquid at the end of the press run, air will be expelled quickly, often causing a splash of liquid.

Once the cell is clean, load 20-30 mL of bacterial pellet resuspension on the cell. Turn the press to medium and allow pressure to stabilize. Then turn to it to high. Slowly open outlet valve in small ¼ turns until flow starts. Using outlet valve, to control flow, do not allow pressure to drop below 1000 psi. Press the total sample three times. Clean the cell by the reverse of the starting procedure 2x 20 mL de-ionized water, 1x 20 mL ethanol. Wipe down all pieces with ethanol. Store all cell pieces in the cold room.
Take the pressed sample and spin down at 15,000 rpm in SA-600 rotor for thirty minutes. Because of the very high speed of the rotor, it is very crucial to ensure samples are balanced. It is recommended to use the digital scale to balance the samples. Upon completion of centrifugation, separate the supernatant from the debris. Check to ensure there is no suspended material. If there is, re-spin or filter using cheesecloth. Run the supernatant over prepared nickel column as soon as possible. Save the precipitate to resuspend in wash buffer with 6 M urea to run SDS-PAGE protein gel in order to ensure most of the protein is in the supernatant and less in the precipitate.

**3.5: HIS TAGGED PROTEIN PURIFICATION**

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**3.5.1: 5X WASH BUFFER (PER LITER)**

Combine 250 mM NaH₂PO₄ 34.50g, 1.5 M NaCl 87.66 g, 100 mM Imidazole 6.808 g, and 0.1 g Sodium Azide. Adjust to pH 8.0 with NaOH. This is prepared in 6 L batches and stored for future dilution.

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**3.5.2: 1X ELUTION BUFFER (PER LITER)**

Combine 50 mM NaH₂PO₄ 6.9 g, 300 mM NaCl 17.53 g, 250 mM Imidazole 17.00 g, and 0.02 g Sodium Azide. Adjust to pH 8.0 with NaOH. This is prepared in 6 L batches and stored for future use.

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**3.5.3: 1X NI-NTA REGENERATION BUFFER (PER LITER)**

Combine 6 M Guanidine Hydrochloride 573.18 g, 0.2 M Acetic Acid 11.4 mL. Adjust to pH 7.4. The guanidine displaces a large volume of water. As a result, begin with 200 mL of hot
de-ionized water and with high stirring, slowly add solids. Once everything has been added, fill to 1 L with additional de-ionized water.

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**3.5.4: GRAVITY COLUMN CELL LYSIS SUPERNATANT PURIFICATION (NATIVE)**

First, ensure the gravity column is clean and recharged, the frit is clean, and the nickel is blue. If the column runs slowly, the frit is most likely clogged. Pull a vacuum to suck 0.1 M NaOH up through the back of the frit, the outlet, and allow it to drain out. Repeat this process multiple times until the liquid flows out very quickly. Then rinse with de-ionized water.

Equilibrate the column with 1x wash buffer by using at least 4 column volumes. Discard the flow-through of the equilibration. Close the column. Load 10-20 mL of lysis supernatant on column. It is recommended to split the total volume of the French Press in half to prevent overloading the column. Stir to ensure maximal binding opportunities. Collect the flow-through in labeled falcon tube. Store the sample on ice.

Close the column. Wash the column with four column volumes of 1x wash buffer. Stir thoroughly. Collect expelled wash in labeled falcon tube. Store it on ice. Close column. **Very gently** add half of a column volume of 1x elution buffer. **Do not stir.** Let the elution on the column sit for at least five minutes. Collect ten fractions of 5 mL elution. You will need to add Additional 1x elution buffer will have to be added after first half column volume travels through the column. Label the fractions or rack. Store the fractions on ice or in the cold room.

Clean column with a quick regeneration protocol.

Run on an SDS-PAGE protein gel lysis debris (resuspended in wash buffer with 6-8 M urea), lysis supernatant, flow-through, wash, elution fractions 1-10 on 4-12% SDS-PAGE gel.
3.6: SDS-PAGE GEL PROCEDURE

Turn on hot block and allow it to reach 85°C and fill wells with de-ionized water. This can be done while collecting samples. Prepare gel samples. If the protein has not been cut, it will likely need to be diluted. An example sample would be 5 uL dye, 5 uL de-ionized water, 5 uL sample. Dye is typically left on the bench, but if none is available, it is stored in the -20°C and can be made following the procedure for preparing Loading Buffer.

