5-2014

ThT and Capillary Electrophoresis to Monitor the Effects of Solutions Conditions on Amylin Aggregation

Michael May
University of Arkansas, Fayetteville

ThT and Capillary Electrophoresis to Monitor the Effects of Solutions Conditions on Amylin Aggregation

ThT and Capillary Electrophoresis to Monitor the Effects of Solutions Conditions on Amylin Aggregation

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biomedical Engineering

By

Michael May
University of Arkansas
Bachelor of Science in Biological Engineering, 2010

May 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council

_____
Dr. Christa Hestekin
Thesis Director

_____
Dr. Suresh Thallapuranam Kumar
Committee Member

_____
Dr. Jeffrey Wolchok
Committee Member

Abstract

Amylin (hIAPP) aggregates have been found in 90% of patients with type II diabetes at autopsy, and are suspected to play a role in the death of islet β-cells[1]. However, this aggregation process is not well understood. Here, we explore methods that utilize capillary electrophoresis (CE) as a means to better understand amylin's aggregation process.

We examined the effects of solutions conditions: agitation, pH, salt, and temperature on amylin aggregation using Thioflavin T, dot blots, and capillary electrophoresis. Thiofalvin T was used to predict the lag time to β-sheet formation. Our results indicated all variables with the exception of agitation were feasible for study with CE. Capillary electrophoresis was then employed to observe the formation of oligomers with dot blots used as a confirmation technique. Overall, results showed all solutions conditions examined promote aggregation, however there was variance between time courses. Conflicting results suggest further study is needed to fully understand the observed amylin aggregation phenomena.

Table of Contents

Chapter 1

Introduction and Background

Amylin and Diabetes

Diabetes mellitus is the seventh leading cause of death in the United States resulting in the death of over 73,000 people every year [2]. It is estimated that over 25 million residents, 8% of the population, have diabetes and an additional 7 million more are undiagnosed. The number of people that are predisposed to diabetes is almost 80 million and this number is growing at a rate of almost 2 million a year. The total healthcare costs for diagnosed diabetic patients is $174 billion dollars [2].

Almost 90% of all cases of diabetes mellitus are considered to be type 2 [3]. Patients with type 2 diabetes mellitus are completely insulin dependent. Their bodies do not produce enough insulin to regulate blood glucose levels. This lack of blood glucose regulation leads to hyperglycemia resulting in multiple complications including heart disease, blindness, kidney failure, neuropathy, and amputation. The primary cause of diabetes can result from various medical conditions including obesity or from a genetic predisposition. However, obesity garners the most attention, accounting for 55% of type 2 cases [22,1]. Between 1995 and 2005 the rate of diabetes diagnoses doubled prompting the CDC to characterize it as an epidemic [3].

Type 2 diabetes is typically due to an insulin deficiency. Insulin is a regulatory protein that is produced in the β – cells of the pancreas. It controls glucose uptake into tissue. Over time, in patients with type 2 diabetes, less and less insulin is produced eventually resulting in no insulin production. This is due to decreased β - cell function and ultimately β - cell death[4]. One possible reason for decreased function and ultimately the death of these β – cells is thought to be the aggregation process of human islet amyloid polypeptide (hIAPP) as it moves toward the

formation of an amyloid plaque. At some point during the development of this plaque the amyloid proteins become toxic to the β – cells [1].

Human Islet Amyloid Polypeptide (hIAPP aka amylin) is a 37 residue protein with a disulfide bridge between amino acids 2 and 7 as seen in Figure 1 [5]. It is secreted in the pancreas by islet β – cells, at the same time as insulin in a ratio of 100 insulin proteins to each amylin protein [6]. Amylin works to reduce the body's demand on insulin. It slows gastric emptying, reduces the intake of food, and inhibits gastric secretion[7]. These actions lead to reduced glucagon production which in turn slows the rate of glucose appearance[8]. Physiological concentrations of amylin are very small (4 – 25 pmol/L) [1].

KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY (Disulfide bridge: 2-7)

Figure 1: Amylin amino acid sequence

In patients with type 2 diabetes, amylin has a propensity to aggregate forming amyloid plaques. These amyloid plaques are found in more than 90% of patients with type 2 diabetes at autopsy [1]. There is a growing trend of thought that believes this aggregation process is harmful to pancreatic β – cells, but the knowledge of this potentially toxic mechanism is not well understood[9]. Recent *in vitro* studies have shown that amylin aggregates are toxic to both human and modified rodent β – cells [10].

Unfortunately, these interactions between amylin and β – cells are not easily identifiable. The general trend of thought is that the aggregates somehow damage the membrane of the β – cells[11]. Further confusion is added when the aggregation mechanism is broken down into subsequent parts. Initially amylin begins as a monomer and then progresses to an oligomeric state, next is a transition state known as a proto-fibril state, followed by a fibril state resulting in the formation of an amyloid plaque. Initially it was thought that the amyloid plaques formed

from fibrils were the cause of β – cell death. However, recent research has indicated this is not the case, instead suggesting that smaller amylin aggregates are more important than large aggregates [12]. The current prevailing view now points toward oligomers and/ or the proto – fibril state as the leading factors for β – cell membrane damage. This coincides with the structural transition of the protein from an α – helix to a β – sheet[13].

The specific mechanism(s) by which amylin destroys β – cells remains unclear. However, there have been several proposed. One possible mechanism is that amyloid plaques impair the flow of glucose to β – cells and the flow of insulin from β – cells [10]. Another possible mechanism is that oligomers or proto-fibrils form small pores in the β – cell membrane. These pores allow various ions into the cell which results in cellular apoptosis [14]. A third mechanism is aggregation due to poor dietary choices. Western diets have been characterized by an increased intake of dietary fat resulting in an increase of free fatty acids in the body. Studies have shown that excess fat impairs β-cell function, decreases insulin production, and can stimulate IAPP aggregation. None of the studies were able to determine the mechanism of amylin aggregation [4, 15].

Thioflavin T

ThioflavinT (ThT) is a florescent dye commonly used to observe the formation of proto-fibrils and fibrils in amyloid proteins. The formation of fibrils involves a structural change moving from α-helix to β-sheet [16]. When ThT is added to a sample of an amyloid protein containing cross β-sheet structures it forms a complex with the fibrils and fluoresces. While by itself ThT exhibits weak excitation and emission wavelengths at 350 and 440 nm [17], when bound

to β-sheet structures the dye has excitation and emission wavelengths at 440 and 490 nm [17, 18]. The method of ThT interaction with amloid proteins is not well understood, however the prominent theory indicates ThT intercalates between grooves of the side chains of the amyloid fibrils. ThT binding within these grooves stiffens the fibril and prevents it from twisting into a less radiative state [17]. ThT binding has been previously used to identify effects of solution conditions on β-sheet formation in amyloid proteins [19, 20]. While studying the effects of rifampicin on β-sheet formation researchers were able to monitor β-sheet formation using ThT [19]. In a separate study, researchers were able to successfully utilize ThT to monitor β-sheet formation while researching the toxicity of β-sheets in amylin. They found that the cytotoxicity of amylin is dependent upon its preparation and the amount of fibrils within the sample [20].

This is a common technique used to monitor β-sheet formation in amyloid proteins. For the current studies, we have used ThT binding to evaluate the effect of solution conditions on the lag time to formation of beta sheets during amylin aggregation and to establish a time-line for fibril formation.

Capillary Electrophoresis

Capillary electrophoresis (CE) is a separation and analysis technique used to differentiate ionic species based upon their charge and size. CE utilizes a voltage to induce a current to pull a species through the capillary. Species with a larger charge will travel more quickly than those with a smaller charge, and species with a larger mass will travel more slowly than those with a smaller mass

Previous work by Pryor *et. al.* has indicated the potential for CE to detect and monitor amyloid aggregation in amyloid β and insulin proteins [21, 22]. Amylin aggregation has been studies via thioflavin T, dot blots, electron microscopy, light scattering, and several other techniques, but has yet to be examined using CE [23-25]. Capillary electrophoresis could provide a better, more affordable alternative for the detection and monitoring of amylin oligomer development and aggregation.

Dot Blots

Dot blots refer to a common technique used for the detection and identification of proteins [10, 19]. Dot blots utilize antibodies to detect the presence of and/or confirm the specific state of a protein. The dot blot assay involves the attachment, or spotting, of a protein onto a membrane followed by the binding of a primary antibody specific for that protein. A secondary antibody then binds to the primary for detection [10]. Dot blots give no specific information about species size. They can only confirm the presence of a specific species[22]. Previous research indicates that dot blots can be used to study amylin oligomer formation and aggregation. In a study by Zhao *et. al* .researchers were successfully able to utilize dot blots to detect the presence of amylin oligomers within the pancreatic islet cells of diabetic patients [25]. In a separate study conducted by Meier *et. al.* to monitor the toxicity of fibrils towards β-cells, dot blots were successfully used to detect the presence of amylin oligomers [19]. In this work, dot bots were used to confirm the presence of oligomers and fibrils in conjunction with capillary electrophoresis.

Purpose of This Work

Previous research in our lab has indicated that amyloid protein aggregation can be affected by various solutions conditions. Here, we have examined the effects of these solutions conditions on amylin aggregation using Th-T, CE, and Dot Blots. Chapter 2 summarizes a study that used ThT to identify the time at which β-sheet formation occurred for each solution condition. In Chapter 3, we described the use of CE as to examine oligomer formation and confirmation of the analysis through dot blots.

Chapter 2

Beta Sheet detection using ThT

Introduction

Benzothiazole dye thioflavin T (ThT) fluorescence is a well-known, commonly used technique for the identification of β-sheets in amyloid proteins [17, 23, 26]. When amyloid proteins transition from an oligomer state to a fibril state there is a shift to a β-sheet formation [16]. When ThT is added to solutions containing β-sheet rich structures it experiences a shift in fluorescence excitation (350 to 440 nm) and emission (438 to 490 nm) maxima [17]. It is thought that ThT intercalates within the grooves formed by amino acid side chains of the amyloid fibril[17]l. ThT does not bind to monomers or oligomers, it only binds to β-sheet rich structures [26]. ThT has been previously used to identify the effects of solution conditions on β-sheet formation in amyloid proteins [19, 20].

A ThT assay involves the dilution of fibril forming amyloid samples into buffered ThT followed by taking a fluorescence reading [20]. Fluorescence time courses generally have the shape of a sigmoidal curve. In the beginning there is a lag phase followed by an increase after which there is a leveling off. The beginning of the sharp increase is indicative of β-sheet formation and the leveling or tapering off indicates fibril/ β-sheet saturation. In this chapter we used ThT to both monitor and to determine the threshold of β-sheet formation for amylin within our sample solutions conditions. We examined the effects of both increased and decreased

agitation, increased pH, salt, and temperature, as well as a combination of these variables. These are controllable conditions that are known to affect amylin aggregation speed.

Materials and Methods

Amylin Preparation

Amylin (Anaspec, Freemont CA, Lot #88089), was stored at -80^{o}C. To ensure samples were monomeric a 3.91 mg/ml stock of amylin was prepared in Hexofluoro-2-propanol (HFIP) [27]. Amylin was aliquotted into vials containing 0.0625 mg of amylin and the HFIP was allowed to evaporate overnight. For ThT studies, amylin was prepared to a concentration of 50 μM under the various conditions specified in Table 1. All samples were examined in triplicate. Tris buffer was chosen based upon previous work in our lab with amyloid – β. Other buffers, such as phosphate buffer, could provide more physiological conditions, however tris provides a more stable environment for separation within CE.

Table 1: Solutions conditions. Changes from the Control are highlighted in red.

Sample	pH	T (C)	Agitation (rpm)	NaCl (mM)
Control	8	25	400	5
pH	7	25	400	5
Salt	8	25	400	140
Agitation1	8	25	0	5
Agitation2	8	25	800	5
Temperature	8	37	400	5
Multiple Variables	8	37	0	140

Fluorescence Measurement

To measure fluorescence we used a Shimadzu RF-Mini-150 Fluoremeter (Columbia, MD) (excitation filter = 460 nm, emission filter = 490 nm). ThT solution was initially prepared to a 1 M concentration with Milli Q water and then diluted to a 5:1 concentration ratio of 35.73 µM ThT and 50 µM amylin using tris – HCl. Volume ratio was 7:1 (175 µL ThT to 25 µL_ Amylin. Time points' fluorescence readings were taken a minimum of 2 hours and a maximum of 6 hours apart. As part of the purpose of this study was to establish conditions that would be suitable for future CE analysis, only aggregation occurring at times greater than 2 hours or less than 24 hours were evaluated. Based on previous work with amyloid β and capillary electrophoresis, β-sheet formation occurring in less than 2 hours does not allow enough time for a time course study. β-sheet formation after 24 hours was considered undesirable for being able to effectively capture all in between time points.

Statistical Analysis

Results were analyzed with an unpaired t test using GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/ttest1.cfm). We inputted the mean, standard deviation, and sample size to calculate each time point's p value in relation to the zero hour time point. The first time point statistically significant in comparison to the zero hour time point was deemed to represent β-sheet formation. Time points were considered to be statistically significant when their p value became less than 0.05.

Results and Discussion

The effect of solution conditions on lag time to β-sheet formation was investigated. β-sheet formation was determined to have taken place when a time point was deemed to be statistically different from the zero hour time point. A confidence interval of 95% was used to determine when a time point was significant in comparison to the zero time point.

Control Conditions

The control conditions were selected based upon previous work performed with another amyloid forming protein, amyloid-β [28]. While there are other more physiological conditions available; these conditions have been found to work best with our chosen buffer, tris. As shown in Figure 2, time points were taken at 0, 6, and 10 hours followed by a time point every 2 hours up to 20 hours. In comparison to the zero hour time point at 6 hours fluorescence readings had hardly increased, but at 12 hours fluorescence had increased by 67%. This was followed by a general leveling off with fluorescence where readings remaining generally around 500 for the next 10 hours. Based on a t test with a 95% confidence interval it was found that the control conditions exhibited a significant difference in fluorescence at 12 hours which indicated this to be the lag time to β – sheet formation.

