

Unfolding Forces of NLeC, a Type III Secretion Protease

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This project's main goal was to profile a Type III Secretion Protease, NLeC, secreted by bacteria cells that unfolds to enter a cell and then refolds back into its active state inside the target cell. This experiment was done in the Summer of 2013, as part of the REU program in Physics run by the University of Colorado at Boulder, funded by the National Science Foundation.

Abstract

Bacterial cells have the capability to infect other cells and cause illnesses in a variety of species. One of the ways in which infections are spread is through the use of a Type III Secretion System. This is a system of a few proteins on bacterial cell membranes that allows them to attack target cells. A key feature of the system is a protein needle that directly connects the bacterial cell to the target cell. Having this connection allows the bacteria to secrete effectors that infect the target cell. One of these effectors is NLeC, a Type III Secretion Protease. It, along with a few other bacterial effectors of the same class of Secretion Proteases as NLeC, enters cells and disrupts the normal functioning of the NF-kappaB complex. This NF-kappaB complex communicates with the nucleus of the target cell to start an inflammatory response. However, when cells are infected and NLeC is present, this complex cannot function, and it effectively inhibits part of the inflammatory response in target cells [1].

The particularly interesting piece about this is that NLeC is a large and intricately folded molecule in the bacteria cell before being injected into target cells. There are two cases of bacteria that make and use NLeC, one which bacteria have evolved and mutated to have this Type III Secretion System, and its ortholog, where the system is not present. However, in both situations, NLeC is involved. In the first, NLeC must fit through this secretion needle and in order to do so must unfold, travel through

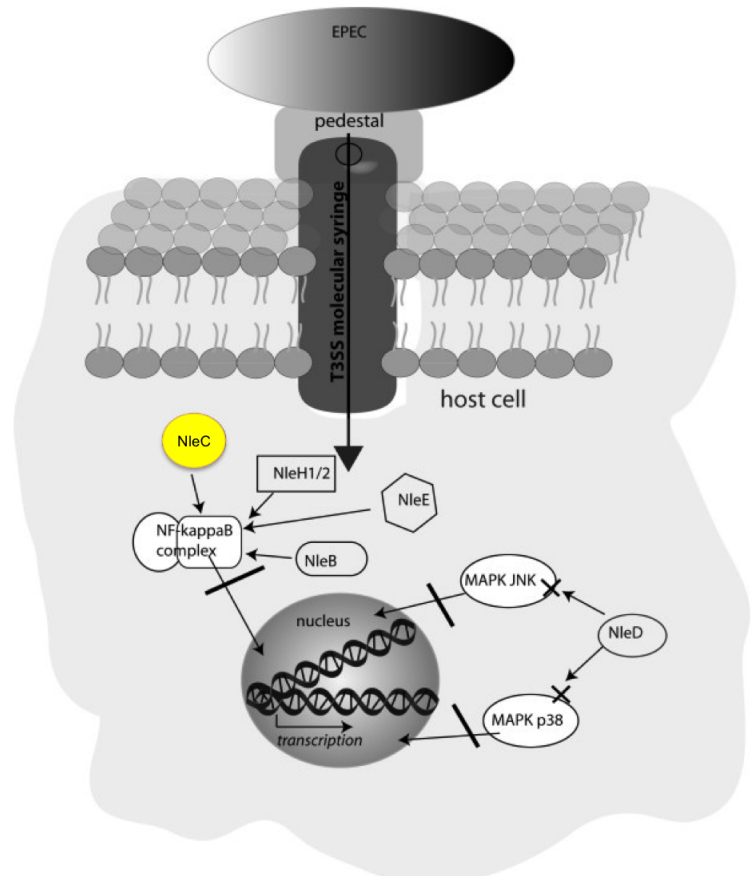


Figure 1: EPEC, enteropathogenic *E. coli*, infects cells via the use of a Type III Secretion System and needle. NLeC, a Type III Secretion bacterial effector, inhibits the inflammatory response in target cells by interfering with the NF-kappaB complex [1]

the needle, and refold into its active state inside the target cell. The other case, NLeC can enter target cells similar to other molecules entering cells, by transport, without a needle. The two main questions in this study are: 1) Does NLeC unfold at low forces, as to allow the bacteria to save energy and time? or 2) Does NLeC refold with fast kinetics once in the target cell, again to save time?