Heat samples at 85°C for five minutes. If the sample turns yellow, it is slightly acidic, but it will turn blue again when running on the gel. It should not be an issue. Centrifuge for 4-5 sec to ensure sample is all at the bottom of the eppindorf tube.

Acquire SDS-PAGE gel from cold room; remove comb holder, and white tape. Insert into gel box with labeling facing the outside. Ensure running buffer is cold and not above room temperature. Add or replace running buffer with running buffer stored in cold room. Fill the middle well to top; if buffer doesn't stay in center, ensure the white clamp is tight and the spacer is in place.

Add 12 uL of samples to 14 wells and 8 uL of molecular weight marker to one well. If running multiple gels, strategically placing weight marker at the end on one and middle on the other will quickly differentiate gels.

Turn on the power supply and connect the lid; the voltage should be approximately 200 mV and the gel will run for approximately thirty minutes. You want to run the dye to the bottom without having it run off. If it does run off, it is possible to determine if the gel will still show the results of interest by comparing the location of the weight markers of interest with location on the gel.
Turn off the power; unclamp and remove the gel. Crack gel by using the pallet knife and twisting at each corner. Remove the gel by using the pallet knife to pry one of the lower corners away, and then push knife through slot.

Place the gel in a 1 L beaker and cover with stain. Cover the beaker with cling wrap. Microwave the beaker for thirty seconds. Place it on agitator for ten minutes.

Pour the stain back into bottle. Rinse the gel with water and cover it with destain. Replace the cling wrap. Microwave it for thirty seconds. Agitate for ten minutes.

Discard destain. Rinse the gel with water. Cover the gel with destain. Replace cling wrap. Microwave it for thirty seconds. Agitate for ten minutes.

Discard destain. Rinse the gel with water. Cover with de-ionized water. Replace cling wrap. Agitate for two hours. Results may be visible immediately.

3.7: DIALYSIS PROCEDURE

First prepare dialysis buffer. Select a stir bar, but do not use the largest bars as they are more likely to remove dialysis clips or tear tubing. Wear gloves when handling tubing; water contain 0.05% Azide, and gloves will also prevent enzymatic degrading of protein and tubing. Remove dialysis tubing from the cold room; cut an acceptable length (9.3mL/cm for larger tubing). Remember to allow space for air, water expansion, and four clips. Ensure there is liquid in dialysis storage bag. Seal it and return it to the cold room.

Rinse the outside of the cut dialysis tubing. Clamp fingers over one end and fill with de-ionized water to ensure there are no leaks and release fingers, letting water rinse the inside.
Clamp one end with two clamps. White and pink clamps are for labeling only, not for holding the tubing shut. Alternate the direction of the clamps to maximize likelihood that one clamp will not fall off.

Using a small funnel, fill the dialysis tubing. Work over a Pyrex dish to catch liquid if the tubing slips or overflows. Clamp the top of the tube, allowing air inside the tube to help with buoyancy.

Place the tube in the prepared dialysis buffer. Ensure the buffer is labeled and covered to prevent debris for entering. Place on stirrer in cold room for at least six hours. Protein will degrade if left for long periods, so do not dialyze over a weekend.

### 3.8: CUT PPROTEIN MINI-BIO-RAD PURIFICATION

Ensure the column is clean without air on the column or lines and the nickel is blue. If there is air on the column, remove from the mini-bio-rad and place the inlet and outlet lines in de-ionized water. Seal the bottom of the column using a black fitting. Unscrew the top of the column, which is the end with the plunger. The cap is double-threaded; the lower half screws clockwise onto the threads of the glass column whereas the top threads are counter-clockwise in order to push the plunger down and compress the column. Pull the unscrewed plunger assembly off the glass walls of the column. Be very careful to pull straight up, as applying even very little force to the sides of the glass threads will cause them to break.