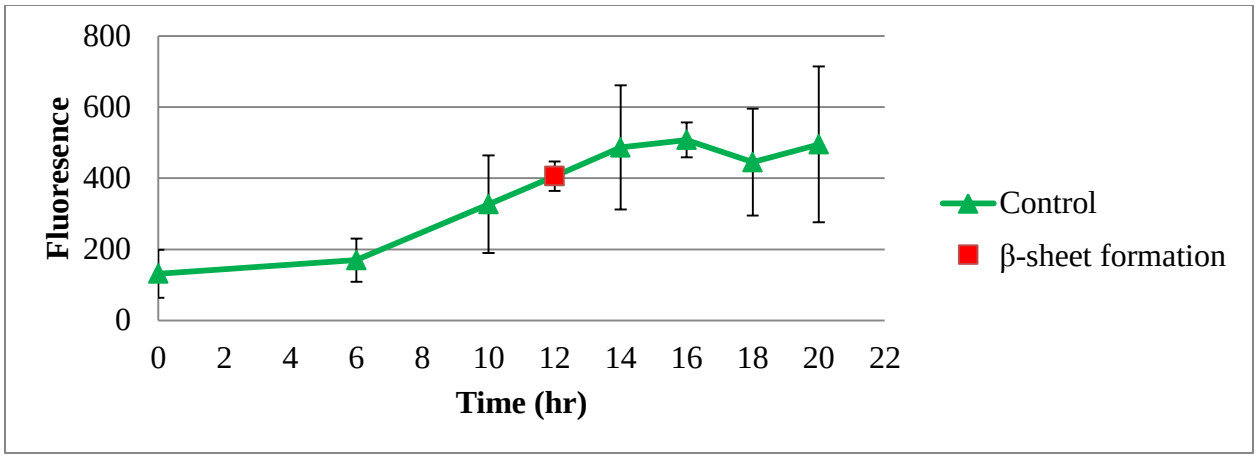


Figure 2: Change in fluorescence indicating β – sheet formation in amylin samples under Control conditions. The red square indicates the first statistically significant point.

Study of Agitation

In this study we chose to examine the effects of agitation by evaluating no agitation (0 rpm), medium agitation (400 rpm), and high agitation (800 rpm). Time points for 0 rpm were taken at 0, 6, 12, 16, 18, 20, 22, and 24 hours. Time points for 800 rpm were taken every 2 hours for 12 hours. Time points for each agitation sample set were different because we expected higher agitation would increase the rate of aggregation and decrease the sample lag time requiring us to measure fluorescence sooner.

Figure 3 shows the results of the ThT study for the varying aggregation conditions. The 0 rpm agitation study did not show statistically significant β-sheet production until the final 24 hour time point. The 800 rpm agitation study produced its first statistically significant time point at 2 hours. An interesting occurrence to note is the amount of fluorescence detected in the 800 rpm agitation study. The first significant time point had a fluorescence reading of 218. This was followed 2 hours later by a fluorescence reading of 2000, an increase of 89%. The fluorescence

stayed around 2000 for the next 6 hours. In comparison to our other ThT studies, this is a much greater amount of fluorescence. The maximum fluorescence detected in the control study was 500, and the maximum fluorescence detected in our other studies never exceeded 1500. ThT binds to β – sheets so therefore the 800 rpm agitation assay resulted in a much higher concentration of β – sheets. In comparison to the control conditions, which exhibited significant β-sheet formation at 12 hours, it is reasonable to say that a lack of agitation can slow the *in-vitro* aggregation time of amylin. In respect to the 800 rpm study it can be said that an increase in agitation can increase the *in-vitro* aggregation time of amylin. This is consistent with the results of a recent study by Tiiman *et al*. who observed that an increase in agitation of another amyloid forming protein, amyloid β, resulted in a dramatic increase in fluorescence in comparison to those without agitation [29]. Therefore agitation sample concentrations one could draw the conclusion that increased agitation or lack thereof can have a profound impact on the aggregation of amyloid proteins *in vitro*.

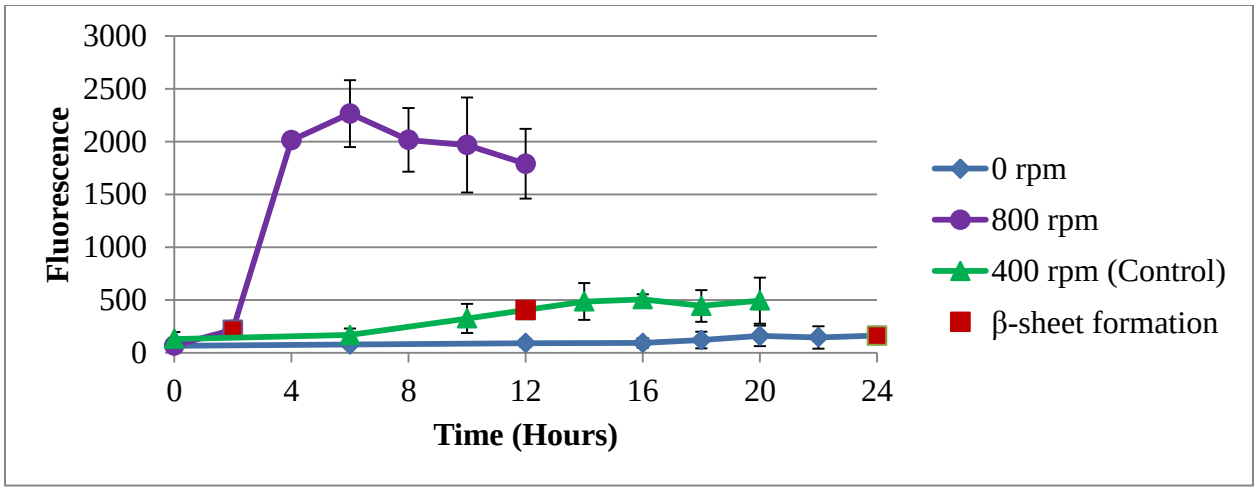


Figure 3: Change in fluorescence indicating β – sheet formation in amylin samples with 0 rpm agitation and 800 rpm agitation in comparison to Control conditions 400 rpm. The red square indicates the first statistically different point.

Study of pH

The pH study was selected to investigate the effects of a more acidic pH in comparison to the control pH of 8. We initially planned to observe the effects of a more basic and a more acidic pH on amylin aggregation. However, we were limited due to the range of our chosen buffer, pH 7.1 – pH 9, and by the isoelectric point of amylin which is at a pH of 8.9. When a protein is at or near its isoelectric point it will most likely aggregate [30]. Performing our study at a pH higher than the control's pH of 8 would have been too close to amylin's isoelectric point. It was therefore decided to investigate the effects of more acidic conditions on amylin aggregation. Our pH study was conducted at a pH of 7.1. Time points were taken every 2 hours for 8 hours, and then every 4 hours until 24 hours was reached. Fluorescence results can be seen in Figure 4. The pH condition had a lag time to significant β-sheet formation of 4 hours. In comparison to the control conditions our pH study did form β-sheets more quickly as evidenced by the faster

increase in fluorescence. This time point compares similarly to the increased agitation assay though it is different. The largest fluorescence reading is just over 500. However when graphed, the time points, instead of forming a typical sigmoidal curve, formed more of a parabolic shape. It would be expected for the fluorescence to increase and then level off, however, after 12 hours there was a decrease in fluorescence. This could be due to a rapid increase in β – sheet formation followed by the fibrils falling out of solution.

Our study suggests that a decrease in pH will result in a decrease in aggregation time. This is contrary to a previous amylin study which found that amyloidosis achieved a maxima at pH 9.5 and the rate of fibrillation was similar for the range of pH 6.5 to pH 8.5 [24] Their study varied in that it used a different aggregation procedure, disoving their sample in DMSO, utilizing higher concentrations of amylin up to 150 μM. They also had increased salt, up to 200 μM, as well as lower temperature, 4 and 22^{o}C, and a different buffer. The differences in our studies could be related to these variations.

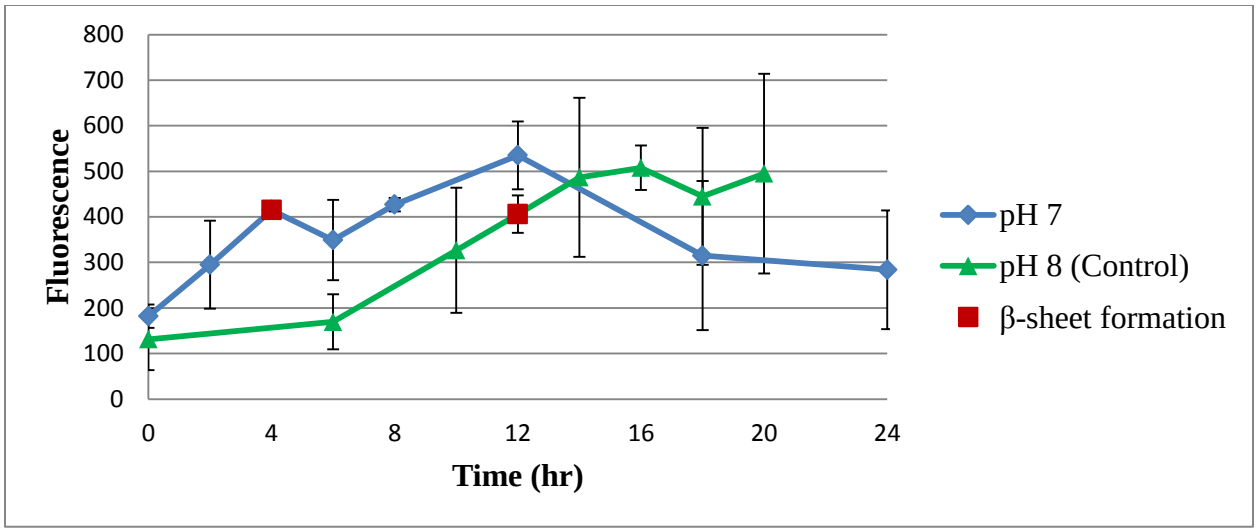


Figure 4: Change in fluorescence indicating β – sheet formation in amylin samples under pH conditions. The red square indicates the first statistically significant point of β-sheet formation.

Study of Salt

The salt conditions were selected to investigate the effects of the addition of a high concentration of salt in comparison to the control conditions. A concentration of 140 mM was chosen to satisfy the requirement of a high salt concentration, which was partially selected since it is comparable to the concentration of salt within the human body [31]. Time points were recorded every 2 hours for 12 hours with a final time point occurring at 24 hours. Time course results can be seen in Figure 5. The first significant fluorescence occurred at 4 hours. It achieved a fluorescence of 1000 at 8 hours which it maintained for 12 more hours. Though taking longer to develop a significant amount of β-sheets than the increased agitation condition, the increased salt condition developed β-sheets much faster than the control condition. From this we determined that an increase in salt concentration will result in a decrease in aggregation time. This is in agreement with a previous study by Jain *et al.* which monitored the effects of

increased salt on amyloid fibril formation. They monitored fluorescence in the presence of 120, 130, and 150 mM NaCl and found that an increase in salt concentration resulted in an increase in the rate of β-sheet formation. A similar study by Pryor *et al*. showed a decrease in lag time with increased salt formation for insulin [21].

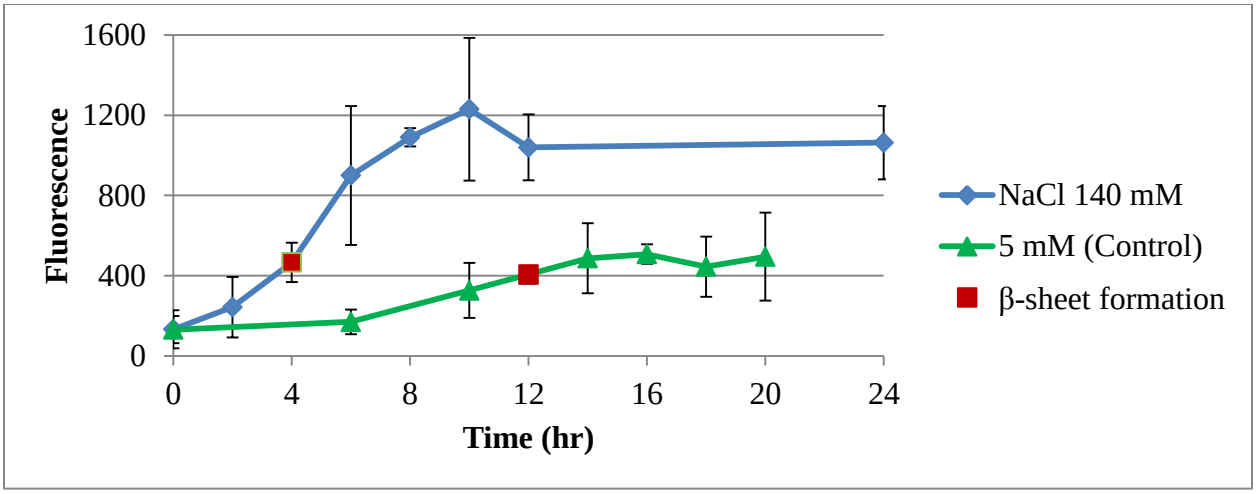


Figure 5: Change in fluorescence indicating β – sheet formation in amylin samples under Salt conditions.

Study of Temperature

Temperature conditions were selected to investigate the effects of increased temperature in comparison to the control conditions. For the study of increased temperature it was determined to raise the temperature to 37°C in comparison to the control's 25°C. This satisfied the requirement of an increase in temperature and also replicated the average temperature of the human body [32]. Time points were taken every 2 hours for 12 hours and a final time point was taken at 24 hours. Figure 6 depicts the fluorescence assay results. The lag time to significant β-sheet formation was detected at 6 hours. After 4 hours, fluorescence had increased above 1000 and stayed there for the next 20 hours. This is indicative of β – sheet formation occurring very

quickly by the 4 hour time point. This is in agreement with previous work by Kudva *et al*. which proposes amyloid aggregation is "directly related to temperature" [24]. They examined amylin aggregation under temperatures of 4, 22, and 37°C respectively and found with each increase in temperature there was an increase in β-sheet formation. In comparison with the control condition, our results along with previous research indicate an increase in temperature will result in an increase in β-sheet formation.

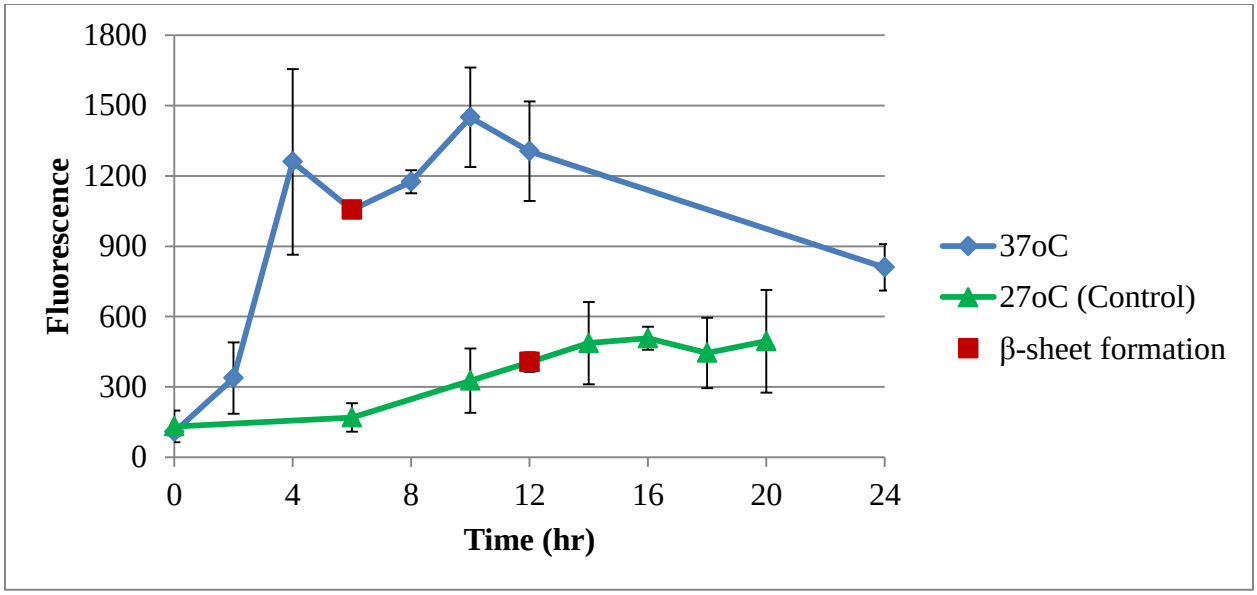


Figure 6: Change in fluorescence indicating β – sheet formation in amylin samples under Temperature conditions.

