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Cross-linking Amyloid Forming Proteins for Improved Understanding of Early Aggregation

Gram Booth
University of Arkansas, Fayetteville

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An Undergraduate Honors College Thesis by Gram Booth

Department of Chemical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

Abstract

Alzheimer's disease is the only disease in the ten leading causes of death in the United states that cannot be slowed, prevented, or cured. Alzheimer's dementia and type II diabetes are the top two diseases caused by improper protein folding, aggregation, and deposition of fibrillar plaques in tissues. These plaques, originally thought to be the cause of these diseases, have been discovered to be mostly benign and representative of the later stages of the disease. The smaller, more soluble oligomeric aggregates are responsible for the death of pancreatic and neural cells. Many oligomeric species are unstable and exist only for a short period of time, which makes them difficult to study. Photo-induced cross-linking of unmodified proteins (PICUP) is a method that allows researchers to take "snapshots" of an aggregation as it progresses. In this study, PICUP is used to study two proteins, amyloid beta and amylin, which are involved in the continuation of Alzheimer's dementia and type II diabetes. Both proteins were successfully cross-linked and visualized on polyacrylamide gels using silver staining techniques. Amyloid beta aggregation in sodium phosphate and HEPES buffers were also compared and showed similar aggregation between the two.

1. Introduction

1.1 Alzheimer's and Type II diabetes

The six FDA approved Alzheimer's drugs on the market today serve to temporarily alleviate symptoms of the disease by increasing the amount of neurotransmitters in the brain (3). These treatments neither slow or stop the progression of the disease. As it is a degenerative brain disease, Alzheimer's is the leading cause of dementia, or a group of symptoms including difficulties with memory, language and other cognitive functions (1-2). While 1% of cases are classified as genetically onset, the largest factor for Alzheimer's diagnosis remains age. One in ten people age 65 or older has Alzheimer's dementia, and these patients account for 96% of all cases of Alzheimer's. The fact that 32% of people age 85 or older have Alzheimer's dementia illustrates just how large a role the aging brain plays in the disease (6). The concern looking forward is that the number of Americans surviving past 65 years of age is expected to grow drastically due to medical advances and improvements to social and environmental conditions. By 2030, the U.S. population age 65 and older will increase to a projected 74 million people; accounting for over 20% of the total American population (8). In the state of Arkansas alone, an increase of 21.8% of people with Alzheimer's dementia is expected by 2025 (7).

Other than Alzheimer's disease, type II diabetes is the most prevalent disease caused by incorrect protein folding with more than 422 million people affected world-wide (9). In the U.S. alone, 29.1 million people had been diagnosed with diabetes by 2014, 90-95% of which representing type II. Type II diabetes is characterized by the reduced production of insulin and thereby suppressed regulation of excess extracellular glucose. This decreased insulin production is associated with death of pancreatic beta cells whose cause is similar to that of brain cells in Alzheimer's dementia.

1.2 Amyloidogenic diseases

Both type II diabetes and Alzheimer's disease are classified as amyloidogenic diseases due to their similar pathology. Deposits of fibrillary plaque due to the misfolding, polymerization, and deposition of amyloid protein are characteristic of a patient who has been diagnosed with an amyloidogenic disease. Amyloid proteins are proteins normally expressed in the body which have a tendency to misfold from their normal conformation, which leads to individual proteins interacting and sticking together to form clusters of protein called aggregates (16). The smaller groups of protein remain soluble in blood and tissue. However, as the aggregates become larger they become insoluble fibrils, precipitating out of solution and forming protein deposits first discovered by physician Rudolf Virchow in patients with Alzheimer's dementia. These deposits were first mistaken to be starch due to the inaccurate iodine-staining methods used by Virchow at the time. Amylum is the name for starch in Latin, therefore the name Amyloid was developed to refer to any other starch-like protein deposits (14).

In order to properly treat this disease, understanding of the underlying mechanism for protein aggregation has been an important topic of study. A primary goal within the Hestekin lab is to better understand amyloid aggregation through the use of a variety of separation and staining techniques. Results from our lab could allow for the creation of better models to represent aggregation in Alzheimer's and type II diabetes, which could be extended to other amyloidogenic diseases like Parkinson's and Huntington's disease. The improved understanding of the aggregation mechanism could also lead to treatments for the reduction or inhibition of aggregation.