Once the nickel column is accessible, add degassed de-ionized water and stir to remove air within the resin. Gently fill the column completely to the top with degassed de-ionized water. Let the column sit carefully until the nickel has settled to the bottom. The glass has no grooves to help hold it with clamps, so be very careful if clamping it to hold it in place.
When the nickel is settled at the bottom, put on gloves and work over the sink. Take the plunger and carefully align it so it is directly above the opening of the column. Apply pressure so the plunger is inserted straight and does not apply force to the sides of the column. Liquid will squirt out the top of the plunger assembly; as long as the frit on the top of the assembly is intact, the liquid should only be de-ionized water and whatever buffer the nickel was previously in. Use manual pressure to force the plunger down as far as possible without expelling all the liquid above the nickel. If at any time air bubbles appear it is possible to try and press them out, but often starting over will be necessary. For the last liquid above the nickel, use the plunger threads and threads on the glass to force down the plunger the final amount without compressing the nickel.

When the column is together and in the mini-bio-rad without air, equilibrate it with 1x degassed wash buffer, using at least four column volumes. Turn on the lamp and ensure it is zeroed in order to visualize absorbance. Discard the equilibration flow-through. Load cut protein and collect output in a labeled falcon tube once absorbance begins to rise. Label the tube “low concentration”.

Store on ice. This fraction contains a low concentration of protein that does not have a His6 tag on it and therefore does not stick to the column. The remainder of the fraction should be wash buffer.

Switch to a second labeled falcon tube when absorbance is above 0.1. Label the tube “high concentration”. Store on ice. Continue collecting in high concentration tube as the absorbance plateaus. If after the absorbance plateaus it begins to increase again, switch to a new falcon tube; label “overloaded”. The high concentration fraction should contain high concentration of protein that does not have a His6 tag on it and therefore does not stick to the
column. The remainder of the fraction should be wash buffer. If an overloaded fraction is needed and collected, this is indicative of running such a high volume of protein over the column that the His6 tags and protein that still has His6 tags attached have run out of binding opportunities; thus are visible in the absorption by the increase after the plateau. This sample is important to keep separate from the high concentration sample as it will need to be rerun over the column to separate the His6 tags and un-cut protein.

When approaching the end of the protein, ensure no air is pulled into pump. It is possible to add a small amount of wash to ensure all protein is put on column. Once all protein is on column, wash with 1x degassed wash buffer. Continue collecting output in either the high concentration falcon tube or overloaded falcon tube until absorbance is below 0.1.

Collect output below 0.1 in a low concentration falcon tube until the absorbance appears to plateau. If the column was overloaded, ensure the low concentration is separated from the initial low concentration, as the later fraction will also need to be rerun over the column. Absorbance may not return to zero.

When the absorbance appears to have plateaued at a low value, load 1x degassed elution buffer on the column. When the absorbance begins to spike, collect output in a falcon tube labeled “fusion protein”. Once absorbance plateaus off near zero, run a quick column regen protocol and discarding output.

Ensure lamb for absorbance is off.

Run an SDS-Page protein gel with the following fractions: lysis debris, lysis supernatant, pre-cut, cut, low concentration, high concentration, overloaded, and fusion protein.
Switch valve to Purge (P) by clicking resize button to reveal valve control. Begin by checking that the pumps are pumping normally by allowing both pumps to pump individually for 2-5 minutes while observing the pressure read out. If there are major fluctuations in pressure, do not use; seek assistance. Make sure you have enough buffer volume to run the purification; more than 500 mL each. All new buffers are to be vacuum-filtered to remove particulate and to de-gas. De-gas each buffer for twenty minutes.

Switch valve to Load (L) by clicking resize button to reveal valve control. The nickel column is equilibrated with two column volumes of 1X wash buffer. French pressed and spun down supernatant is loaded onto nickel column using the auxiliary pump with a loading speed 1-1.5 mL/min. Turn on the quadtec detector from biorad manual screen.

Switch valve to Inject (I) by clicking resize button to reveal valve control. Using the auxiliary pump, add 1X wash buffer to column until A280 absorbance drops to 2.5-2.0. Fill one rack completely with tubes. If running overnight, add eleven to second rack. In the biorad manual screen, click file open, select program Gradient.4, and select new run. Label and date both fields. Click the green play symbol.