Study of Multiple Variables

Since the human body is a complex system, we chose to investigate a condition were multiple variables (salt, temperature, aggregation) varied from the control conditons and were more in line with mimicking the human body. We chose to increase the salt concentration to 140 mM, to increase the temperature to 37°C, and decrease agitation to 0 rpm while keeping the pH

at 8.0. Time points were measured every 2 hours for 12 hours and a final time point was taken at 24 hours. Results for this study can be seen in Figure 7.

The first statistically significant point of fluorescence occurred at 4 hours. Fluorescence increased to above 1000 after 12 hours and maintained fluorescence above 1000 for the final 12 hours. Our previous studies on the effects of varying salt and temperature indicated increases in both will decrease the aggregation time for β-sheet formation. This study is consistent with those previous, although they did not appear to act synergistically. Our results indicate statistically significant β-sheet formation at 4 hours which is similar to the salt study, but less than the temperature study.

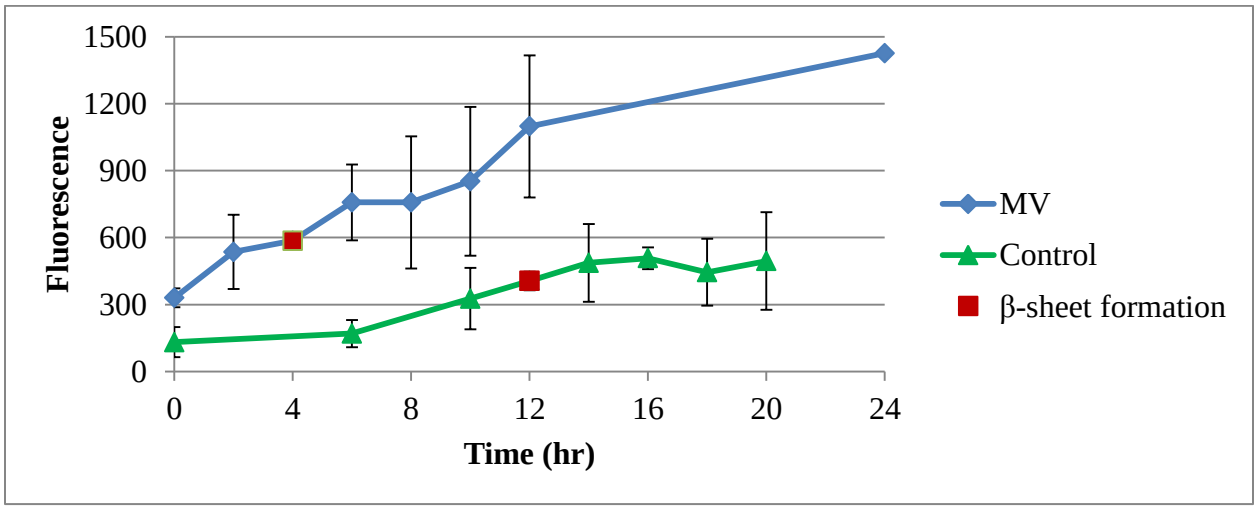


Figure 7: Change in fluorescence indicating β – sheet formation in amylin samples under multiple variables.

Discussion

In this study we monitored the effect of solution conditions on β-sheet formation in amylin. After reviewing data collected the following general statements can be made concerning the effect of agitation, temperature, salt, and pH on amylin fibril formation time. An increase in agitation will result in a decrease in fibril formation time. An increase in temperature will result in a decrease in fibril formation time. An increase in salt (NaCl) concentration will result in an increase in fibril formation time. A decrease in pH will result in a decrease in fibril formation time. Conditions mimicking the body will result in a decrease in fibril formation time. Using statistical analysis, for each individual study we found the first time point that statistically significant from the zero hour time point. We used this statistically significant time point to indicate the lag time before beat sheet (fibril) formation for each study. All expected initial fibril formation lag times are summarized in Table 2.

Table 2: Expected lag time of beta sheet/ fibril formation based upon a 95% confidence interval.

Study	**Expected Lag Time (hr), p value < .05**
Control	12
Agitation 1	24
Agitation 2	2
pH	4
Salt	4
Temperature	6
Multiple Variables	4

Chapter 3: Capillary Electrophoresis

Introduction

Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) is a separation technique used to separate species based on their charge and size. It employs the use of an applied voltage to generate a current resulting in species migration. The speed of migration is based upon the electrophoretic mobility of the species, which is determined by its mass and charge. Analytes that have a larger charge will move faster than those with a smaller charge. Analytes with a greater mass will move slower than those with a lesser mass. In capillary electrophoresis the capillary is filled with a buffer solution. When an electrical field is applied to the buffer filled capillary it induces a flow of electrolytes toward the cathode. Depending on the analyte's charge, this flow acts to either enhance or impede its mobility within the capillary. This flow is further enhanced with a process known as electro osmotic flow (EOF). EOF occurs when the charge on the inside of the capillary creates a more concentrated layer of ions which acts to creaste a bulk flow. To utilize EOF we left the capillary uncoated. The inside of the siliconized capillary is negatively charged. As the positive ions in the buffer solution migrate towards the cathode they stick to the negatively charged capillary walls. Other positively charged ions are pulled towards the capillary wall but are unable stick, creating a mobile concentrated layer. Because there are now less negative ions to impede it, this concentrated layer moves more easily toward the cathode and acts to create a bulk flow of ions enhancing the speed of of species migration [33].

For this study, CE was used to detect and monitor hIAPP (amylin) aggregation under different solutions conditions, similar to those monitored by ThT in Ch.2. At a pH of 8 amylin

has a charge of +1 and therefore migrates toward the cathode. Therefore, its mobility is enhanced by the presence of EOF.

Dot Blots

Dot blots are a traditional method used to detect the formation of oligomers and fibrils in amyloid forming proteins. Dot blots work by employing antibodies to detect the presence of a protein or specific conformations of a protein. Both A11 and OC antibodies are commonly used for the detection of amyloid aggregation conformations [25]. The A11 antibody has been correlated with toxic oligomers in amyloid proteins. The OC antibody recognizes amyloid fibrils. AB18018 was used as a control to recognize human amylin 1-37. AB18018 is specific for the full length 37 aa human amylin sequence. We chose to use dolt blots with primary antibodies AB18018, A11, and OC for the detection of amylin oligomers and fibrils in our CE study.

Materials and Methods

Table 1 depicts the solution conditions we analyzed using CE and dot blots. We varied pH, salt, and temperature, and then combined high salt, increased temperature, and no agitation for a multiple variable condition. Our control solution condition was based on previous work with other amyloid forming proteins, insulin and amyloid β [28].

Table 1 was developed using the solutions conditions used for the ThT study in Chapter 2. Sample conditions, Agitation 1 and Agitation 2 were removed due to their aggregation lag times (Chapter 2, Table 2). Agitation 1 and Agitation 2 had lag times of 2 hours and 24 hours, respectively. A lag time of 2 hours does not allow enough time for a study with capillary electrophoresis and 24 hours is to long for a feasible study.

Table 1: Solution conditions to be used in Capillary Electrophoresis.

Sample	**pH**	**T (C)**	**Agitation (rpm)**	**NaCl (mM)**
Control	8	25	400	5
pH	7	25	400	5
Salt	8	25	400	140
Temperature	8	37	400	5
Multiple Variables	8	37	0	140

Amylin Preparation

Human amylin 1-37 (Anaspec, Freemont CA, Lot #88089), was stored at -80^{o}C. In order to ensure samples were monomeric a 3.91 mg/mL stock of amylin was prepared in hexofluoro-2-proponol (HFIP). Amylin was aliquotted into vials containing 0.0625 mg of amylin and the HFIP was allowed to evaporate overnight. For CE studies, amylin was prepared to a concentration of 50 µM. All samples were examined in triplicate. Samples were initially dissolved in 5 mM NaOH for 10 minutes. Following that tris buffer and salt were added accordingly to make conditions described in Table 1. Samples were then subjected to temperature and agitation specified by their solution condition in Table 1.

Dot Blots

Dot blots were performed by spotting 2 µl of 50 µM amylin at each time point onto a nitrocellulose membrane (Pall, Port Washington NY). Primary antibodies used were AB18018 1:10000 dilution (Abcam, Cambridge MA), A11 1:2000 dilution (Invitrogen, Camarillo CA), and OC 1:4000 dilution (Millipore, Hayward CA). Secondary antibody used was goat anti rabbit

alkaline phosphatase (Pierce, Rockford IL). Dot blots were developed using NBT/BCIP developing solution (Thermo Scientific, Waltham MA).

Capillary Electrophoresis

Capillary Electrophoresis was performed using a Beckman Coulter P/ACE MDQ Glycoprotein System (Fullerton, CA) (214nm filter). The machine was interfaced with an IBM computer and Beckman Coulter 32.0 Karat software (V 5.0). Samples were pressure injected at 0.5 psi for 8 seconds and separated at 7 kV for 60 minutes in a glass capillary of length, 31 cm. The capillary was rinsed with deionized water for 10 minutes at 50 psi and then 100 mM Tris-HCl for 2 minutes at 20 psi between each time point. The capillary was changed between each time course to prevent contamination from protein adherence to the capillary wall.

Data Analyzation

Capillary electrophoresis data was analyzed using Origin 8.6 (Origin Lab, Northampton Ma) and Microsoft Excel 2010 (Microsoft, Redmond Washington). CE data was imported into Origin 8.6 and analyzed for peak area and elution time. The data obtained was then exported to excel where it was graphed vs. time.

Results

Control Conditions

Results from CE were plotted vs. time to develop electropherograms. Electropherograms allow for determination of when an analyte passes the window of a capillary. They are characterized by a flat baseline interrupted by a positive peak and then a continuation of the baseline. The peak is generated as the species migrates past the UV window. The peak's area

and elution time can be used in comparison to other CE runs to evaluate aggregation changes to the species. If the species is larger it will take longer to migrate through the capillary and will therefore result in an increase in elution time. An increase in the amount of species is typically related to an increase in peak area. Electropherograms can then be plotted against each other to show changes in between each CE run to develop a time course.

In capillary electrophoresis a negative peak appears in the presence of a UV transparent sample or zone [34]. Interestingly all five conditions (control, pH, salt, temperature, and multiple variables) exhibited at least one negative peak in each run. If the peak consistently had the same elution time and area during every run we could simply call it a system peak [34]. However, as the sample aggregation time increased so did the elution time of the negative peak. Figure 1 depicts a set of electropherograms forming a time course for the control condition as analyzed by CE. This negative peak occurred in all five solutions conditions, which indicates an increase in size of the migrating species affecting this negative peak. Previous research has shown negative peaks to be associated with zones of varying ionic strength [34]. With this in mind, we calculated the ionic strength for both our separation buffer and our sample solution, Table 2. We found that there were differences in ionic strength for both buffers. This could have contributed to the appearance of negative peaks.

Table 2: Sample Ionic Strengths

Sample	Buffer (M)	Sample (M)
Control	0.058	0.052
pH	0.092	0.066
Salt	0.058	0.212
Temperature	0.058	0.045
Multiple Variables	0.058	0.18

The control condition, Figure 1, exhibited this negative peak in all three time course repeats. The elution time for the peak did increase as aggregation time increased (Figure 2A). Time course 3 exhibited 2 negative peaks, 3A and 3B. Both of these peaks consistently eluted within a short amount of time of each other for the entire time course.

The control condition exhibited an increase in the negative peak's area for all 3 time courses (Figure 2B). The peak area increases for time courses 1 and 3A and 3B were similar until the final time point. The increase in peak area for time course 2 was greater than that of time courses 1 and 3. The control condition did not develop a positive peak.

While there was variance between individual time courses for a given sample condition, all sample conditions showed a general increase in negative peak area with an increase in aggregation time. The variance between each time course could be due to protein sticking within the capillary. Capillaries were changed between each time course to avoid contamination. With a new capillary being used for each time course there could have been variation in the amount of protein sticking in each time course.

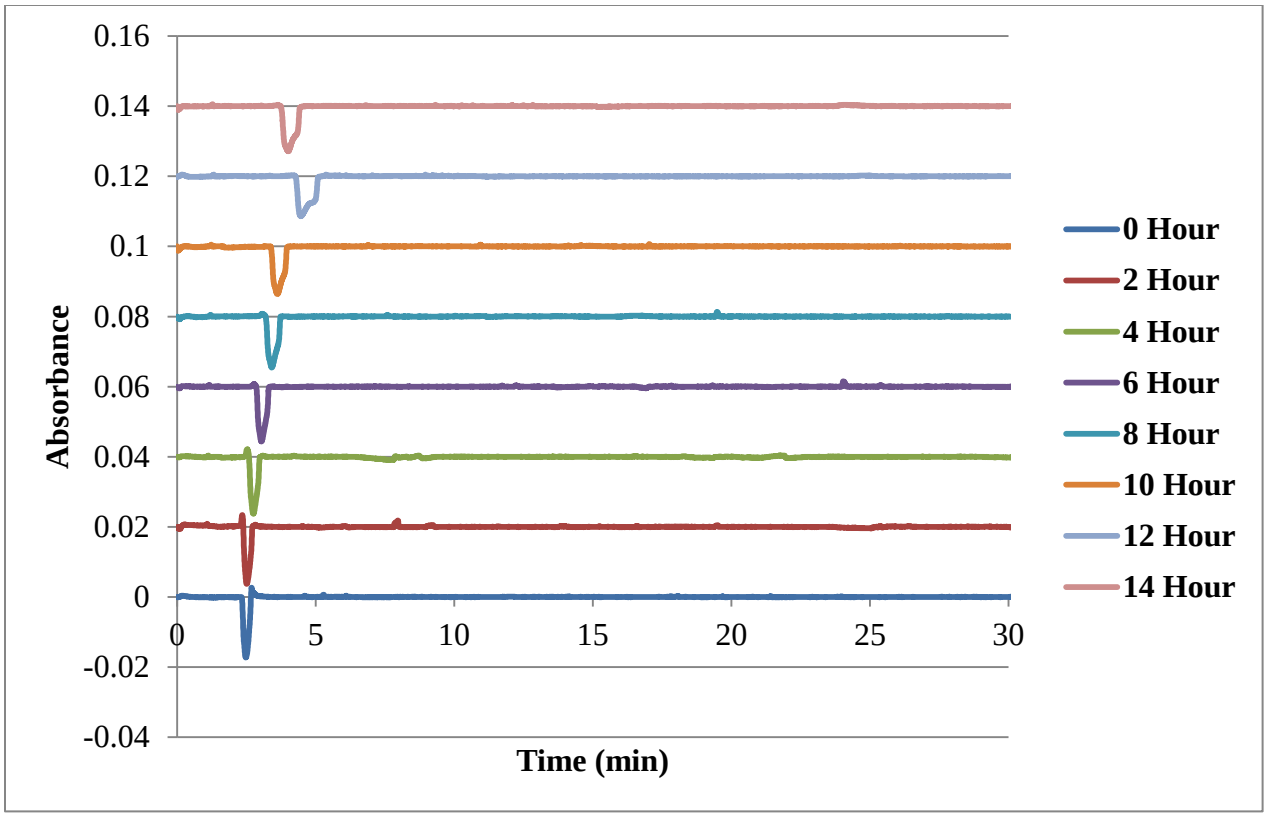


Figure 1: Electropherogram of a Control CE run. Peak of interest is the negative peak occurring between 0 and 10 minutes.