1.3 Amyloid Beta

Years after the initial discovery of cerebral amyloid deposits in dementia patients by Alois Alzheimer, it was discovered that these deposits were actually composed of protein instead of starch (15). This protein was named amyloid beta due to the abundance of beta sheet in its structure. Amyloid beta is formed after being cleaved from a much larger protein located in the synapses of neurons in the brain called amyloid precursor protein (APP). APP is integral in neuronal development, signaling and repair (18). One of two metabolic pathways lead to the formation of amyloidogenic amyloid beta from APP, which is the main target for many Alzheimer's disease therapeutics (19). Once formed, amyloid beta most commonly exists as a 42 peptide chain with the sequence given below in Figure 1.

Figure 1: Amyloid beta's Primary Structure (Figure created by Booth)

D A E F R H D S G Y E V H H Q K L V F F A E D V G S N K G A I I G L M V G G V V I A

The natural confirmation of amyloid beta is an alpha helix, which can be converted into a beta sheet due to the stabilizing effects of hydrogen bonding and aromatic peptide π-π interactions. Around 25% of the surface of the structure is hydrophobic which enhances the protein-protein interactions that lead to aggregation.

1.4 Amylin

Amylin or islet amyloid polypeptide (IAPP), another amyloid of interest, has been indicated as a key player in the development and continuation of type II diabetes. Amylin (IAPP) and insulin are both produced by pancreatic beta cells from the same 89-amino acid long precursor protein

referred to as preProIAPP (10). Since amylin and insulin are synthesized together at a 1:20 molar ratio, an increase in demand for insulin leads to an increased production of amylin (11). At relatively high concentrations, misfolded amylin protein tends to form aggregates which have been shown to be toxic to pancreatic beta cells, even causing cell death (12). The primary structure of the 37 residue long protein is shown below in Figure 2. Besides peptides 1-7, the entirety of amylin's peptide chain has been shown to exhibit amyloidogenic properties (13).

Figure 2: Amylin's Primary Structure (Figure created by Booth)

K C N T A T C A T Q R L A N F L V H S S N N F G A I L I S S T N V G S N T V

Similar to amyloid beta, hydrophobic interactions induce the formation of beta sheets which are then stabilized by hydrogen bonding. These beta sheets are the main culprit for amylin aggregation, as they act as a nucleation site for the progression towards higher order aggregates like oligomers, proto-fibrils, and insoluble fibril deposits (20).

1.5 Fibrils Vs. Oligomers

The insoluble, fibrillary deposits had been previously considered to be the main key to these diseases as well as the most cytotoxic form of amyloid protein. It has been more recently accepted that the smaller, more soluble oligomeric species of protein are more toxic and fibrils more benign (17). The mobility of oligomers allows them to move around in the blood and fluid between neurons, causing further damage. Oligomers also tend to be more unstable than the monomer or fibril form, making them a lot harder to study over a period of time.

1.6 Methods Used to Study Oligomers

a) Photo-induced Cross-linking of Unmodified Proteins

Photo-induced cross-linking of unmodified proteins (PICUP) was used to study these early, unstable stages of protein aggregation by allowing us to take "snapshots" of the protein aggregation as it progresses. PICUP allows researchers to collect data about the smaller, more toxic stages that are still soluble and therefore more maneuverable in the body. The cross-linking method shown in Figure 3 was first developed by the Kodadek laboratory at the University of Texas as a way to study proteins that form stable oligomers (12).

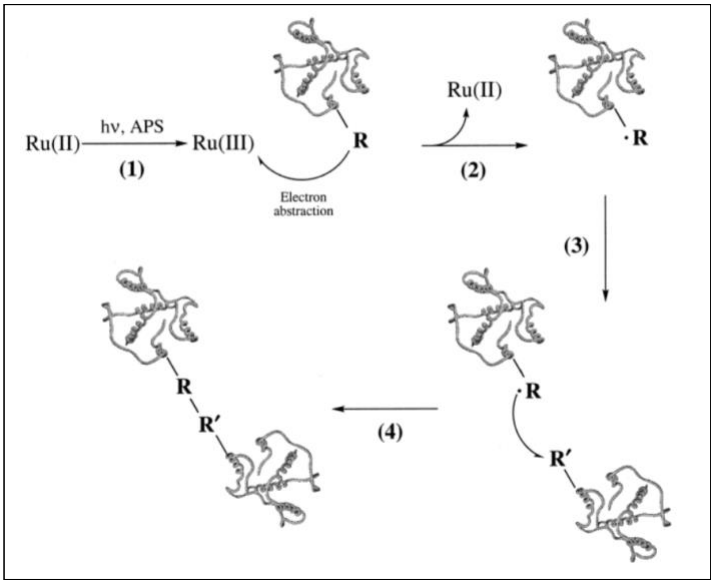

Figure 3: Photo-induced Cross-linking Reaction

PICUP relies on the photo-oxidation of tris(2,2'-bipyridyl)dichlororuthenium(II) complex or Ru(II) to Ru(III) in the presence of an electron acceptor, commonly ammonium persulfate (APS). The Ru(III) formed acts as a strong oxidizer, removing a single electron from any nearby proteins and creating free radicals, recycling back to Ru(II). Formation of radicals on proteins