When collection is over, go to post run to print the read-out. Combine the tubes that are under the peak. If multiple peaks or if it is the first time running a protein over the column, a gel should be run to determine where the protein of interest came off.

Clean the pumps of buffer by purging the pumps with de-ionized water and shoot 5 mL de-ionized water into wash ports on top of the pump heads.
3.10: TEV CUTTING OF HIS TAGGED PROTEINS

Collect dialyzed protein in 250 mL screw top bottle. Save dialysis tubing for after cut. Remove a 10 μL sample and set aside for a SDS-PAGE protein gel. Add 0.5 M DTT stock solution to protein solution to make final DTT concentration of 1 mM. Add 2% V/V TEV enzyme. Allow the TEV enzyme to cut at room temperature for a minimum of six hours with nutation. Alternatively, the solution can cut overnight in the cold room with gentle stirring.

Run an SDS-Page gel comparing pre-cut sample set aside earlier and a sample that has been cut. Put cut protein solution to dialysis tubing and dialyze into 4 L 1X wash buffer. This will remove the excess DTT and save the nickel column from being reduced by DTT.

3.11: BIO-RAD CUT PROTEIN COLLECTION

Follow starting procedure for cell lysis purification in BIO-RAD purification. Once the pumps have been primed and verified to be functioning properly and the column has been equilibrated, select program cut protein collection. Label and date both fields. Click start button. The program will run wash buffer for ten minutes. Get sample ready by collecting it into 50 mL tube(s) or 250 mL flask. Using auxiliary pump, run cut protein over column no faster than 1.5 mL/min. If more than eighty minutes is needed, pause the program while continuing to pump sample onto the column with auxiliary pump. Watch the A280 read-out and collect into protein fraction as it comes off.

Run wash buffer through column. Clean column by rinsing with elution buffer then washing with wash buffer. Turn off quadtec detector. Clean pumps of buffer by purging the
pumps with de-ionized water and shoot 5 mL de-ionized water into wash ports on top of the pump heads.

3.11.1: GENERAL PROCEDURE FOR USING BIO-RAD DUAL FLOW FPLC

Make sure that you have the right buffers that have been vacuum-filtered to remove particulates and to de-gas. Ensure that all fittings are finger tight. Check manufacturer’s instructions for pressure and flow rate settings for the column being used. To change buffers, pull 10 ml through the priming port with the 35 mL syringe to prime the pumps with the new buffer. This is done by inserting the syringe, twisting the port open one turn, then pulling the plunger to 10 mL; while still inserted, close the port to finger tight.

FIGURE 36: FRONT SCHEMATIC OF BIO-RAD

Using the manual screen, change valve to purge (P). Press the resize button to view the valve controls and pump 1 mL/min pump A for about two minutes to check for stable pump pressure. Repeat for pump B.

Set valve selector to Load (L) and turn on quad-tec detector. Make sure the column is equilibrated properly-- usually 2-3 column volumes of buffer A. Set up rack of tubes and/or separate collection container. Select program to be run-- Gradient.4: is for collection of his
tagged protein, TEV Purification 1: for Harvesting TEV enzyme, Cut Protein Collection.1: for collecting cut protein.

Name the run with the sample type and date in both fields. In the notes, enter the name of the sample and any other relevant information. This can be done after the run is started. Click the green play button at the top right to start the program. The stop button will end the program early and save the read-out. The pause button will stop the read-out and the pumps. The hold button will keep the read-out going but keep the pumps at their position until it is pressed again. After collection of sample, wash the column with correct buffer. Set valve to purge and connect pumps A and B to degassed de-ionized water bottle. Prime the pumps with water. Run 50/50 pumps A and B for ten minutes. Use the syringe to run 5mL of degassed de-ionized water into pump head to wash out ports. If the last two steps are not done, it will lead to salt build-up on the pump heads and non-functioning of this equipment.
3.12: RECHARGING A NICKEL COLUMN

The Quigen handbook claims each column can complete five runs before needing recharging. From experience, the columns can actually do more. When resin changes from light blue to brownish grey, then it is time to recharge the nickel.