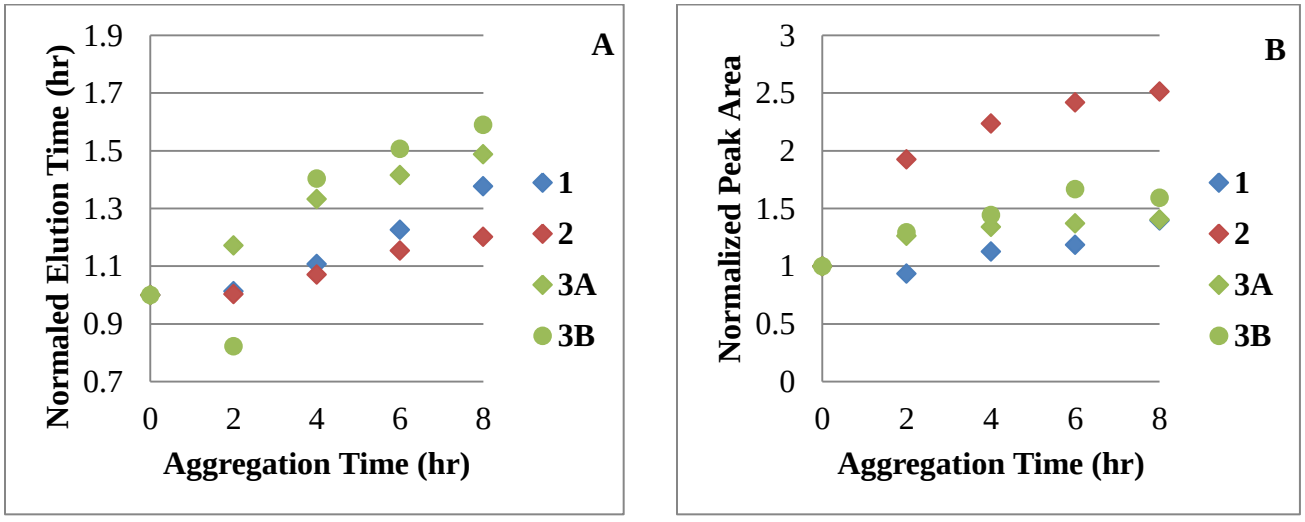


Figure 2: A. Negative peak normalized control elution times vs. aggregation time. B. Negative peak normalized control peak area vs. aggregation time.

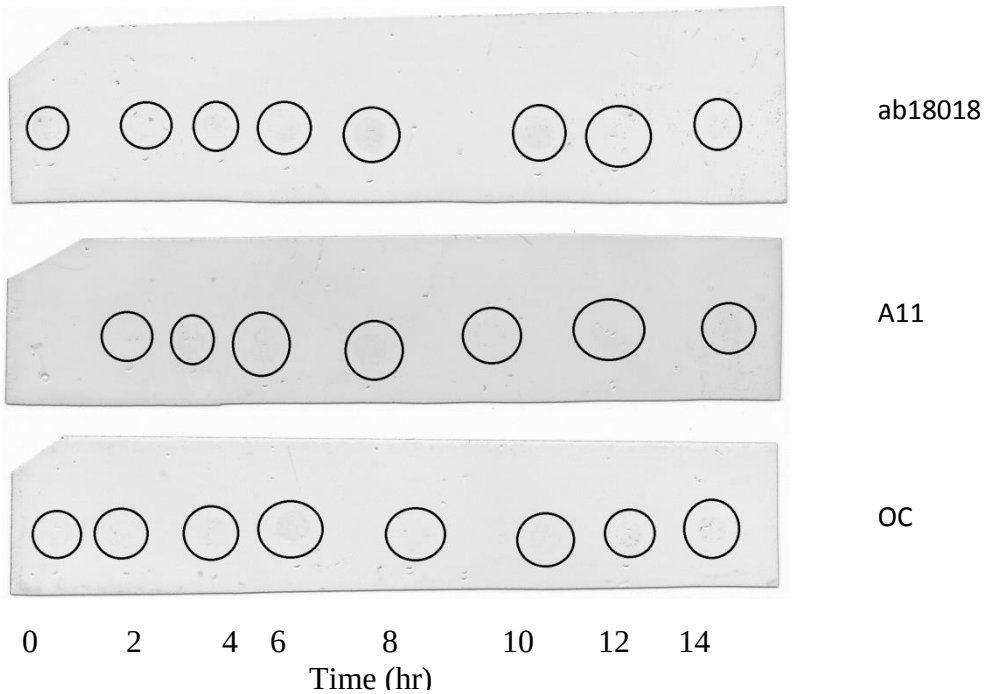


Figure 3: Dot blot of Control condition. The presence of a dot confirms the presence of amylin (ab18018), an oligomer (A11), or fibril (OC). Positive dot blots have been circled because of difficulty in obtaining the proper contrast in photographs.

Dot blots confirmed the presence of oligomers at the 2 hour time point. The presence of oligomers was confirmed for the rest of the time course. The presence of fibrils was detected at all time points. Dot blot results can be seen in Figure 3. While the dots are visible to the eye, they can be difficult to visualize in an image and therefore circles have been added to indicate the presence of a dot. The presence of both fibrils and oligomers at time 0 indicates aggregation was occurring very quickly within our control sample which could explain why no new peaks were detected.

Study of pH

Amylin's isoelectric point is at pH 8.9. When a protein approaches its isoelectric point it has a propensity to aggregate [30]. With this knowledge we assumed a lower pH than our control would result in an increase in aggregation time. However, results indicated a lower pH resulted in lower elution times than the control. At a pH of 7 the charge on amylin should be close to +2. In the control conditions with a pH of 8 the charge of amylin is +1. At pH 7 the charge on amylin is twice that of the control condition. With a larger charge we expected it to migrate more quickly than the control.

The lower pH resulted in an increase in elution time for negative peaks but was variable between each time course. Figure 5A depicts the negative peak elution times. Time course 1 initially had a decrease in elution time, but finished with a final elution time of 1.3 minutes. Time course 2 at first had a slow increase in elution time but after a 4 hour elution time of 1.15 minutes it increased sharply to 2.5 minutes for the 6 hour time point.

The pH condition showed an increase in negative peak area, but was inconsistent between each time course as seen in Figure 5B. Time courses 1 and 2 were consistent until the last time point of which time course 1 had a peak area of 1.2 and time course 2 a peak area of 2.5. Time course 3 had a greater initial increase than 1 and 2, and finished with a peak area of 2 for the 6 hour time point. Time courses 2 and 3 both finished the 6 hour time point with greater peak areas than the control.

As seen in Figure 5C, pH elution times for the positive peak were not consistent. The elution times at the 2, 4, and 6 hour time points for time course 1 were all less than the initial elution time. Time course 2 showed an initial decrease for time points 2 and 4 followed by an increase for the 6 hour time point. The CE was unable to detect a peak after 2 hours for the third time course.

The pH conditions exhibited an overall decrease in peak area for all three time courses. Peak area for time course 1 decreased to 0.8 at 6 hours. Peak area for time course 2 decreased to 0.2 by 6 hours Peak area for time course 3 decreased to 0.8 at 2 hours and then was no longer detectable. Figure 5D depicts positive peak area for all three time courses.

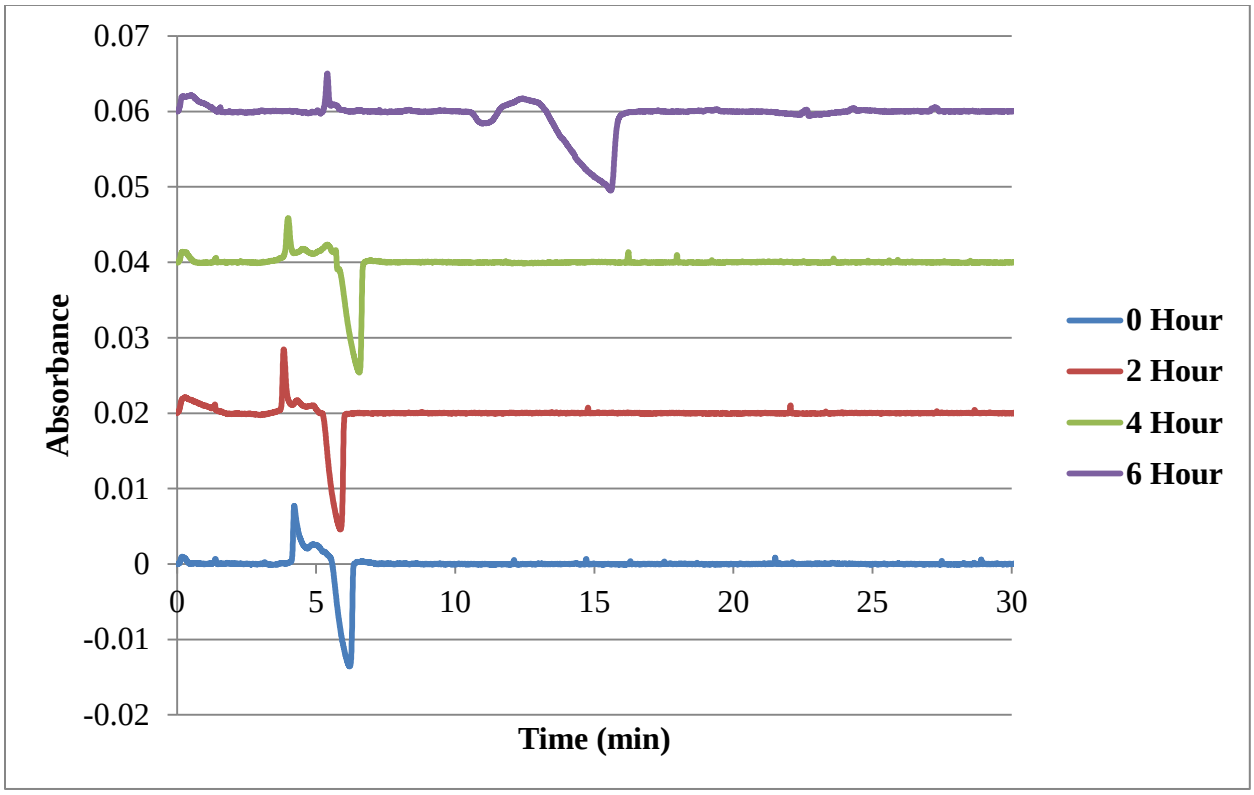


Figure 4: Electropherogram of a pH run. Peaks of interest are the initial positive peak which is followed by the negative drop peak.

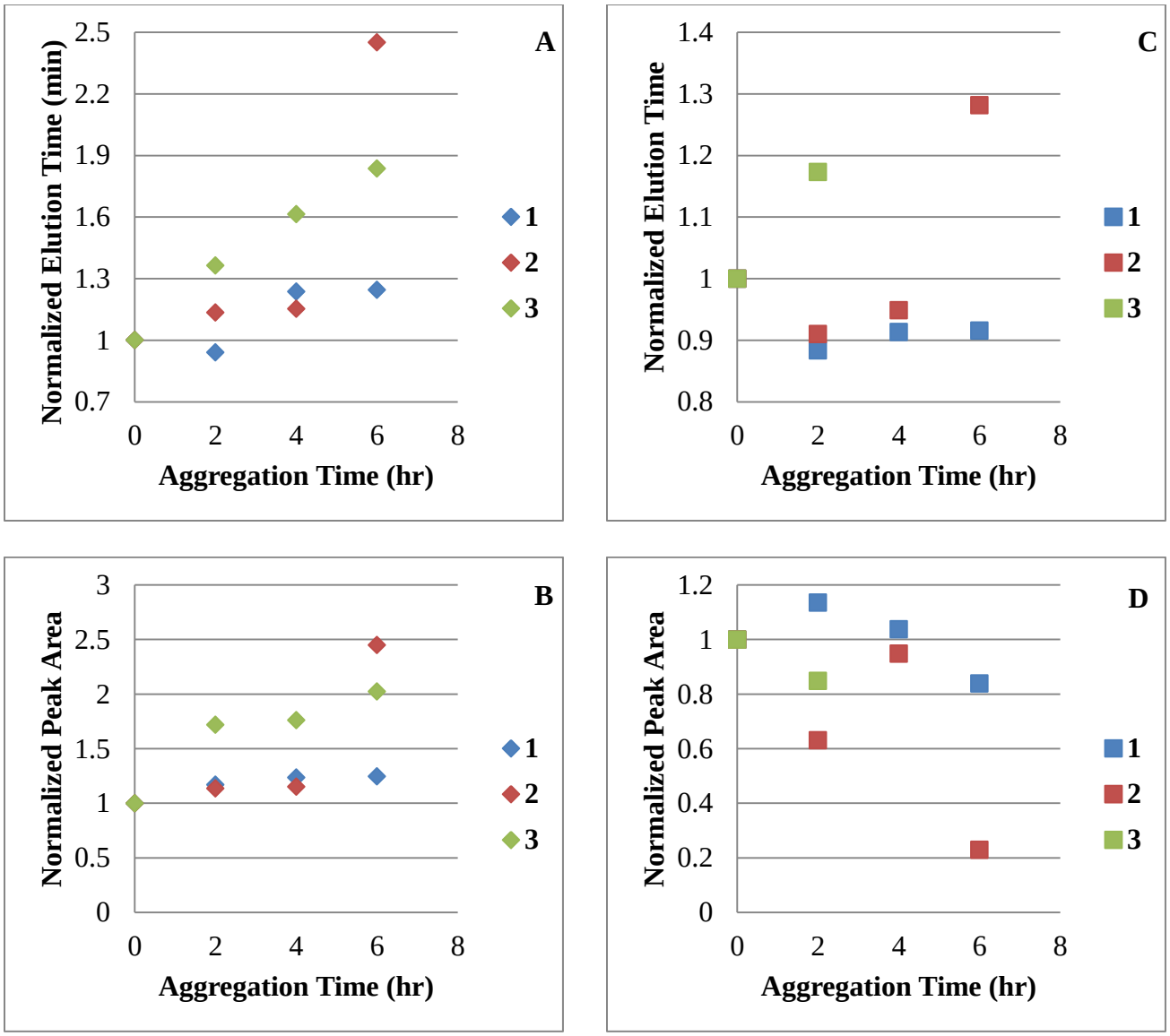


Figure 5: A. Negative peak normalized pH elution times vs. aggregation time. B. Negative peak normalized pH peak area vs. aggregation time. C. Positive peak normalized pH elution time vs. aggregation time. D. Positive peak normalized pH peak area vs. aggregation time.