can form anywhere, however the areas where stabilization through aromatic side chains are preferred. Once formed, the radical is either stabilized through an intramolecular covalent cross-link with a neighboring protein or reaction with the surrounding medium. The reaction is stopped with the addition of 2-Mercaptoethanol (BME), a biological antioxidant. The cross-linking reaction takes place in under 1 second, which is a desirable trait of this method. An exposure to light of around 2 seconds has been shown to be optimum for the study of oligomeric proteins. Further aggregation after PICUP can be separated using a reagent such as sodium dodecyl sulfate (SDS), leaving the covalent cross-linkages in place. This allows for the later study of aggregate samples taken over a long interval of time by removing the problem of instability in small aggregate species. PICUP allows these early protein aggregates to be analyzed for characteristics including size distribution and relative abundance of the different sizes.

b) Protein Separation and Visualization

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most prevalent methods used to separate proteins. SDS is a denaturant that helps unfold and unbind proteins to allow for separation based on size. Gel electrophoresis is used to create an electrical force field across a porous polyacrylamide gel, creating a flow of molecules through the pores of the gel. The larger the molecule, the slower the movement throughout the gel, and therefore a separation based on size is achieved. Charge density is also a factor for migration speed, however SDS helps eliminate differences in surface charge.

Out of all of the methods used to stain proteins in polyacrylamide gels, silver staining was chosen due to its sensitivity even at the nano-gram range and relatively low cost. Since the amount of protein used in aggregation assays is on the order of micro-molar, the final amount of

protein in a single band on a gel can be rather hard to detect. Silver stain is sensitive down to a very low nano-gram range which made it perfect for our needs. The method is also completed using common and relatively cheap reagents which allowed us to perform all of our experiments without having to replace the original reagent kit. The basis for silver staining stems from the fact that proteins bind silver ions, which can then be reduced into silver metal which builds a visible image of the silver-bound protein. There are five main steps used in a silver staining assay: i) fixation to immobilize the protein within the gel and remove interfering compounds; ii) sensitizing and water washes to increase sensitivity of staining and remove molecules that may increase background noise; iii) silver ion solution diffusion into gel, binding to protein is diffusion limited; iv) water washes and development to create visual image; v) stopping solution which ends development at desired intensity.

2. Materials and Methods

2.1 Protein Preparation

In order to prepare our protein for aggregation, amyloid beta (lot and company) and amylin (lot and company) were treated with hexofluoro-2-propanol (HFIP) in order to produce monomeric samples of a specific size. HFIP was placed on ice in a chemical fume hood for around 15 minutes to cool before use. A solid sample of amyloid protein received by the manufacturers was removed from the -80°C freezer and placed on ice in the chemical fume hood. Enough HFIP was added to the solid protein to bring the final protein concentration to 1 mM. The HFIP and protein mixture was well mixed by pipetting the solution in and out 10 times around the storage vial. The solution is then well sealed to trap the volatile HFIP and allowed to rest on ice for 60 minutes. If the solution was anything other than clear and colorless, a note that the peptide quality could be

poor was made and the manufacturer contacted. The volume of 1 mM solution necessary to produce the desired amount of protein/vial was calculated. This volume of solution was quickly pipetted into non-stick micro-centrifuge tubes. The open tubes were allowed to evaporate in the fume hood overnight, producing a thin clear film at the bottom of the tube and then stored at -80°C. White or chunky peptide indicated poor peptide quality as well. The number of tubes produced is compared to the theoretical number based on the initial volume of HFIP added to the protein. If the number of tubes differed by more than four then further analysis was required to determine the actual amount of protein in each tube.