1 Introduction

The goal of the experiment is to profile the unfolding forces this Type III Secretion Protease, NLeC. There are two main steps involved, each with sub steps. These two main phases are the preparation of the surface and atomic force microscopy to measure the forces. The first set of steps, is a multi-step process to functionalize the glass slides for proper adherence of the chemicals and molecule in question. Important parts of the project include: the use of different chemicals and buffers, as well as lab techniques (such as use of micropipettes, centrifuge, tweezers), Atomic Force Microscopy (AFM) of the slide with the protein and Analysis of results looking for the correct features from the pulling, in IGOR.

2 Methods

As stated, there are two main methods that are used to fully complete an experiment in the lab. The first is the preparation of the surface, from a clean glass slide to a functionalized sample with the protein in question loaded onto it. The second is the actual experimental part where use of an Atomic Force Microscope (AFM) is used to measure the unfolding forces of the sample. This is done by tapping the surface with the cantilever tip, hopefully pulling a protein up and “popping,” or unfolding, each piece to see a force curve on the computer due to the calibrations set. Finally, this data is analyzed to determine how this protein works and to test the two hypotheses proposed.

2.1 Surface Preparation

The slides that the are eventually used to hold the protein and gather data in the AFM start out as

simple 1.5 cm squared disks no more than a millimeter or two thick. In order to avoid sources of error, these first steps are crucial in completely cleaning the surface. Working on the single molecule scale means that even a speck of dust could ruin an otherwise decent sample. Potassium hydroxide (KOH), a strong base, is used to do the first cleaning of the glass. This is very good at ridding all organic substances that could be on the surface.

Once the surface is rid of these contaminants, the functionalizing begins. The first step is pouring a certain type of silane on the slides. This will make the slides more hydrophobic and it also includes an amine group that allows adherence of the next molecule. Next the NHS-PEG-Maleimide layer is added. This is a solution of a few different chemicals that will act as an in-between layer for the silanes and the protein that will be added.

Although this experiment is concerned with a single protein, NLeC, other proteins in a polymer chain must be used a handles to signal the graph is in fact one including the protein. In this chain are four NuG2s and a cysteine on the end to bond to the NHS-PEG-Maleimide layer. NuG2s are proteins of which the force profiles and contour lengths are known.

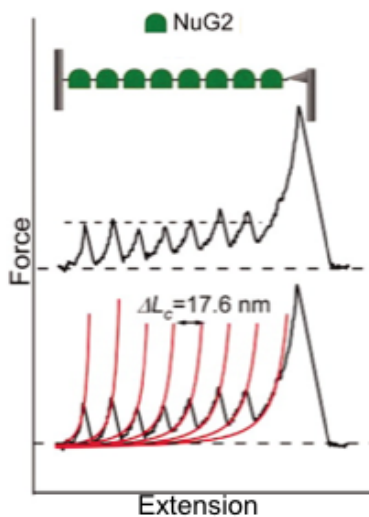


Figure 2: The diagram shows a Force vs. Extension curve of an NuG2 polymer. It has a characteristic sawtooth pattern. It also has a contour length of around 18 nm. This will be an important detection marker for the construct used in the experiment [3].

These are helpful in determining if a graph with some sort of pull means something or if it means there was some other contaminant or source of error. The NuG2s have been profiled to have a contour length of roughly 18 nm. In looking at the final results, if there are force curves with the similar shape as NuG2 as well as another curve not

previously known and same contour length, then a good pull has been recorded and further analysis may be done to determine more information about the protein in question.

The cysteine on the end of the protein construct with NuG2s and NLeC joins the chain with the now functionalized glass surface. This should, ideally, properly orient the protein construct for the best pulling results.

2.2 Procedure

1. Place two glass slides in petri dish under UV light for 15-20 min.
2. Silanize step: separate slides into two different petri dishes and cover with Silane, let sit for 30 minutes.
3. Place glass slides in teflon holder and rinse by dunking in two beakers of isopropanol and two beakers of nano pure water.
4. Use pure nitrogen air gun to dry the slides completely.
5. Place glass slides in a petri dish and incubate in the oven, in a vacuum, for one hour.
6. Add borate buffer to the petri dish containing both samples, place on rocker for at least an hour (may leave overnight).
7. Remove NHS-PEG-Maleimide from freezer, while it is thawing, use pure nitrogen air to dry the slides from the borate buffer.
8. Centrifuge the NHS-PEG-Maleimide with a counterbalance of Borate Buffer, pipette 50 microliters of the NHS-PEG-Maleimide onto one glass surface. Flip the other slide and place on top so that both functionalized sides face inward in a "Maleimide Sandwich". Let incubate for one 1 hour.
9. Place metal disks on a hotplate and set temperature to 110 degrees Celsius. Dot wax on around 100 degrees Celsius. Add glass slides, functionalized side up to the metal disks.