3.12.1: QUICK CHARGE

A quick charge begins by running two column volumes of de-ionized water through the column. Next, two column volumes of 100 mM NiSO4 are run through the column. The nickel sulfate is washed off with two column volumes of de-ionized water. Next, two column volumes of regeneration buffer are run through the column. Finally, two column volumes of wash buffer are run through the column to prepare it for use.

3.12.2: FULL CHARGE PROTOCOL

A full charge begins with thoroughly washing the column, using five column volumes of de-ionized water. Next, three column volumes of 2% SDS are run through the column; then one column volume of 25% ethanol, one column volume of 50% ethanol, one column volume of 75% ethanol, one column volume of 100% ethanol and one column volume of de-ionized water. Next, five column volumes of 100 mM ETDA pH 8.0 are run over the column. Then, two column volumes of 100 mM NiSO4 and two column volumes of de-ionized water are run through the column. Finally, the column is equilibrated with two column volumes of wash buffer.

3.12.3: NATIVE LYSIS BUFFER (PER LITER)
Combine 50 mM NaH₂PO₄, 6.9g, 300 mM NaCl, 17.54 g and 10 mM imidazole, 0.68g.

Fill to 1 L with de-ionized water. Adjust to pH 8.0.

3.12.4: NATIVE BUFFER A (WASH) (10X PER LITER)

To make a 10x volume of buffer, combine 0.5M NaH₂PO₄, 69g, 3 M NaCl, 175.4g, and 200 mM Imidazole, 13.6g. Fill to 1 L with de-ionized water. Adjust to pH 8.0 with NaOH.

3.12.5: NATIVE BUFFER B (ELUTION) (5X PER LITER)

To make a 5x volume of buffer, combine 0.25 M NaH₂PO₄, 34.5g, 1.5 M NaCl, 87.7g, and 1.25 M Imidazole, 85.0 g. Fill to 1 L with de-ionized water. Adjust to pH 8.0 with NaOH.
3.13: DNA GELS

3.13.1: 50X TAE BUFFER (TRIS-ACETATE-EDTA)

Combine 242 g Tris base (2 M), 57.1 ml acetic acid and 100 ml 0.5 M EDTA. Adjust to pH 8.5. Adjust final volume to 1 Liter with de-ionized water.

3.13.2: AGAROSE GELS

Pour 550 ml 1x TAE buffer into 1 L beaker. Add 1.2% or 2% (W/V) of agarose and swirl. Microwave for two-minute intervals until all agarose is dissolved. Wear protective gloves. Seal the gel box with tape and insert combs. Add 200 uL Ethidium Bromide. Be sure to wear nitrile gloves, as this is bio-hazardous. Pour into gel box; check that all combs are properly placed.

3.13.3: 10X DNA LOADING BUFFER

Combine 50% glycerol and 50% 10X TAE buffer, then add enough bromophenol blue and xylene cyanol to make it stain dark blue. Any solids that came in contact with DNA gels are bio-hazardous and must be properly disposed in the bio-hazardous waste containers.

3.14: BUFFERS

3.14.1: BORATE BUFFER: 4X(PER LITER)

Add 0.5 M KOH. Add this base first or the boric acid will not dissolve. Then add 1.0 M Boric Acid and 0.4 M KCl. Adjust to pH 9.0.
3.14.2: TEV WORKING BUFFER 20X (PER LITER)

Combine 1 M Tris-HCl, 121.1 g, and 10 mM EDTA, 3.72 g. Adjust to final volume to 1 L. Adjust to pH 8.0.

To use, dilute to 1x and add 0.5M DTT to make the final concentration 1 mM DTT.

3.14.3: LYOPHILIZATION BUFFER

Combine 20 mM Ammonium Bicarbonate and 2 mM glucose or sucrose. Adjust pH to 7.5.
3.15: TEV PROTOCOL

The foundation for this protocol can be found in BioTechniques, volume 30, page 544.

3.15.1: RESUSPENSION BUFFER1:10X (PER LITER)

Prepare 0.5 M Tris-Cl, 60.55 g, and 3 M NaCl, 175.4 g to a volume of 1 L with de-ionized water. Adjust to pH 8.0 with HCl.