2.2 Nano-drop Analysis

During certain HFIP treatments, pipetting error and premature evaporation of HFIP lead to unknown amounts of protein/vial. Nano-drop analysis was required to confirm the amount of protein per aliquot directly before use in experiments. A ThermoScientific NanoDrop Fluorospectrometer was used to perform this analysis. For the proteins used in this study, a wavelength of 280 nm was chosen to determine the concentration of our protein samples. (explain why) The surface of the measuring pedestal was cleaned by placing 2 µL of water on the window, then wiped away using a Kim wipe.

A water measurement is required first by the program in order to calibrate the reading. A 2 µL water blank was placed directly centered over the window on the measuring pedestal. The upper arm was lowered and the measurement was taken. After taking the measurement, a Kim wipe was used to thoroughly clean the upper and lower windows. Repeat this procedure with an alternating series of one water blank and three buffer blanks until the water absorbance is fairly flat (around 0.05 to 0.01). If the absorbance radically fluctuated, the surface was dirty and the

buffer helped to remove any material on the surfaces. In order to take a measurement, a 2 µL of sample of protein is placed on the window and measured. Three sample measurements are conducted and averaged for concentration calculations. If the three sample readings vary a lot from one another, another 1-2 readings were added to that group. Between each group of sample measurements, a water measurement is conducted and compared to the original reading to confirm that the window was clean. To calculate the sample absorbance, the background noise of the water and buffer were subtracted from the sample's absorbance.

2.3 Aggregation

A protocol for protein aggregation was adapted from by Bitan et al. (21). Sodium Phosphate and sodium chloride buffer was used to follow physiological conditions as well as facilitate aggregation. A 40 mM sodium phosphate with 5 mM sodium chloride was used to bring the lyophilized protein to 20 µM at the beginning of an aggregation. A more concentrated sodium chloride solution was then added to bring the total concentration of NaCl to 50 mM. The solution was left on ice for 1.5 hours, while the cross linking reagents were prepared. After the time on ice, the sample was taped down to a temperature controlled shaker at 400 rpm and 25°C with eight 25 µL samples removed during the five-hour aggregation. Mixing the aggregate solution before removing a sample was critical as the protein tended to settle at the bottom of the solution, and could lead to minimal amounts of protein in the sample. Through testing, it was determined that pipetting from the bottom to the top of the solution twice then pulling from the middle was sufficient to mix the solution and not significantly change the aggregation.

2.4 Photo-induced Cross-linking of Unmodified Proteins

The equipment used for the photo-oxidation of Ru(II) (Figure 4) included a Carl Zeiss Xenon arc lamp XBO 75W/2 with power supply 120V/120 W and a Mamiya/Sekkor 500 DTL camera.