10. Add TCEP and NLeC to the sample in a 3.5 to 1 ratio. Let incubate for 1 hour.
11. Use sample or put in humidity chamber in refrigerator until use.

2.3 Atomic Force Microscopy

Atomic force microscopy is key in this experiment to actually measure the forces it took to pull and unfold this polymer. In this case, a stable, top of the line commercial AFM was used in order to limit noise and make the most precise measurements possible. This is particularly important for the experiment because the single molecule scale can encounter many issues that may otherwise seem fine in other cases. The machine is able to do this because of what goes on inside its door. The sample in question is mounted onto a metal disk that is held securely in place on the stage via magnetism.

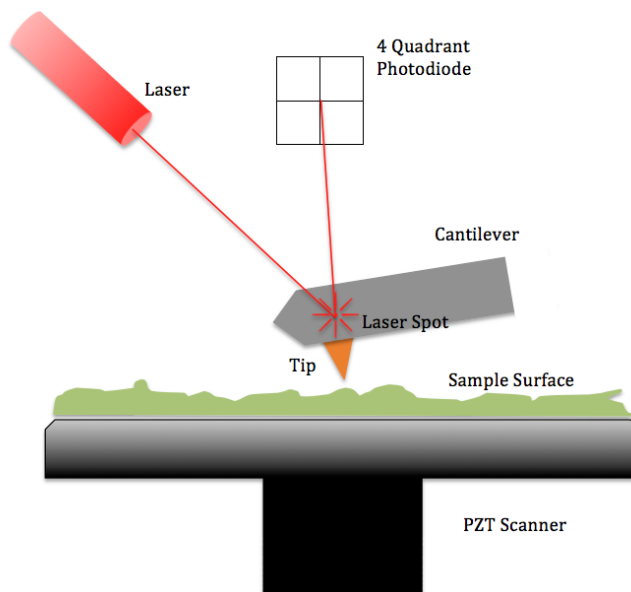


Figure 3: A schematic drawing of the basics of an AFM. The tip on the cantilever approaches the surface, pushes down and pulls back up, if a protein is attached, the cantilever will bend causing the deflected laser spot on the photo diode to measure changes in voltage resulting in a graph with a force profile.

In order to pull on the surface, the first requirement is a tip. This is situated at the end of a cantilever, which there are four of on a chip. This cantilever will approach the surface and push into

it with the tip. The cantilevers used in this experiment are Olympus BioLever Longs. They measure $100\ \mu\text{m}$ in length, $30\ \mu\text{m}$ in width, and $180\ \text{nm}$ in thickness; all measurements include the gold coating. The cantilevers also have a tip that protrudes about $7\ \mu\text{m}$ and is $30\ \text{nm}$ wide[2].

The tip will touch down to the surface at varying pulling speeds, according to what calibrations are entered into the computer. Speeds have varied from $500\ \text{nm/s}$ to $2\ \mu\text{m/s}$. Force at which the tip is pressing into the surface also varies, from $100\ \text{pN}$ to $400\ \text{pN}$. These can be varied, as different graphs will result.

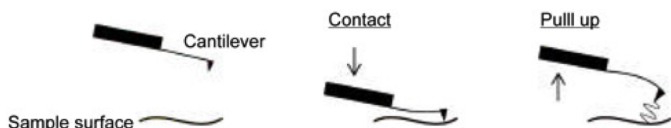


Figure 4: A cantilever and tip approach a surface, make contact, and pull away with a polymer attached. It is clear the bend that happens in the cantilever from these pictures, this bend causes the laser beam deflected off of the cantilever to shift off the center of the photodiode to correctly determine the deflection to calculate the forces [2].