3.15.2: RESUSPENSION BUFFER2: (INCLUSION BODY RESUSPENSION)PER LITER

Combine 6 M guanidine-HCl, 573 g, 100 mM NaH$_2$PO$_4$, 13.80 g, and 10 mM Tris-Cl, 1.211 g. Adjust to final volume of 1 L. Adjust to pH 8.0 with NaOH.

The following three have the same ingredients at the same concentrations. Make 1 L of EQ buffer, do not pH it, and then pH 0.5 L to 8.0 for the EQ buffer, 0.25 L to 6.3 for wash buffer, and 0.25 L to 4.5 for the Elution Buffer.

3.15.3: EQUILIBRATION BUFFER(EQ BUFFER)PER LITER

Combine 6 M urea, 363.6 g, 100 mM NaH$_2$PO$_4$, 13.80 g, and 10 mM Tris-Cl, 1.211 g.

As per the note above, separate 0.5 L and adjust to pH 8.0 with NaOH.

3.15.4: WASH BUFFER(PER LITER)

Combine 6 M urea, 363.6 g, 100 mM NaH$_2$PO$_4$, 13.80 g, and 10 mM Tris-Cl, 1.211 g.

As per the note above, using 0.25 L separate from 1 L made of EQ buffer, adjust to pH 6.3 with NaOH.
3.15.5: ELUTION BUFFER (PER LITER)

Combine 6 M urea, 363.6 g, 100 mM NaH₂PO₄, 13.80 g, and 10 mM Tris-Cl, 1.211 g.

As per the note above, using 0.25 L separated from 1 L made from EQ buffer, adjust to pH 4.5 with NaOH.

3.15.6: STORAGE BUFFER: MAKE 2 L, 1 L WILL BE GLYCEROL (PER LITER)

The amounts given here are per liter; to make this buffer, you will need to make 2 L, so double the amount added. In 200 mL of de-ionized water, combine 0.1 M Tris-HCl, 12.11 g, 0.5 M NaCl, 29.22 g, and 0.5 mM EDTA, 0.1861 g. Many of these stocks are in solution form at higher concentrations and can be added as a liquid to eliminate time waiting for solids to dissolve. Once the solution is uniform, add 1 L of glycerol. Fill remainder to 2 L with de-ionized water. Adjust to pH 8.5.

Place the buffer in the cold room, stirring and covered. Just before adding TEV, add 5 mM DTT in solid form. This will not dissolve immediately, but with stirring, it will not impede function.

3.15.7: STEPS FOR TEV PRODUCTION

TEV is a natural protein and a protease that self-cleaves, so care must be taken to keep all protein solutions cold at all times. All solutions, including the final dialysis buffer, should be at 4°C before use and the enzyme should always be on ice if out of the freezer.

Grow *E.coli* with TEV genes included in 5 mL of LB with 5 uL of ampicillin for at least six hours during the day. Use sterile technique to transfer the 5 mL into a 100 mL LB overnight
growth with 100 uL ampicillin. Transfer 100 mL into a 2 L TPP with 2 mL ampicillin, and glucose and metals and vitamins, at concentrations consistent with standard TPP growths.

*E. coli* is expressed until OD600 is approximately 1.0. Then induce with 400 uM IPTG, 0.1905 g for 2 L. Let the *E.-coli* induce for four to five hours. Centrifuge at 10,000 rpm for ten minutes.

Resuspend the pellets in 25mL of 1x Resuspension Buffer 1 per liter of culture. Production can be stopped at this point by flash freezing in liquid nitrogen and storing at -80°C.

Thaw frozen pellets in cool water and add 500 ug/mL lysozyme, 200 ug/mL DNase1, 50 ug/mL pefabloc, 20 mM MgSO₄, and 2 mM CaCl. Stock solutions of MgSO₄ and CaCl can be found on the molecular biology bench. The solids generally are not measured out but rather a micro-spatula tip of lysozyme, DNase, and pefabloc are directly added because of their high activity.

Incubate the pellets for thirty minutes with agitation at 4°C. Lyse the cells by three freeze-thaw cycles. Freeze the solutions in liquid nitrogen until solid, then place the frozen samples in a cool water bath to thaw them. This process is repeated three times.