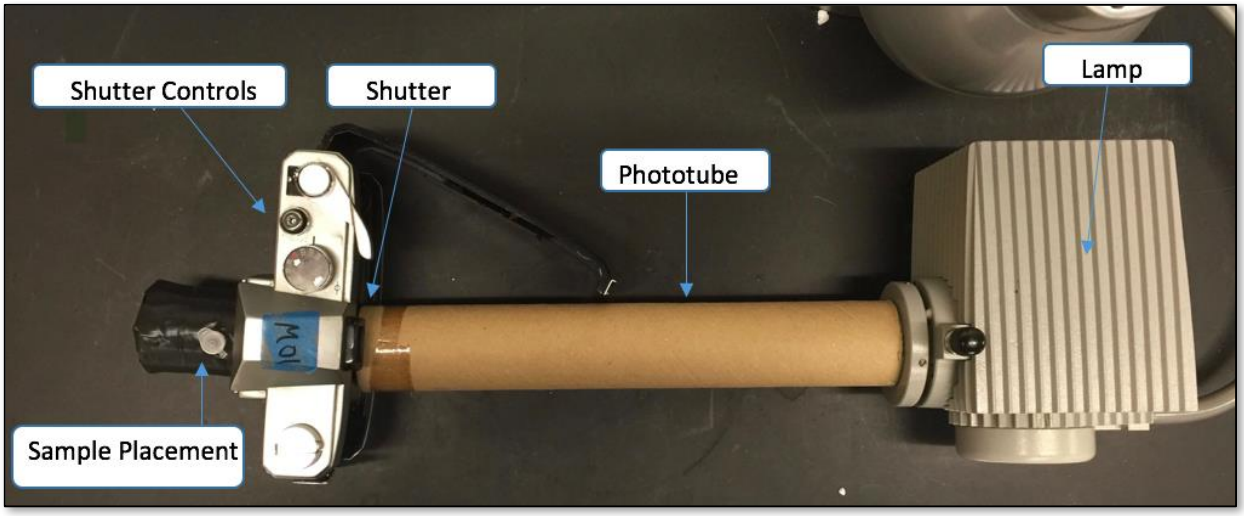

Figure 4: Photo-Oxidation Equipment

Due to the photo-sensitive nature of Ru(II), PICUP had to be conducted in a dark room. The protocol for PICUP used was modified from the protocol used successfully by Bitan et al. (21). Light from the lamp traveled through a 10-inch cylindrical tube to the back of the camera shutter door. The shutter on the camera was used to fix the sample's exposure time to one second. The shutter was activated twice during PICUP to ensure a two second exposure. 6 µL of each 25 µL sample of amyloid protein at 20 µM was added to a 0.5 mL non-stick micro-centrifuge tube along with 2 µL of the aggregation buffer (40 mM NaPh, 50 mM NaCl). In a dark room, 1 µL of 10 mM Ru(II) and 1 µL of 200 mM APS are added to the sample and exposed to 2 seconds of light. The cross-linking reaction is terminated with the addition of 5 µL of β-mercaptoethanol (BME). For each 25 µL of sample, three repeats of the PICUP process are performed for a total

of three 15 µL of cross-linked samples per (time-point). After PICUP, all samples are stored at -80°C.

2.5 Gel Electrophoresis

SDS-PAGE was used to separate the protein aggregates after PICUP. After some testing, 12.5% polyacrylamide gels were determined to show the best distribution of amyloid aggregates. The dye used for gel electrophoresis of amyloid protein was BioRad 4X Laemmli protein sample buffer mixed with a 10:1 volumetric ratio of BME. Cross-linked protein was prepared for electrophoresis by adding 5 µL of 4X Laemmli sample buffer to the 15 µL PICUP samples. A 1:10 by volume protein standard ladder was produced by combining 10 µL of Precision Plus Protein™ Dual Xtra unstained Protein Standards, 25 µL of 1:10 4X Laemmli buffer, and 65 µL of the aggregation buffer in a 0.5 mL non-stick micro-centrifuge tube. Before use, the tube containing the 1:10 standard was submerged half-way in a 100°C water bath for 5 minutes, then transferred to a -20°C freezer for 1 minute. This process helped separate the standards, improving the separation of the ladder during electrophoresis. A total volume of 6 µL of 1:10 standard was determined to give the closest final silver stain saturation to the 20 µL protein samples and was therefore used in all experiments.

The gels in the Hestekin lab were hand-cast before each round of electrophoresis. In order to produce 12.5% polyacrylamide gels, a small stock solution of 30% acrylamide was produced regularly with the help of safety coordinator Dr. Tammy Rechtin. Stock solutions of 1.5 M TrisCl (pH 8.8), 1.0 M TrisCl (pH 6.8), and 10% sodium dodecyl sulfate (SDS) were also produced to form the base of the gel. The polymerization of the gels was activated with 10%

ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). The exact gel recipe used in the Hestekin lab is given by the table below (Table 1). Once the activation reagents were added to the resolving gel, the gel was quickly transferred into the glass slides before polymerization. After 10 minutes of polymerization, the same procedure was used with the stacking gel with an added step of inserting the well-forming combs into the top of the glass slides. After an additional 10 minutes, the combs were removed gently and any excess gel material was cleaned from the wells using gel loading pipette tips before loading.

Table 1: 12.5% Polyacrylamide Gel Reagents

	Resolving Gel	**Stacking Gel**
30% Acrylamide	4.875 mL	830 uL
1.5 M TrisCl, pH 8.8	3.75 mL	----------
1.0 M TrisCl, pH 6.8	----------	630 uL
ddH20 water	6.225 mL	3.4 mL
10% SDS	0.15 mL	50 uL
10% APS	0.15 mL	0.10 mL
TEMED	45 uL	15 uL

Loading samples into the gel wells was performed with Corning gel-loading pipette tips (end of tip thickness of 0.5 mm) to reduce pipetting error. These tips also allowed for loading into dry wells which improved the quality of the final images greatly. Increased speed when loading the gels with sample and starting electrophoresis cut down on diffusion perpendicular to the electroosmotic flow and into the walls separating the wells. This reduced streaking caused by the lagging protein moving down the gel. Gels were run for around an hour at a constant 100V and 20 µL of sample per well. To determine exact run times for the gels, the blue 4X Laemmli buffer was tracked throughout the electrophoresis and cued the end of the run when it reached the

bottom green gasket holding the gel in place. The silver staining process immediately followed electrophoresis as polyacrylamide gels dry out easily and the protein is free to diffuse until fixed.