However, it is important to note that the computer and machine do not just know there is a protein attached, and this is why a laser is involved. There is a laser aimed and focused on the cantilever that deflects onto a four-quadrant photodiode. This photodiode measures deflection of the laser spot. If the laser spot moves too far off of the center of the diode, it is clear that the tip has something attached and the cantilever is bending in response. This produces a graph on the computer that may be a protein, or could be a contaminant, which is unfortunately common because it is easy to contaminate surfaces dealing with single molecules.

In order to capture the right data, a few calibrations in IGOR Pro and Asylum Research software must be done. First, calculate the inVOLS; this finds distance/volts. Second, capture thermal data and fit the curve to find stiffness and the resonant frequency of the cantilever. Finally, force/volts can be found by combining the results of these first two steps. Combining these two pieces gives the k , spring constant, value of the cantilever and tip

pulling system [3]. This approach to the surface, push in, and pull back repeats some one to two thousand times. It is automated by the software on the computer.

3 Results

Although 30,000 pulls were done this summer, only about 30 of them were actually useable in the final data set. However, these graphs that were acceptable showed promising results. The overall goal of this project was to determine if it was even plausible to pull on this protein and see unfolding forces; the two hypotheses about the protein's folding and refolding patterns were just—hopefully—the next step.

The outcome of this experiment is actually very encouraging. On the single molecule scale it is common to see many errors and not actually pull on the correct polymer. However, on the pulls that were decent, the data showed more about the protein, NLeC, in question.

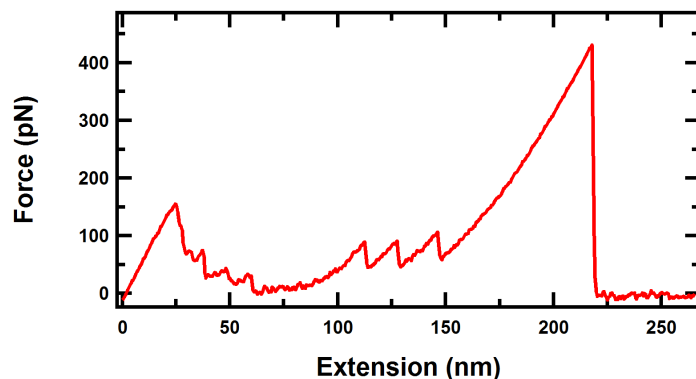


Figure 5: An actual Force vs. Extension curve. The tip pushed into the surface with $150\ \text{pN}$ of force at $1\ \mu\text{m/s}$. There is a visible large well which, based on other data collected, is considered to be NLeC. Following the Type III Secretion Protease are two NuG2s (see Fig. 2) and then a large rupture force once the peptide is fully extended and breaks from the surface.

The unfolding of this peptide is similar to pulling a rope and having a knot untie. When pulling the rope, it becomes taught, then after a certain amount of force, the knot unties, and the rope goes slack. But the pulling speed has not changed so after a moment, when the rope ends extend further,

the rope will become taught again.

Another example shows that the protein (NLeC) is clearly much larger than the NuG2 proteins in the peptide chain and also has a different force profile.

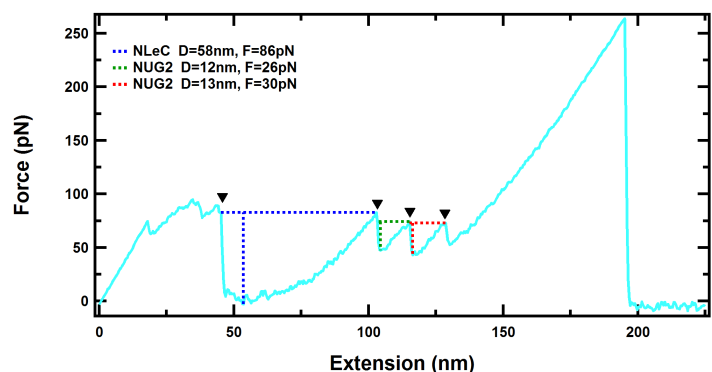


Figure 6: Another Force vs. Extension curve. Black arrows label beginnings and ends of peaks. The dotted lines show the change in extension and the change in force. Also labeled at the top of the graph are the numbers associated with the lines. This will be useful in plotting the change in force against the change in extension of each protein to try to find clusters that show similarity in the molecules unfolded.

A main part of the preliminary step of this project is to see these clusters develop. If present, they show that there is consistency in the findings, further proving that the large well seen is the protein in question, and not just a different contaminant each time.