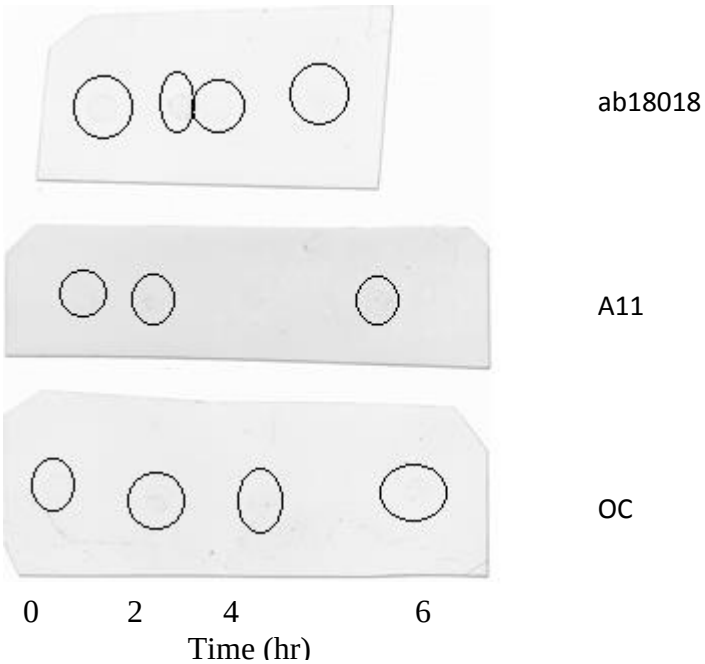


Figure 6: Dot blot of pH condition. Antibody ab18018 confirmed the presence of amylin. A11 confirmed the presence of oligomers. OC confirmed the presence of fibrils.

For the dot blot assays, ab18018 confirmed the presence of amylin at all the time points (Figure 6). Oligomers were detected at the 0, 2, and 6 hour time points. Although a positive oligomeric dot is not present as 4 hours, oligomers should be present and therefore it is most likely an experimental error. Fibrils were detected at all time points. Our dot blot assay confirmed the presence of fibrils and oligomers at time zero which indicates the positive peak represents amyloid oligomers/ fibrils.

Our depicts increases in positive peak elution time and decreases in positive peak area. However these contradict each other. At a pH of 7 amylin has a predicted charge of +2. The more charge a protein, the less likely it will aggregate [30]. Previous work has shown as pH decreases so too does amylin's aggregation rate. Researchers found that the rate of amylin aggregation had maxima at pH 9.5 and that from 8.5 to 5.5 it decreased. [24]. Our research along

with Kudva *et al*. indicates a lower pH has more complex effects on aggregation making it less desirable to use as a study condition due to its inconsistency.

Study of Salt

Salt condition, Table 1, caused an increase in elution times and peak area for all time courses. The three time courses were fairly consistent until the final 6 hour time point. Figure 7 depicts an electropherogram of the Salt condition. Time courses 1 and 3 displayed negative peak elution times close to 1.6 minutes. Time course 2 however, resulted in a decrease from the four hour time point, dropping from 1.3 minutes to 1.1 minutes. A comparison of negative peak elution times can be seen in Figure 8A.

The salt condition resulted in an initial increase of negative peak area for the three time courses, but following the 4 hour time point, time courses 2 and 3 decreased as shown in Figure 8B. Time course 1 had a slight increase between the 4 and 6 hour time points. While 2 and 3 followed the same trend, there were no similarities in increases of peak area between the time courses. At the 6 hour time point time course 2 decreased in peak area to less than 1. This decrease could be due to the transition from an oliogmeric state to a fibril state. The increase in peak area indicates the presence of more species which could be amylin aggregating from a monomer to an oligomeric state.

Positive peak elution times can be seen in Figure 8C. The salt condition resulted in consistent increases in positive peak elution times for all 3 time courses until the final 6 hour time point. At 6 hours, time course 2 showed a decrease in elution time. The 6 hour elution times for time courses 1, 2, and 3 were 1.7, 1.1, and 1.6 minutes. The consistent increases in elution time are indicative of the development of larger species.

Figure 8D depicts positive peak areas. Salt conditions showed an increase in positive peak area for all three time courses. Time course 1 finished with a peak area of 6.3. Time course 2 finished with a peak area of 3.3. Time course 3 finished with a peak area of 3. While there is variance in peak area, the overall trend of increasing peak area is similar to that of the positive peak elution time indicating the development of larger species.

Previous work has shown that increased salt concentration can enhance amyloid aggregation. A study by Kudva showed an increase from 0 to 100 mM NaCl increased the rate of amylin aggregation by close to 100% [24]. A separate study found that aggregation of amyloid proteins rises with increased salt concentrations up to 500 mM [35]. Both our positive peak elution time and peak area grew with each time point which indicates the presence of an aggregating species. Salt induced protein aggregation is believed to be caused by what is known as the hydrophobic effect [36]. This occurs when the addition of salt induces the folding of the nonpolar regions of a protein, promoting enhanced oliogomer and fibril formation [37].

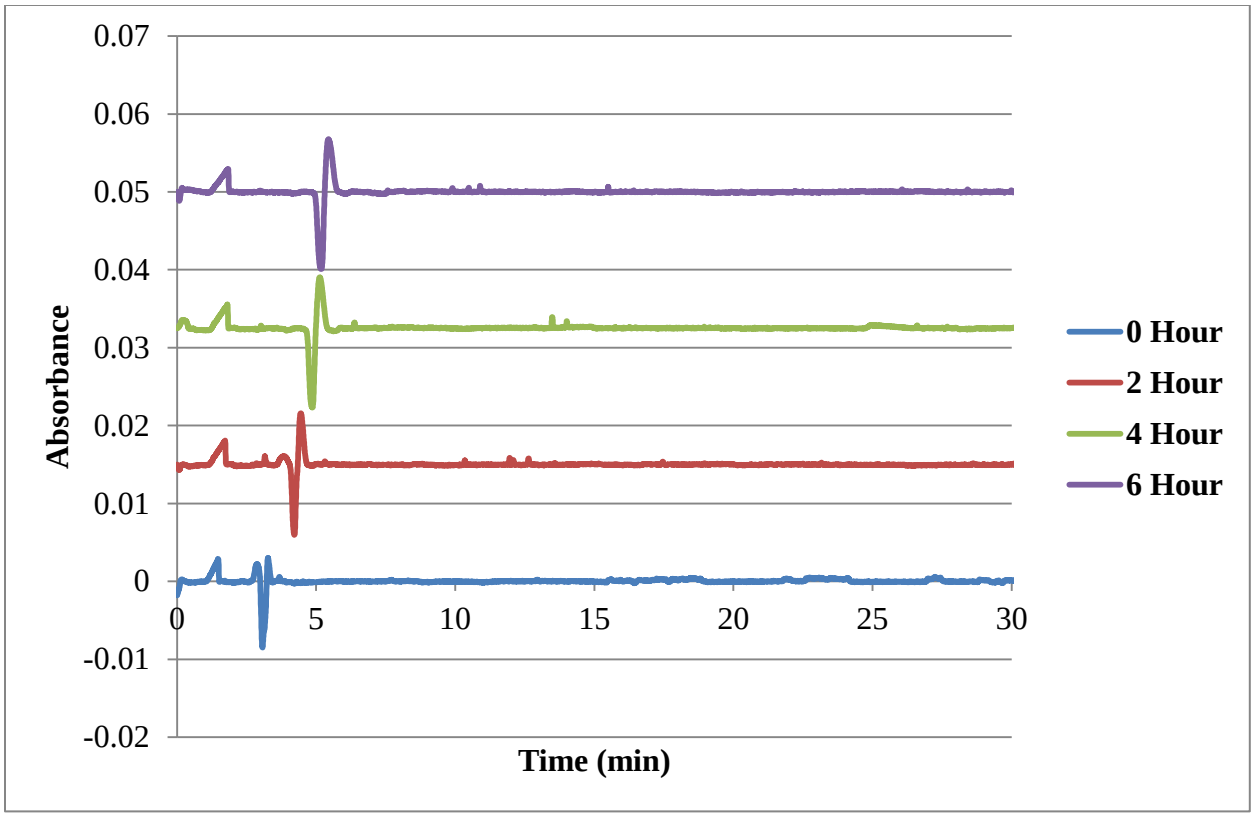
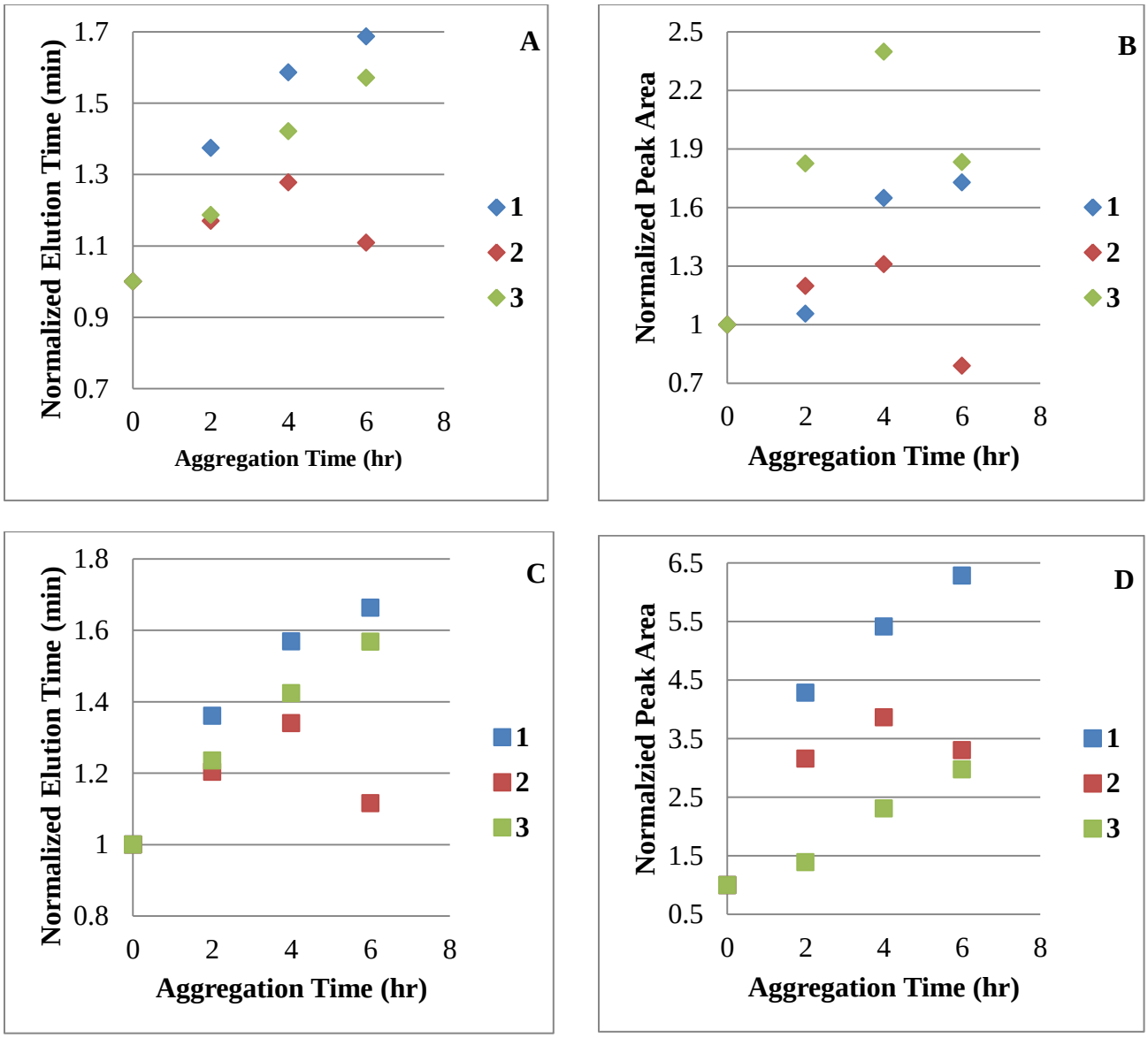


Figure 7: Electropherogram of Salt condition. There is a system peak at 2 minutes and then a negative peak. The negative peak is immediately followed by a positive peak.



Figures 8: A. Negative peak normalized salt elution times vs. aggregation time. B. Negative peak normalized salt peak area vs. aggregation time. C. Positive peak normalized salt elution time vs. aggregation time. D. Positive peak normalized salt peak area vs. aggregation time.

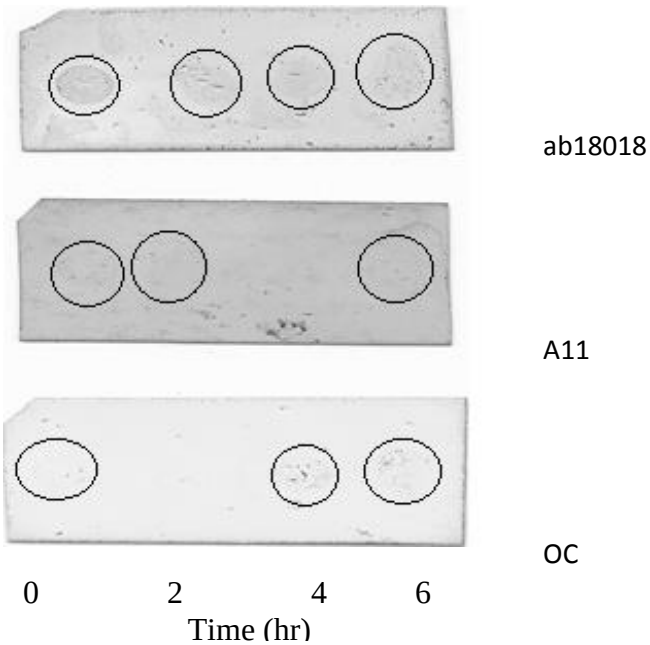


Figure 9: Dot blot of Salt condition. Antibody ab18018 confirmed the presence of Amylin. A11 confirmed the presence of oligomers. OC confirmed the presence of fibrils.