2.6 Silver staining

The GE Healthcare Life Sciences PlusOne Protein Silver Staining Kit was used to visualize the protein after gel electrophoresis. Similar to PICUP, a majority of silver staining must be performed in a dark room due to the light sensitivity of many of the reagents: most of all being silver nitrate. After the completion of SDS-PAGE, the gel(s) were removed from their housing and immediately placed into a fixing solution which had already been prepared in the hour before. The fixing solution, comprised of 7.5 mL of 95% ethanol, 2.5 mL glacial acetic acid and 15 mL of water, immobilized the protein to the gel to prevent further molecular diffusion. After a 1-hour minimum time spent in the fixing solution, the gels could be transferred to a sensitizing solution. This solution was comprised of 7.5 mL of 95% ethanol, 1 mL of 5% sodium thiosulfate, 1.7g of sodium acetate, 125 µL of 25% glutaraldehyde, brought to 25 mL with water. The gel(s) needed to be submerged in this solution for a 1-hour minimum as well. Both the fixing and sensitizing solutions could contain the gels for up to 24-hours if an overnight stopping point was required. Three water washes of 15 minutes each helped remove any reagents from the gel besides the protein, which reduced background staining during the silver staining step. All of the methods used after this point were performed in a dark room due to the light sensitive nature of many of the reagents, most of all the silver nitrate. The silver staining solution used in the Hestekin lab was composed of 2.5 mL of a 2.5% silver nitrate solution mixed into 22.5 mL of water. Silver stain was performed for an hour, followed by two, one-minute water washes to remove the silver stain from the gel but not the protein. Once the water washes were complete,

the developing solution was added to the gels and shaken for 4-15 minutes until the protein was visible enough to image. At that point, the developing solution was immediately swapped for the stopping solution and left to fully expose for around 30 min. The developing solution used comprised of 0.625g of sodium carbonate, 20 µL of formaldehyde, and brought to 25 mL with water. The stopping solution was made from 0.365g of Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na2) with water to bring the solution to 25 mL. Once finished, the gels were ready to be imaged and could be exposed to light.

3. Data Analysis and Results

Amyloid Beta in Sodium Phosphate Buffer

Amyloid beta was first aggregated in a 40 mM sodium phosphate and 50 mM sodium chloride buffer. The result of these aggregations is given by Figure 5 below. The monomer weight of amyloid beta (1-42) is around 4.5 kDa, and can be seen in Figure 5 as the band around the 5 kDa standard throughout the aggregation. It is also shown that the dimer form of amyloid beta is present throughout the aggregation with an increase in intensity around 2.25 hours and again around 4.25 hours. Larger oligomer species, around 12-mer to 13-mer, are present throughout the aggregation but are most concentrated at around the 5 hours.