Add triton X-100 to 1% and vortex for one minute. If this step is skipped or forgotten, TEV purification will also purify a secondary protein that will give the TEV a color. The efficacy of this combination is unsure and will make using the resulting TEV require further purification in order to separate the secondary protein.

Centrifuge the vortexed solution at 10000xg for twenty minutes. Separate the pellets and supernatant. If it is late in the day, this is the place to stop before finishing purification. Pellets can be stored at -80°C. Store the supernatant also, as it will be run on a gel to ensure the protein was in the pellet.
Using Resuspension Buffer2, incubate pellets at 65° until fully dissolved. Fill each centrifuge tube to the maximum allowable. Centrifuge for twenty minutes at 15000xg. While the pellets are incubating, dissolving, and centrifuging, prepare the gravity column and buffers needed. TEV buffers must be prepared fresh, as urea loses functionality over time. Equilibrate column by running at least five column volumes of EQ buffer through the column.

Load supernatant onto equilibrated column. Collect the flow-through in a labeled falcon tube. Wash the column with four column volumes EQ buffer. Collect the liquid exiting the column in a falcon tube labeled “EQ buffer”. Wash the column with three column volumes TEV wash buffer. Collect the liquid in a falcon tube labeled “wash buffer”. Close the column; put 0.5 column volumes on the column and wait five minutes.

Collect the elution in fractions, loading four additional column volumes on the column. If running the Dual Flow Bio-Rad the procedure is named TEV purification.

Make storage buffer and put in cold room so that it is cold when it is needed. Do not add DTT until just before use.

Run a SDS-PAGE protein gel on lysis supernatant, resuspended pellet, flow-through, EQ wash, TEV wash, and fractions. Pool fractions containing TEV and adjust pH to 8.5 with NaOH. Dialyze into storage buffer for four to eight hours. Precipitate is pelleted by centrifugation at 15000Xg and stored at -80°C. Aliquot into 1 mL fractions and snap freeze with liquid nitrogen. Precipitated final pellet is re-dissolved in EQ buffer and dialyzed into fresh storage buffer.
3.16: COMPETENT *E. COLI* BY RBCL METHOD

Care should be taken to ensure that competent cells do not become contaminated. For this reason, all items used in this protocol should be sterile, and hands and work surfaces should be sprayed with ethanol. Solutions 1 and 2 should be filter-sterilized and kept at 4°C.

Inoculate a 3 ml overnight culture with DH5a or *E.-coli* strain of choice. Add 1-5 ml of this culture to 500ml of pre-warmed LB with 2.5 ml 1 M Mg++. Grow until OD600 reaches 0.5. Chill the bacteria in ice water for fifteen minutes. Spin the growth at 3000 rpm for five minutes at 4°C. Resuspend by mild vortex in 30-50 ml buffer I per 500 ml culture. Spin again at 3000 rpm for five minutes at 4°C. Resuspend in10 ml buffer II per 500 ml culture. Aliquot cells into microfuge tubes, either 100 uL or 200 uL aliquots.

Freeze in a dry ice ethanol bath and store at –80°C. Competent cells should be tested before use to check if they are competent or for any contamination.

3.17: TRANSFORMATION

3.17.1: TBUFFER I

To make 1 L of Buffer I combine 12 g of RbCl, 9.9 g of MnCl4H2O, 30 mL of 1 M potassium acetate, 1.5 g of CaCl2H2O and 150 g of glycerol.

To make 100 mL of Buffer I combine 1.2 g of RbCl, .99 g of MnCl4H2O, 3 mL of 1 M potassium acetate, 0.15 g of CaCl2H2O and 15 g of glycerol.

3.17.2: TBUFFER II

To make 1 L of Buffer II combine 20 mL of 1 M MOPS, pH 6.8, 1.2 g RbCl, 11g CaCl2H2O, and 150 g of glycerol.
To make 100 mL of Buffer II combine 2 mL of 1 M MOPS, pH 6.8, 0.12 g RbCl, 1.1 g CaCl$_2$$\cdot$2H$_2$O, and 15 g of glycerol.