Dot blots for salt condition can be seen in Figure 9. Antibody ab18018 confirmed the presence of amylin for all time points. Oligomers were detected at 0, 2, and 6 hours. The presence of fibrils was detected at all time points. The 4 and 6 hour time points were noticeably darker for the fibril nitrocellulose membrane. While A11 and OC both confirmed the presence of oligomers and fibrils the final 6 hour time point for both is more noticeable than the previous points. Combining this with our positive peak area and elution times increases it is reasonable to suggest that we are monitoring amylin aggregation. However, while dot blots did confirm the presence of oligomers and fibrils they did not provide any information about the details of the aggregating species.

Study of Temperature

Temperature conditions showed an increase in negative peak elution time for all time courses which can be seen in the electropherogram in Figure 10. Time courses 1 and 2 results were similar until the final time point. For the 8 hour time point time course 1 had an elution time of 1.6 minutes. Time course 2 had an elution time of 1.3 minutes. Both time courses showed a decrease in elution time from the 6 hour to the 8 hour time point. Time course 3 showed an increase in elution time, however it was not similar to time courses 1 and 2. Time course 3 had an outlier point at 2 hours and did not show a decrease in elution time from the 6 hour to the 8 hour time points. Its 8 hour time point finished with an elution time similar to time course 2, 1.4 minutes. The overall increase in elution time indicates the development of an increasingly larger species migrating through the capillary. The third time course was performed several weeks after the first two, which might explain some of the variation between them. Negative peak elution times can be seen in Figure 11A.

Temperature conditions resulted in an increase in negative peak area for all time courses. Figure 11B depicts negative peak areas. Time courses 1 and 2 showed a decrease in peak area between the 6 and 8 hour time points. There was variance between all three time courses. Time course 3 only showed slight increases in peak area for the whole time course. The peak areas at 6 hours for time courses 1, 2, and 3 were 2.2, 1.5, and 1.2. With the drop of the final peak area, temperature displayed a comparable characteristic with the salt condition. Similarly this could be to the transition from oligomers to fibrils.

The temperature condition exhibited a positive peak in time courses 1 and 3. Time course 1 showed an increase in peak elution time until decreasing at the 8 hour time point. Time

course 3 developed a positive peak at the 6 hour time point; however it showed a decrease in elution time at the 8 hour time point. Figure 11C depicts positive peak elution times.

Temperature conditions resulted in peak area for time courses 1 and 3 and can be seen in Figure 11D. Time course 1 increased to a peak area of 3.1 at 6 hours and then decreased to a peak area 2.8 at 8 hours. Time course 3 developed a peak at 6 hours which increased to a peak area of 2.4 at 8 hours. Time course 2 did not develop a positive peak. The plot of temperature's peak area was similar to its plot of elution time. This decrease could be due to progression from oligomers to fibrils. Previous work by Kudev *et al*. found that there was a direct relationship between temperature and amylin aggregation. They studied amylin aggregation at 4^{o}C, 22^{o}C, and 37 ^{o}C [24]. They found that increased temperature resulted in higher rates of amylin aggregation. The increases of elution time and peak area for time course 1 indicate the development of larger aggregated species, however it is difficult to draw conclusions based on one time course. Time course 2 did not develop a positive peak, and the positive peak for time course 3 developed 6 hours after time course 1.

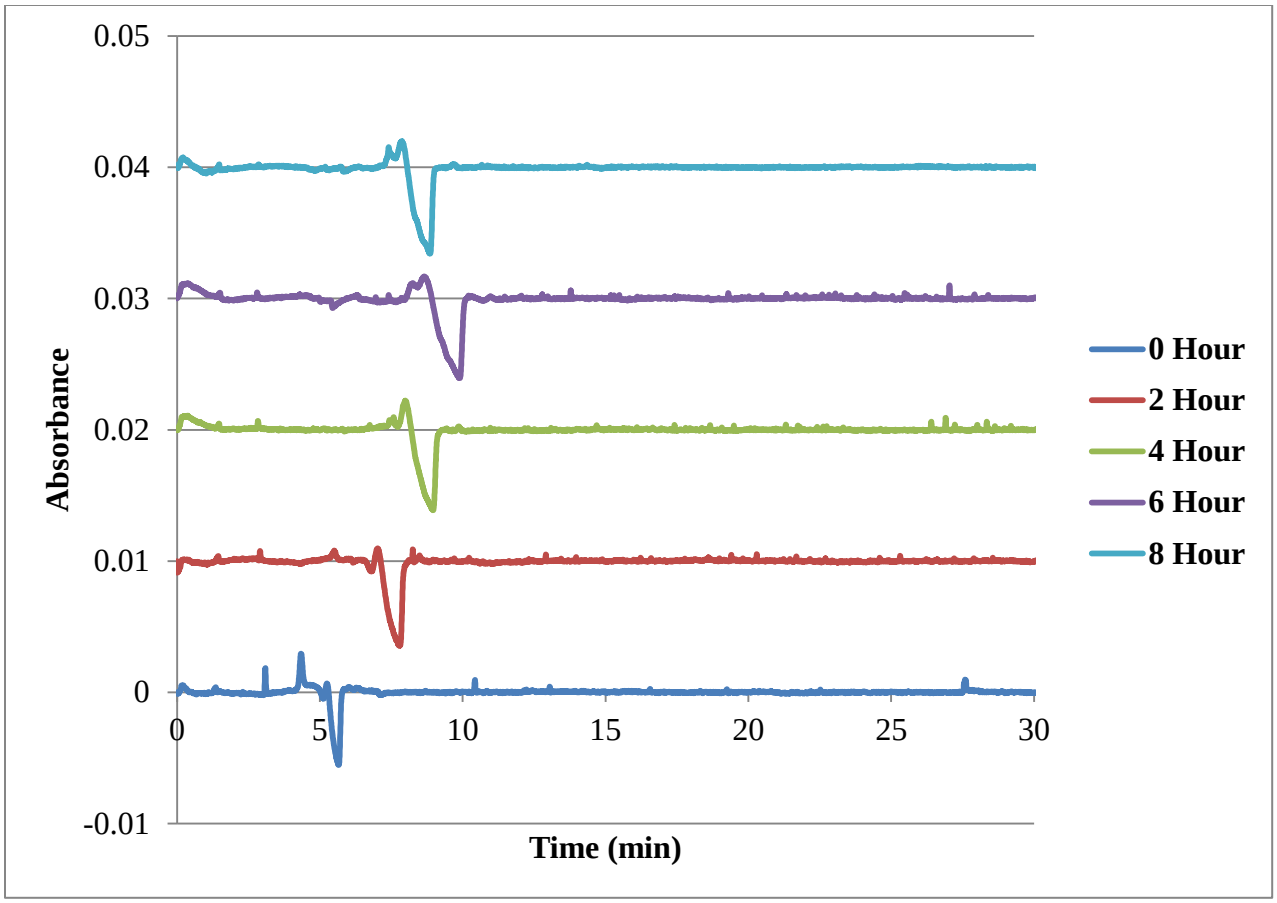
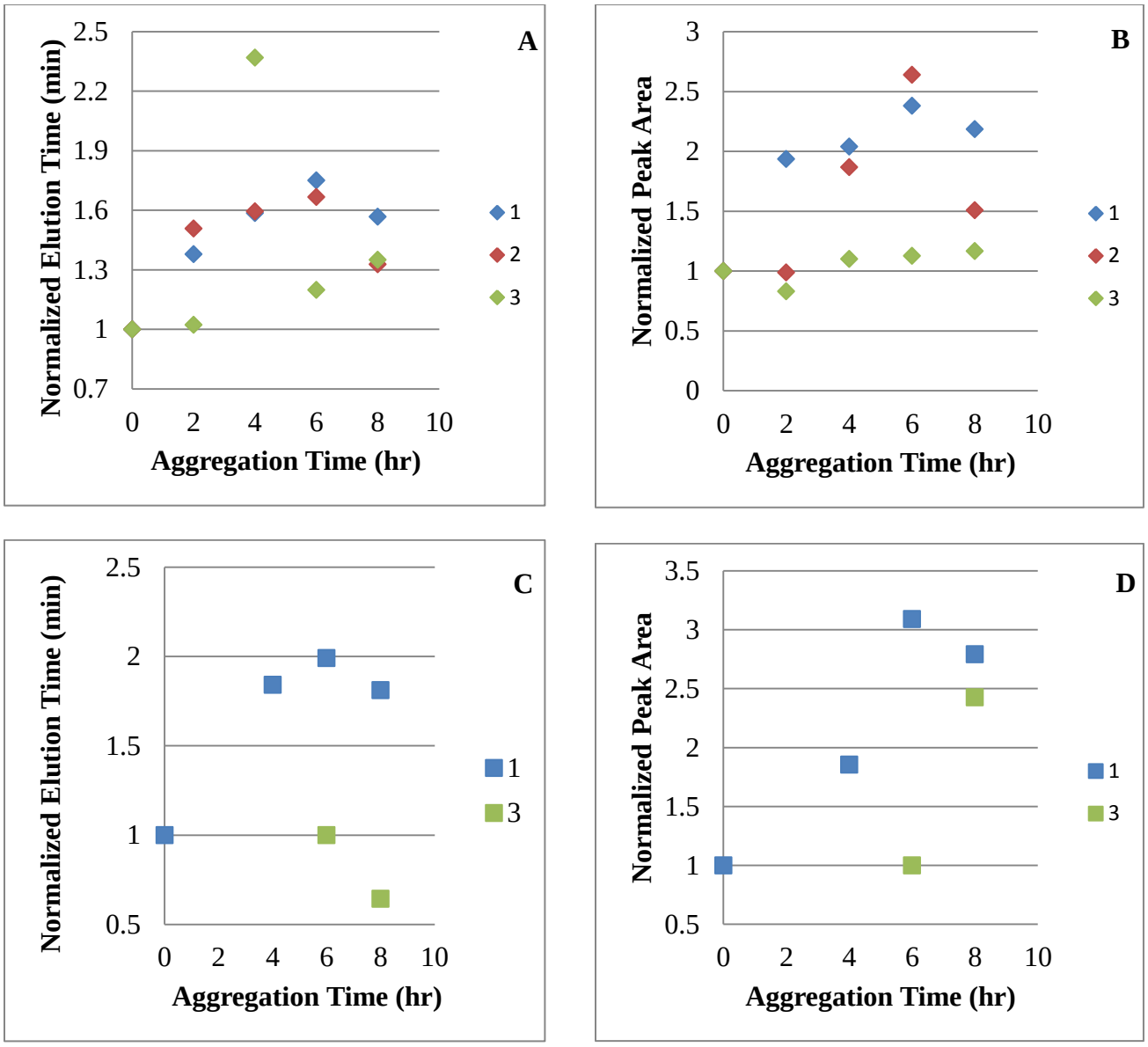


Figure 10: Electropherogram of Temperature condition. The area of interest is between 0 and 10 minutes. Initially there is a positive peak which is closely followed by a negative peak.



Figures 11: A. Negative peak normalized temperature elution times vs. aggregation time. B. Negative peak normalized temperature peak area vs. aggregation time. C. Positive peak normalized temperature elution time vs. aggregation time. D. Positive peak normalized temperature peak area vs. aggregation time.

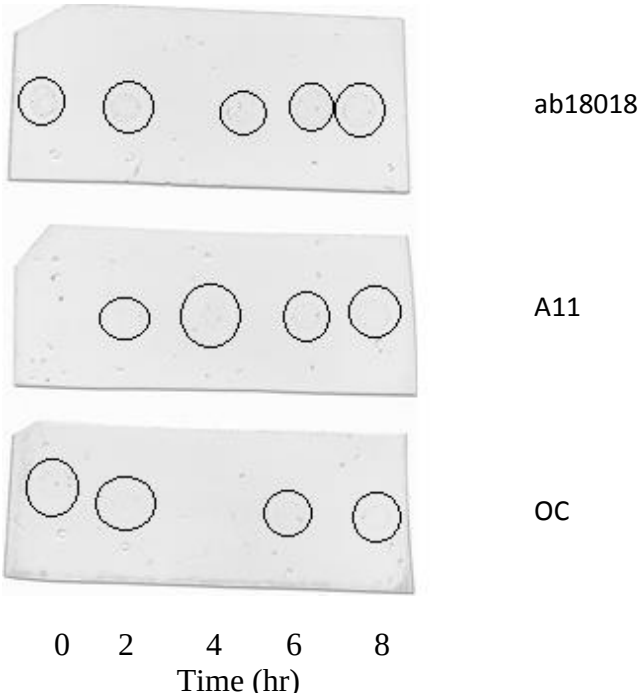


Figure 12: Dot blot of Temperature condition. Antibody ab18018 confirmed the presence of amylin. A11 confirmed the presence of oligomers. OC confirmed the presence of fibrils.

Dot blots can be seen in Figure 12. Antibody ab18018 detected the presence of amylin for all time points. Oligomers were detected at the 2 hour time point and confirmed for the rest of the time course. Fibrils were detected at all time points. The 6 and 8 hour time points were noticeably darker for both oligomers and fibrils. The detection of both oligomers and fibrils at the same time indicates amyloid aggregation has occurred we cannot say capillary electrophoresis was able to monitor oligomer formation. While based on previous studies as well as our work with ThT, it is reasonable to suggest that increased in temperature will result in increased amylin aggregation, we were not able to conclusively monitor amylin oligomer formation [24].

Study of Multiple Variables

A combination of multiple variables (MV) showed an increase in negative peak elution time for each time course as shown in Figure 13. Figure 14A depicts negative peak elution times. Time course 1 showed the greatest increase in elution time with a final elution time of 1.9 minutes at 6 hours. Time courses 2 and 3 finished with elution times close to 1.5 minutes. In comparison to the control conditions, MV conditions behaved fairly similarly, but eluted slightly faster. By 6 hours the elution time was roughly 1.6 minutes while for the control it eluted around 1.3 minutes. The increase could indicate larger aggregated species which could have resulted from the increased salt concentration as well as the increased temperature which.

Negative peak area can be seen in Figure 14B. The MV condition showed an increase in negative peak area for each time course, however there was variance between the time courses for all time points. In comparison with the control at the 6 hour time point the MV condition finished the time course with slightly greater negative peak areas. With its increase in salt and temperature it was expected that the body conditions would have greater peak area than the control yet the control had larger peak areas at the end of its time course.

Figure 14C depicts positive peak elution times. The MV condition showed an increase in positive peak elution time for all time courses. Time course 1 showed much higher increases in elution time than time courses 2 and 3. Time courses 2 and 3 showed similar increases until the 6 hour time point with 2 showing an elution time of 1.2 minutes and 3 an elution time of 1.6 minutes. The 6 hour elution times for time course 1 was 2 minutes. While there was variance between the 3 time courses all showed a general trend of increasing elution time which suggested the development of larger aggregated species.

The development of larger aggregates was further confirmed after observing the results of positive peak area. Positive peak areas can be seen in Figure 14D. MV conditions showed an increase in positive peak area for all three time courses. The increase for time course 2 was much less than time courses 1 and 3 with a final peak area of 1.3 at 6 hours. The peak areas for 2 and 3 were about 3 at 6 hours.