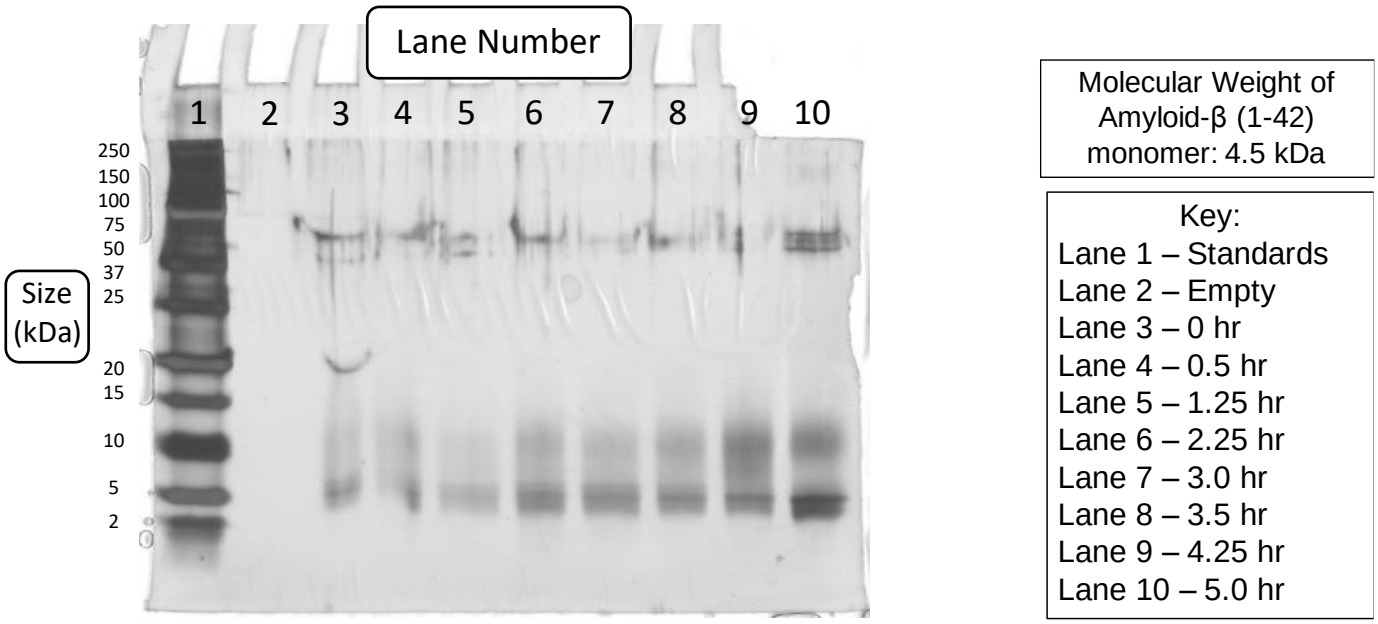

Figure 5: Amyloid beta in 40 mM Sodium Phosphate Buffer

Amyloid Beta in HEPES Buffer

Amyloid beta was also aggregated in 100mM, pH 7.4, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in order to test whether this buffer effects aggregation of amyloid beta. The advantages of using HEPES buffer is that it is better at maintaining a physiological pH than other standard buffers. As shown by Figure 6 the aggregation looks almost identical to amyloid beta in sodium phosphate with monomer, dimer, and 12-mer/13-mer bands present at the same times along the aggregation.