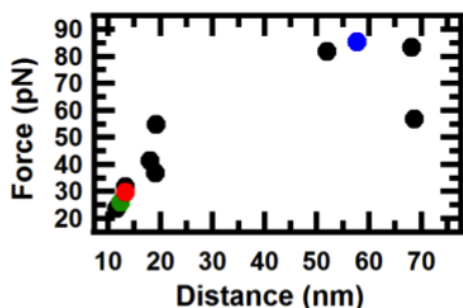


Figure 7: This graph plots, as points, the change in Extension and the change in Force of the proteins unfolded by 800 nm/s pulling rate. There is a clear emergence of two clusters, even in this early data. Only four NLeCs are included in this graph.

Based on these findings, it appears that NLeC,

the protein in question, unfolds at around 85 pN of force and Extends about 65 nm. However, based on pulling at different rates, it has also been noted that pulling rate can affect the force required and extension of the protein. In general, a faster pulling rate will cause the protein to unfold at larger forces and extend longer distances before rupturing. This is because the protein has less time to react, so it can overshoot the distances that it would normally take to unfold and be done at larger forces.

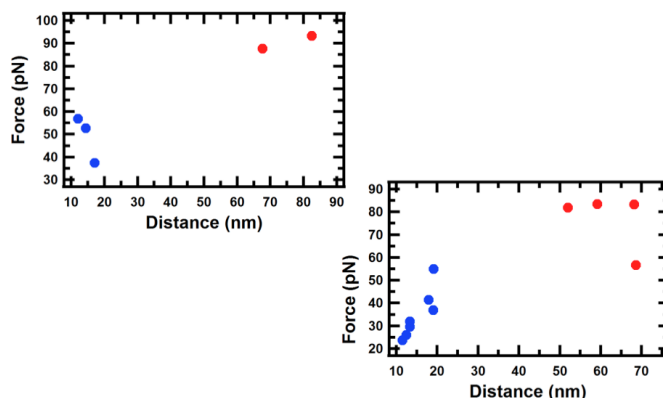


Figure 8: Difference in force required and distance extended based on pulling rate. The graph on the left is of pulling rate $2 \mu\text{m/s}$ while the graph on the right is of 800 nm/s

Examining the graphs above it is apparent that a faster pulling rate does indeed allow the protein to extend longer and under higher forces. In the $2 \mu\text{m/s}$ case, the average force required is about 90 pN and the distance extended is about 80 nm. In the 800 nm/s case, the average force required is about 80 pN and the distance extended is about 65 nm.

4 Future Directions

Much more work is required to fully prove which hypothesis is correct (see Abstract), however this initial part of the project has answered many questions. To improve this experiment, changes to the surface may be in order, such as to leave TCEP; a chemical buffer that prevents the formation of disulfide bridges from forming on the surface, on to prevent NLeC from bonding with the surface itself (NLeC has a cysteine, which has a Sulfur atom in its functional 'R' group).

Other changes could be in the tip approach itself. Adhesion forces have given some trouble in the data collection, so another group member is working on a program that will tell the tip to approach the surface, break the adhesion force, and then go back down to pull up a protein without the large adhesion force at the beginning of a pull.

A huge next step will be the implementation of the second phase of the project, refolding the protein. This is a much more complicated process than simply making a sample and tapping on it with the AFM. It will also be susceptible to more errors because the same sources of contamination apply as in the first phase, but now another error will play in. In order to measure refolding, the tip must pull a protein to full extension, then slam back down to the surface quickly to measure the refolding forces. If in the first phase, our success of a good pulling curve was about 0.1%, the success rate of Phase II, getting a pull then correctly slamming the tip back down to the surface is even lower. More studies about how to approach this project needs to be done.

5 Acknowledgment

I would like to thank my Principal Investigator, Tom Perkins, who allowed me to work in his labs and with his group this summer. Also, my post-doc, Rob Walder, who helped start this project and teach me new techniques, exposing me more to the world of biophysics as well as atomic force microscopy. My lab partner, Ayush Adhikari, who worked alongside me this summer teaching me the surface chemistry steps and having a great partner to learn more with. Huge thanks to Debbie Jin and Dan Dessau, who organized the REU Program at the University of Colorado at Boulder and allowed me to be a part of it, and to the NSF who funded this program.

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