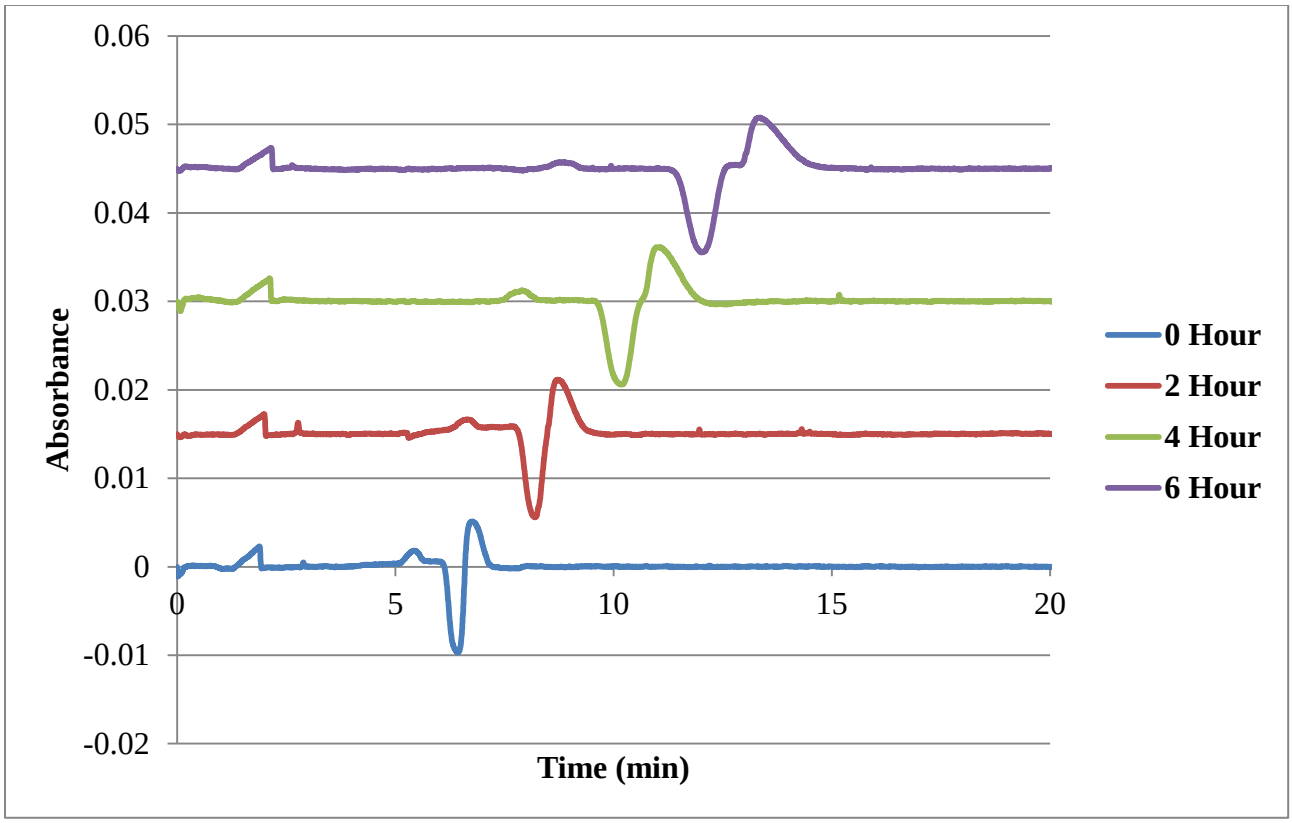
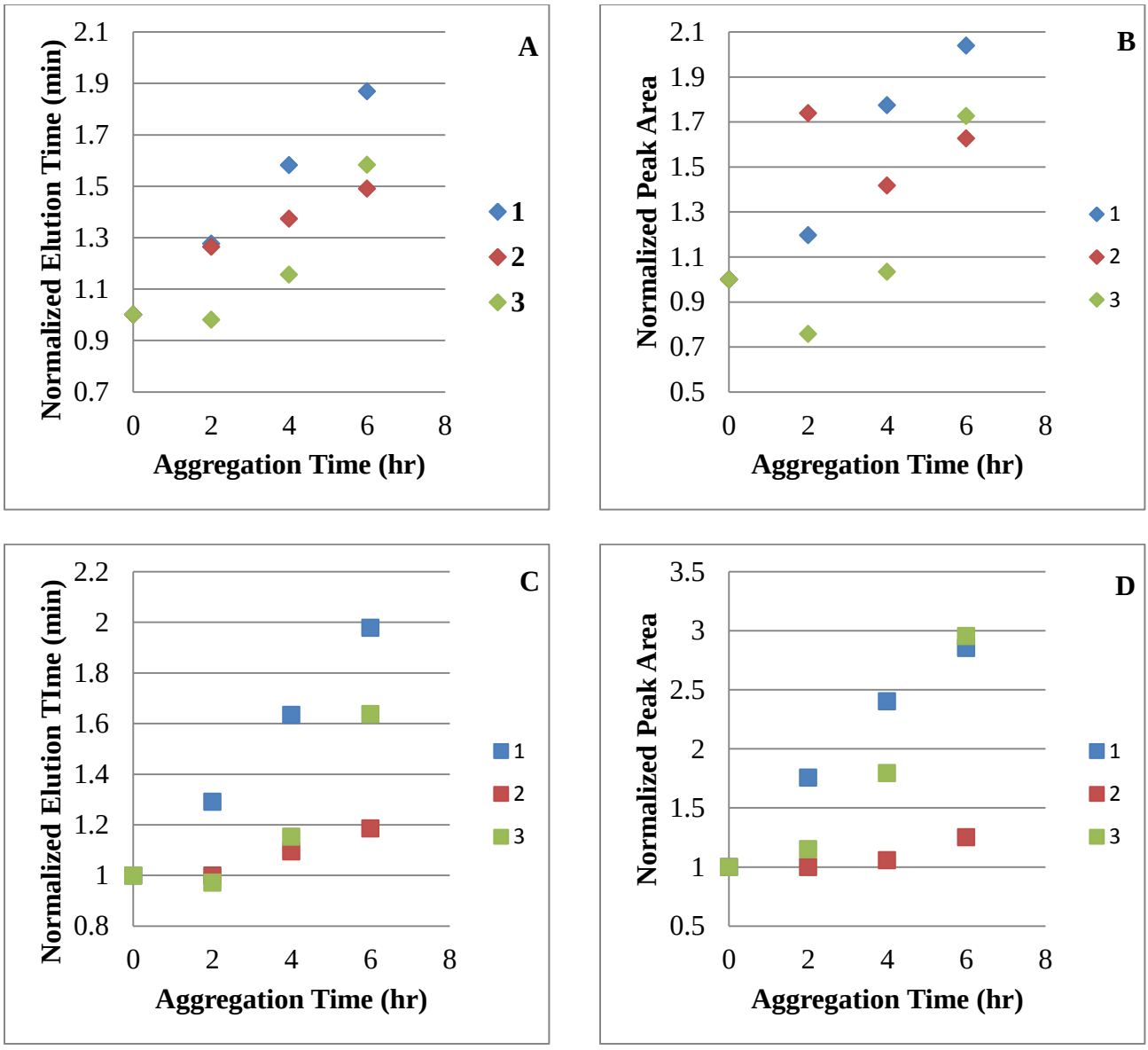


Figure 13: Electropherogram of MV condition. There is a system peak at 2 minutes followed by a negative peak at 7 minutes which is immediately followed by a positive peak.



Figures 14: A. Negative peak normalized MV elution times vs. aggregation time. B. Negative peak normalized MV peak area vs. aggregation time. C. Positive peak normalized MV elution time vs. aggregation time. D. Positive peak normalized MV peak area vs. aggregation time.

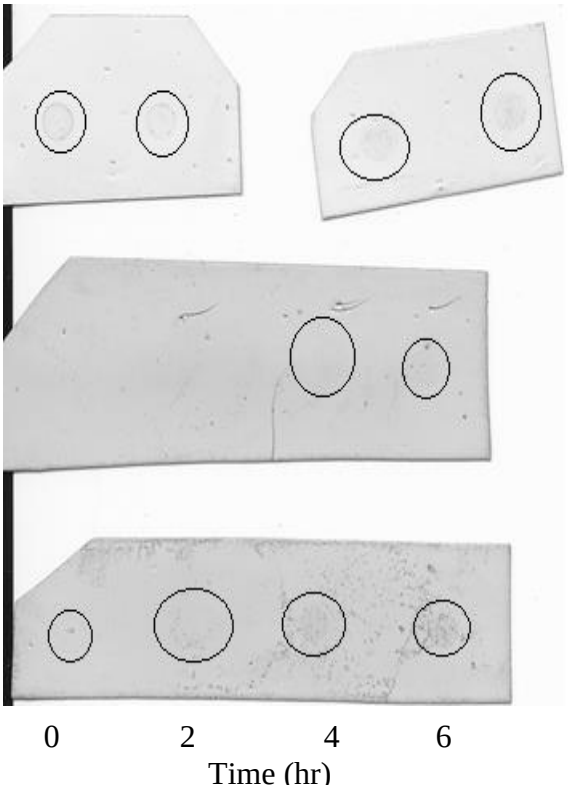


Figure 15: Dot blot of Body condition. Antibody ab18018 confirmed the presence of amylin. A11 confirmed the presence of oligomers. OC confirmed the presence of fibrils.

Dot blots can be seen in Figure 15. Antibody ab18018 confirmed the presence of amylin for all time points. Oligomers were detected at the 4 and 6 hour time points. Fibrils were detected at all time points. The 4 and 6 hour time points were noticeably darker for both oligomers and fibrils. The presence of positive peaks that showed consistent increases in both peak size and peak area indicated the presence of aggregating species. While dot blots did detect oligomers and fibrils, due to the detection of fibrils at all time points we were unable to use them to conclusively monitor the formation of oligomers and determine the identity of the aggregated amylin species within the capillary.

In comparison with the pH, salt, and temperature studies the MV results were more consistent. With the exception of positive peak area for the Salt study, the values obtained for normalized elution time and peak area, both positive and negative, were similar to the other studies. Though there was variance between individual runs, the MV results did result in consistent increases for elution time and peak area. The electropherograms revealed an interesting similarity between the salt and MV studies in comparison to the other three. The negative peak for the salt and MV studies elutes after the positive peak while in the other three studies it elutes before the positive peak. Both salt and MV studies also exhibited a system peak between 1 and 2 minutes. The only similarity between the two studies was the high salt concentration of 140 mM NaCl.

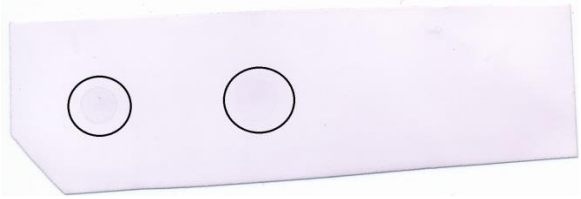
Improvements/ Causes for variance

Our results showed an overall consistent trend that these conditions do promote aggregation, however some of the time courses were inconsistent with each other. It is our belief that a variety of reasons led to this inconsistency. First is the preparation of amylin. Amylin was purchased from Anaspec, and had a peptide content percentage of 83%. Therefore 17% of the sample was not amylin. We do not know the identity of this 17%. It is possible that it may have contained some sort of contaminant. In addition, our preparation method involved the use of HFIP to dissolve the sample and aliquot it out. There are other preparation methods available that might remove this 17% as well as reduce amylin to its monomeric form such as size exclusion chromatography. Size exclusion chromatography separates molecules based on their size and molecular weight [38]. This would allow us to better purify the amylin sample so that only the monomeric form of amylin remains.

Our method of detection/ separation using capillary electrophoresis involved the use of a bare siliconized capillary. The inside of this capillary is negatively charged [39]. Amylin is positively charged which results in "sticking" of the protein on the interior wall of the capillary [40]. Sticking could be the cause of variance between our individual CE runs. To alleviate this sticking a coating polymer can be used to neutralize the interior capillary surface. Unfortunately, preliminary results with a coating polymer, PEO, had inconsistent current. Perhaps this could be improved in the future by examining other coatings.

CE can also be performed with a polymer matrix to enhance the resolution of different species. We performed preliminary tests to determine if amylin could be studied using a polymer matrix. Both the absorbance and currents were inconsistent. We therefore chose to forgo using a polymer matrix. It might be possible to get more consistent absorbance and current if we were to utilize a different polymer for both coating and separation such as PHEA or PVA [41].

Also of concern is the specificity of our primary ab18018 antibody. A control study was conducted to confirm its specificity. We blotted 50 µM amylin along with 50 µM BSA and our sample Tris buffer. We expected to only observe the amylin dot; however the ab18018 detected both the amylin and the BSA which can be seen in Figure 16. The BSA blot was not as strong as the amylin's, but its presence indicates the potential for a false positive. This makes it difficult to use to confirm the presence of amylin within our samples. For future work there will need to be a new, more specific antibody selected.



Amylin BSA Tris

Figure 16: Control dot blot study for the specificity of AB18018 antibody. 50 µM Amylin, 50 µM BSA, and 43 mM Tris sample buffer were blotted onto a nitrocellulose membrane. Positive blots have been circled.

Previous work has revealed amylin aggregation can be concentration dependent. While our 50 µM amylin concentration was acceptable, higher concentrations have been shown to result in a faster aggregation rate [24]. It would be prudent to observe variations in concentrations under our solutions conditions for future work.

References

1. Marzban L, Verchere CB. The role of islet amyloid polypeptide in type 2 diabetes. The Canadian Journal of Diabetes 2004;28(4):39.

2. Diabetes Statistics [Internet]: American Diabetes Association; c2013 [cited 2013 June/4]. Available from: http://www.diabetes.org/diabetes-basics/diabetes-statistics/.

3. Hoyert D, Xu J. Deaths preliminary data for 2011. National Vital Statistics Report 2011;61(6).

4. Grateau G, Kyle RA, Skinner M, editors. Amyloid and amyloidosis. CRC Press; 2004. .

5. Roberts A, Leighton B, Todd J, Cockburn D, Schofield P, Sutton R, Holt S, Boyd Y, Day A, Foot E. Molecular and functional characterization of amylin, a peptide associated with type 2 diabetes mellitus. Proceedings of the National Academy of Sciences 1989;86(24):9662-6.

6. Adeghate E, Kalász H. Suppl 2: Amylin analogues in the treatment of diabetes mellitus: Medicinal chemistry and structural basis of its function. The Open Medicinal Chemistry Journal 2011;5:78.

7. Woods SC, Lutz TA, Geary N, Langhans W. Pancreatic signals controlling food intake; insulin, glucagon and amylin. Philosophical Transactions of the Royal Society B: Biological Sciences 2006;361(1471):1219-35.