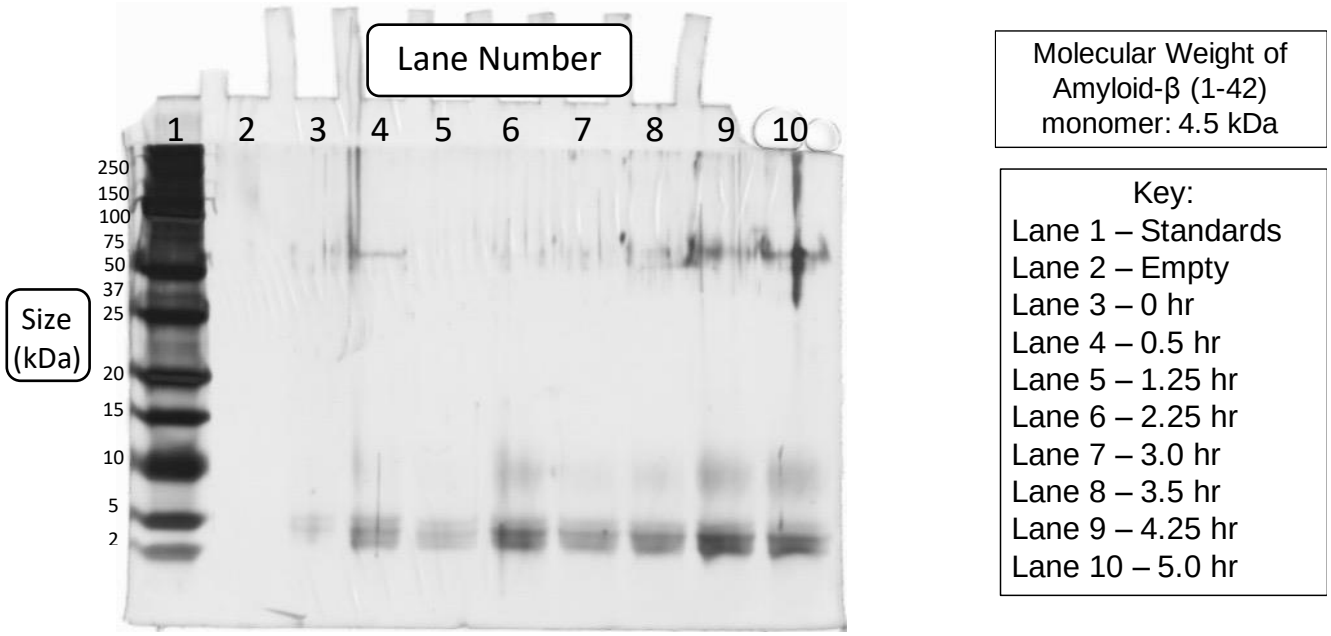

Figure 6: Amyloid beta in 100 mM HEPES Buffer

Amylin in Sodium Phosphate Buffer

There were many challenges associated with using the established PICUP protocol with amylin protein. The different amino acid sequence reduced silver binding, requiring longer developing times (10 minutes longer) in order to visualize the protein, leading to excess background staining of the gel. Samples of amylin protein from the same aggregation were combined in order to boost the amount of protein, therefore reducing development times and background saturation. Figure 7 shows the final result after troubleshooting. It appears that the monomer band around 3.9 kDa is disappearing near 3.5 hours and on, to become higher order aggregates. It is also thought that there are more oligomer species present in the gel, but just at too low of a concentration to visualize using the current techniques.

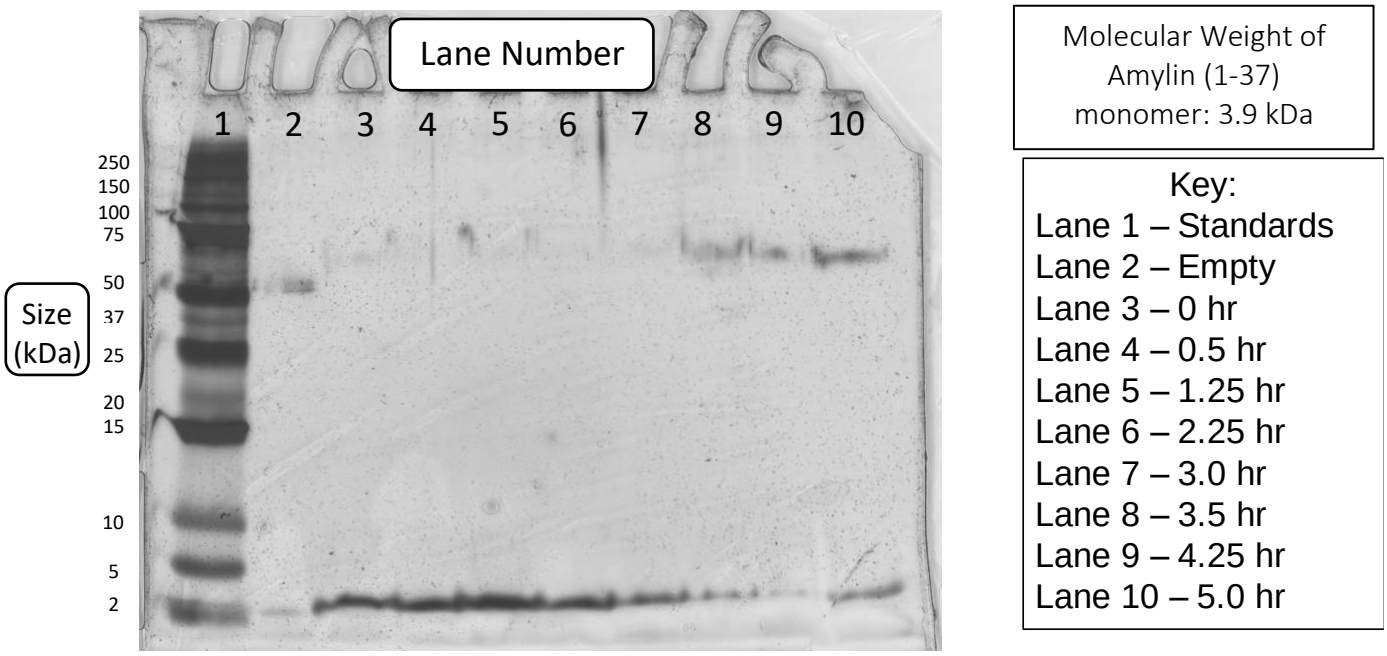

Figure 7: Amylin in 40 mM Sodium Phosphate

4. Conclusion and Future Work

Amyloid beta was successfully cross-linked and visualized in both sodium phosphate and HEPES buffer which allowed our lab to be more comfortable with a transition to preparing samples in HEPES buffer. Amylin was successfully cross-linked; however, more work is to be done to improve the intensity of current staining techniques.

In the future, PICUP could be used to test different sequence variants of amyloid beta and amylin to determine what parts of the protein enhance or inhibit aggregation. Different mutants of these proteins could investigate the impact of increasing or decreasing flexibility or hydrophobicity.

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