3.17.3: PROCEDURE

Thaw competent cells on ice; add 2 uL of DNA to 50 uL competent cells and incubate for thirty minute on ice. Incubate for two minutes at 42°C. Incubate for five minutes on ice. Add 1ml LB without antibiotics. Incubate in shaker for thirty minutes at 37°C. Spread 100 ul if doing regular transformation or all if doing ligation transformation, on a plate with appropriate antibiotic selection. Incubate overnight at 37°C.

This process needs to have controls run simultaneously to ensure that the transformation works. A competent cell aliquot without DNA added should proceed through all the steps in parallel with the competent cell that receives the DNA. When plating the cells, in addition to each antibiotic plate, an LB plate without antibiotics should also be prepared. Thus, for one DNA sequence going into one *E. coli* strain, there should be two Eppendorfs of competent cells and four LB plates, two with antibiotic and two without.

3.18: PYRIDINE HEMOCHROME ASSAY

Note that anything with pyridine is bio-hazardous, and needs to be properly disposed and not put down the sink.

The purpose of this assay is to determine the concentration of heme in heme/DMSO solution.

3.18.1: SOLUTION A
Prepare 20 mM NaOH, 20% Pyridine in de-ionized water. There is a stock of this solution.

3.18.2: SOLUTION B

Dilute 329 mg K$_3$FeCN$_6$ into 10 mL de-ionized water.

3.18.3: PROCEDURE

Dilute approximately 5 mg heme into 5 mL DMSO. Make sure to allow heme to reach room temperature before opening it. Next, it is necessary to blank the UV-VIS spectrophotometer. Using a disposable cuvette, add 958 uL of solution A and 2 uL of solution B to cuvette. Mix solution through inversion. Collect reference along 0.4 cm path length. Next, the oxidized absorbance must be collected. Add 40 uL of the heme solution to the cuvette. Mix through inversion. Record the absorbance and 558 nm. If the absorbance is below 0.1, add a known amount more of heme.

Next, the reduced absorbance must be collected. Cover the tip of a micro spatula with sodium dithionite and add it to the cuvette. The sodium dithionite is kept in a desiccator and it is important to close it right after removing the sodium dithionite. Mix through inversion; the cuvette should turn red. Record the absorbance at 558 nm. The absorbance should be higher than the oxidized value.

Repeat the blank, oxidized, and reduced absorbance measurements two more times after emptying and washing the cuvette. Remember that the pyridine solution is bio-hazardous. Proper personal protective equipment should be worn and the solution should be discarded in the bio-hazardous waste containers.
Calculate the heme concentration using Beer’s Law, \( A = \Delta \varepsilon \cdot L \cdot [c] \). Recall the path length, \( L \) is 0.4 cm. Absorbance \( A \) is the average of the difference of reduced absorbance less the oxidized absorbance. The extinction coefficient, \( \Delta \varepsilon = 27.06 \text{cm}^{-1} \text{mM}^{-1} \). This will allow solving for \([c]\) for the heme concentration in mM.

### 3.19: MAKING GLYCEROL STOCKS

Using sterile technique, add 750 µL of sterile 80% glycerol stock to 2 mL glycerol stock tube that has been labeled on the top and side. Add 750 µL of growth. Gently invert several times to mix the glycerol and bacteria. Store in -80°C.


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I have been taught that all experiences and choices help to shape the person you are currently and who you become. The choices that led me to this page of my life have been vast and often represent the path less taken. That is why family and friends have always played such an important role in my life, helping me to become the scientist and woman I am today. They have helped me to overcome obstacles and best nay-sayers.

As this chapter of my life comes to a close, I am excited for the next to begin. I believe that while I will be taking the path less taken, it is the path I have been working towards all this time, often without knowing it. The skills I have developed expand far beyond laboratory work. I have faith in myself. I know how to learn, how to research, how to struggle with a problem and not give up. I am capable of nearly anything given the resources. I can work independently or in a group. I am resourceful. I can explain myself clearly and thoroughly. I can put my thoughts down coherently so others can understand and follow my reasoning. I know how to hypothesize and systematically test my hypothesis. These are only some of the skills I have learned beyond the laboratory specific skills and they will continue to serve me well.