8. Young A. Roles of amylin in diabetes and regulation of nutrient load. Nutrition 1998 6;14(6):524-7.

9. Kinnunen P. Amyloid formation on lipid membrane surfaces. The OPen Biology Journal 2009;2:163.

10. Trikha S, Jeremic AM. Clustering and internalization of toxic amylin oligomers in pancreatic cells require plasma membrane cholesterol. J Biol Chem 2011;286(41):36086-97.

11. Guillo C, Roper MG. Simultaneous capillary electrophoresis competitive immunoassay for insulin, glucagon, and islet amyloid polypeptide secretion from mouse islets of langerhans. Journal of Chromatography A 2011 7/1;1218(26):4059-64.

12. Marzban L, Park K, Verchere CB. Islet amyloid polypeptide and type 2 diabetes. Exp Gerontol 2003 4;38(4):347-51.

13. Weise K, Radovan D, Gohlke A, Opitz N, Winter R. Interaction of hIAPP with model raft membranes and pancreatic β-Cells: Cytotoxicity of hIAPP oligomers. ChemBioChem 2010;11(9):1280-90.

14. Engel M, Khemtemourian L., Kleijer C, Meeldijk H, Jacobs J, Verkleij A, De Kruijff B, Killian JA, Hoppener JWM. Membrane damage by human islet amyloid polypeptide

through fibril growth at the membrane. Proceedings of the National Academy of Sciences 2008;195(16).

15. Ma Z, Westermark G. Effects of free fatty acid on polymerization of islet amyloid polypeptide (IAPP) in vitro on amyloid fibril formation in cultivated isolated islets of transgenic mice overexpressing human iapp. Molecular Medicine 2002;12:8863.

16. Liu G, Prabhakar A, Aucoin D, Simon M, Sparks S, Robbins KJ, Sheen A, Petty S, A., Lazo N, D. - Mechanistic studies of peptide self-assembly: Transient α-helices to stable β-sheets. - J Am Chem Soc 2010;132(- 51):- 18223.

17. Hudson SA, Ecroyd H, Kee TW, Carver JA. The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds. FEBS Journal 2009;276(20):5960-72.

18. Jha S, Patil SM, Gibson J, Nelson CE, Alder NN, Alexandrescu AT. Mechanism of amylin fibrillization enhancement by heparin. J Biol Chem 2011;286(26):22894-904.

19. Meier JJ, Kayed R, Lin C, Gurlo T, Haataja L, Jayasinghe S, Langen R, Glabe CG, Butler PC. Inhibition of human IAPP fibril formation does not prevent β-cell death: Evidence for distinct actions of oligomers and fibrils of human IAPP. American Journal of Physiology-Endocrinology and Metabolism 2006;291(6):E1317-24.

20. Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJ. The aggregation potential of human amylin determines its cytotoxicity towards islet β-cells. Febs Journal 2006;273(15):3614-24.

21. Pryor E, Kotarek J,A., Moss MA, Hestekin C,N. Monitoring insulin aggregation via capillary electrophoresis. International Journal of Molecular Sciences 2011;12(12):9369-88.

22. Pryor NE, Moss MA, Hestekin CN. Unraveling the early events of amyloid-β protein (Aβ) aggregation: Techniques for the determination of Aβ aggregate size. International Journal of Molecular Sciences 2012;13(3):3038-72.

23. Jain S, Udgaonkar JB. Salt-induced modulation of the pathway of amyloid fibril formation by the mouse prion protein. Biochemistry 2010;49(35):7615-24.

24. KUDVA Y, MUESKE C, BUTLER P, EBERHARDT N. A novel assay in vitro of human islet amyloid polypeptide amyloidogenesis and effects of insulin secretory vesicle peptides on amyloid formation. Biochem J 1998;331:809-13.

25. Zhao H, Sui Y, Guan J, He L, Gu X, Wong HK, Baum L, Lai FM, Tong PC, Chan JC. Amyloid oligomers in diabetic and nondiabetic human pancreas. Translational Research 2009;153(1):24-32.

26. Goldsbury C, Goldie K, Pellaud J, Seelig J, Frey P, Müller S, Kistler J, Cooper G, Aebi U. Amyloid fibril formation from full-length and fragments of amylin. J Struct Biol 2000;130(2):352-62.

27. Ryan TM, Caine J, Mertens HD, Kirby N, Nigro J, Breheney K, Waddington LJ, Streltsov VA, Curtain C, Masters CL. Ammonium hydroxide treatment of Aβ produces an aggregate free solution suitable for biophysical and cell culture characterization. PeerJ 2013;1:e73.

28. Pryor E. Capillary and microchip electrophoresis for the monitoring of disease causing amyloid proteins. University of Arkansas; 2012.

29. Tiiman A, Noormägi A, Friedemann M, Krishtal J, Palumaa P, Tõugu V. Effect of agitation on the peptide fibrillization: Alzheimer's amyloid-? peptide 1-42 but not amylin and insulin fibrils can grow under quiescent conditions. Journal of Peptide Science 2013;19(6):386-91.

30. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: Mechanism and driving forces in nonnative protein aggregation. Pharm Res 2003;20(9):1325-36.

31. National Agricultural Laboratory U. Sodium and chloride. USDA: .

32. White-Ziegler CA, Malhowski AJ, Young S. Human body temperature (37 C) increases the expression of iron, carbohydrate, and amino acid utilization genes in escherichia coli K-12. J Bacteriol 2007;189(15):5429-40.

33. Kirby B. Micro-and nanoscale fluid mechanics: Transport in microfluidic devices. New York: Cambridge University Press; 2010. .

34. Beckers J. UV detection in capillary zone electrophoresis peaks or dips-that is the question. Journal of Chromatography A 1994;679(1):153-65.

35. Klement K, Wieligmann K, Meinhardt J, Hortschansky P, Richter W, Fändrich M. Effect of different salt ions on the propensity of aggregation and on the structure of Alzheimer's Aβ(1-40) amyloid fibrils. J Mol Biol 2007 11/9;373(5):1321-33.

36. Spolar RS, Ha J, Record MT. Hydrophobic effect in protein folding and other noncovalent processes involving proteins. Proceedings of the National Academy of Sciences 1989;86(21):8382-5.

37. Date MS, Dominy BN. Modeling the influence of salt on the hydrophobic effect and protein fold stability. COMMUNICATIONS IN COMPUTATIONAL PHYSICS 2013;13(1):90-106.

38. Barth HG, Jackson C, Boyes BE. Size exclusion chromatography. Anal Chem 1994;66(12):595R-620R.

39. Landers J. *Handbook of capillary and microchip electrophoresis and associated microtechniques*. 3rd ed. Boca Raton: CRC Press; 2008. .

40. Deyl Z, Mikšík I, Charvatova J, Eckhardt A. Comparison of the electrophoretic separation of proteins in capillaries with different inner diameter. Journal of Chromatography A 2003;1013(1):233-8.

41. Doherty EA, Meagher RJ, Albarghouthi MN, Barron AE. Microchannel wall coatings for protein separations by capillary and chip electrophoresis. Electrophoresis 2003;24(1-2):34-54.

Appendix

Mixing Table for Amylin at 50 µM

Samples	5mM NaOH (ul)	40mM Tris –HCl (ul)	5mM NaCL (ul)	140mM NaCl (ul)
Control	13.66	259.32	.295	0
pH 1	13.66	259.32	.295	0
Salt 1	13.66	250.93	0	8.68
Agitation 1	13.66	259.32	.295	0
Agitation 2	13.66	259.32	.295	0
Temp1	13.66	259.32	.295	0
Body 1	13.66	250.93	0	8.68

Tris Buffer Preparation

pH at 5°C	pH at 25°C	pH at 37°C	Grams/liter for 0.05 M Tris HCl	Grams/liter for 0.05 M Tris Base
7.55	7	6.7	7.28	0.47
7.66	7.1	6.8	7.13	0.57
7.76	7.2	6.91	7.02	0.67
7.89	7.3	7.02	6.85	0.8
7.97	7.4	7.12	6.61	0.97
8.07	7.5	7.22	6.35	1.18
8.18	7.6	7.3	6.06	1.39
8.26	7.7	7.4	5.72	1.66
8.37	7.8	7.52	5.32	1.97
8.48	7.9	7.62	4.88	2.3
8.58	8	7.71	4.44	2.65
8.68	8.1	7.8	4.02	2.97
8.78	8.2	7.91	3.54	3.34
8.88	8.3	8.01	3.07	3.7
8.98	8.4	8.1	2.64	4.03
9.09	8.5	8.22	2.21	4.36
9.18	8.6	8.31	1.83	4.65
9.28	8.7	8.42	1.5	4.9
9.36	8.8	8.51	1.23	5.13
9.47	8.9	8.62	0.96	5.32
9.56	9	8.7	0.76	5.47
9.67	9.1	8.79	0.69	5.53

Buffer was made to 43.56 mM concentration so that upon dilution with amylin, NaOH, and NaCl buffer concentration would be 40 mM.

Procedures:

40 mM pH 8

Made at 25^{o}C for use at 25^{o}C

At 25^{o}C pH should be 8.0

1. Weigh out 0.7736 mg Tris – HCl crystals (0.0049 moles)
2. Weigh out 0.4617 mg Tris Base crystals (.0038 moles)
3. Add to .2 L deionized H_2O
4. Titrate with Tris Base or Acid to reach appropriate pH
5. Filter into container

100 mM pH 8

Made at 25^{o}C for use at 25^{o}C

At 25^{o}C pH should be 8.0

1. Weigh out 1.776 g Tris – HCl crystals (0.0113 moles)
2. Weigh out 1.06 g Tris Base crystals (0.00875 moles)
3. Add to 0.2 L deionized H_2O
4. Titrate with Tris Base or Acid to reach appropriate pH
5. Filter into container

40 mM pH 8

Made at 25^{o}C for use at 37^{o}C

At 25^{o}C pH should be 8.3

1. Weigh out 0.535 g of Tris – HCl crystals (0.00339 moles)
2. Weigh out 0.645 g of Tris Base crystals (0.00532 moles)
3. Add to 0.2 L of deionized H_2O
4. Titrate with Tris Base or Acid to reach appropriate pH
5. Filter into container

40 mM pH 7

Made at 25^{o}C for use at 25^{o}C

At 25^{o}C pH should be 7.0

1. Weigh out 0.3171 g Tris – HCl (0.0021 moles)
2. Weigh out 0.0020 g Tris Base (1.69 E -5 moles)
3. Add to 0.05 L deionized H_2O
4. Titrate with Tris acid to reach appropriate pH
5. Filter into container

100 mM pH 7

Made at 25^{o}C for use at 25^{o}C

At 25^{o}C pH should be 7.0

1. Weigh out 0.728 g Tris – HCl (0.00462 moles)
2. Weigh out 0.0047 g Tris Base (3.879E -5 moles)
3. Add to 0.05 L deionized H_2O
4. Titrate with tris acid to reach appropriate pH
5. Filter into container

HFIP Preparation

Monomerization by HFIP and storage of Amylin peptide

Stock Solutions:

A. **Assay Buffer:**
HFIP (1,1,1,3,3,3-hexafluoro-2-propanol)

B. **Amylin**: 1mg/vial Anaspec

Procedure:

1. Amylin is stored as a solid at -80C. Remove and place on ice when ready to prepare stock peptide films.
2. Place 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) on ice in the hood and allow to cool. HFIP is highly corrosive and very volatile. Add enough HFIP to Amylin such that the final peptide concentration is 1mM (e.g. 256 ul cold HFIP to 1 mg Amylin). Rinse vial thoroughly.
3. Incubate at room temperature for 60 min, keeping vial closed. Solution should be clear and colorless. Any traces of yellow color or cloudy suspension indicate poor peptide quality and should not be used.
4. Place peptide—HFIP solution back on ice for 5–10 min.
5. Separate the HFIP into vials with 0.0625 mg/vial. That means each vial has 16 µL stock.
6. Aliquot solution into non-siliconized microcentrifuge tubes. Do not close tubes.
7. Allow HFIP to evaporate overnight in the hood at room temperature.
8. All traces of HFIP must be removed. The resulting peptide should be a thin clear film at the bottom of the tubes. The peptide should not be white or chunky.
9. Store dried peptide films over desiccant at -80C. These stocks should be stable for several months.

Sample Preparation

Agitation 1

1. Remove vial with .0625 mg amylin from -80oC freezer
2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 259.3 µL of 43.56 mM Tris – HCl pH 8 (This will depend on which condition you are using.)
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 0.295 µL, total volume should now be 273.27 µL)
6. Place on shaker at 0 rpm and 25^{o}C.
7. Take time points according for the condition you are using.

Agitation 2

1. Remove vial with .0625 mg amylin from -80oC freezer
2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 259.3 µL of 43.56 mM Tris – HCl pH 8 (This will depend on which condition you are using.)
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 0.295 µL, total volume should now be 273.27 µL)
6. Place on shaker at 800 rpm and 25^{o}C.
7. Take time points according for the condition you are using.

Body 1

1. Remove vial with .0625 mg amylin from -80oC freezer
2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 250.9 µL of 43.56 mM Tris – HCl pH 8 for use at 37^{o}C (This will depend on which condition you are using.)
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 8.679 µL, total volume should now be 273.27 µL)
6. Place on shaker at 0 rpm and 37^{o}C.

7. Take time points according for the condition you are using.

Control 1

1. Remove vial with .0625 mg amylin from -80oC freezer
2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 259.3 µL of 43.56 mM Tris – HCl pH 8 (This will depend on which condition you are using.)
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 0.295 µL, total volume should now be 273.27 µL)
6. Place on shaker at 400 rpm and 25^{o}C.
7. Take time points according for the condition you are using.

pH 1

1. Remove vial with .0625 mg amylin from -80oC freezer
2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 259.3 µL of 43.56 mM Tris – HCl, pH 7.0
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 0.295 µL, total volume should now be 273.27 µL)
6. Place on shaker at 400 rpm and 25^{o}C.
7. Take time points according for the condition you are using.

Salt 1

1. Remove vial with .0625 mg amylin from -80oC freezer
2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 250.3 µL of 43.56 mM Tris – HCl pH 8 (This will depend on which condition you are using.)
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 8.679 µL, total volume should now be 273.27 µL)
6. Place on shaker at 400 rpm and 25^{o}C.
7. Take time points according for the condition you are using.

Temp 1

1. Remove vial with .0625 mg amylin from -80oC freezer

2. Prepare 50µM concentration using a proportion of 5 mM NaOH (add 13.66 µL of 5 mM NaOH to vial)
3. Let dissolve for 10 minutes
4. Dilute into appropriate tris buffer by adding 259.3 µL of 43.56 mM Tris – HCl pH 8 for use at 37^{o}C (This will depend on which condition you are using.)
5. Add enough NaCl to solution such that the final NaCl concentration is 5 mM (add 0.295 µL, total volume should now be 273.27 µL)
6. Place on shaker at 400 rpm and 37^{o}C.
7. Take time points according for the condition you are using.

Dot Blot Protocol

Procedure

AB 18018 primary antibody

1. Use only tweezers when handling nitrocellulose membranes
2. Cut nitrocellulose membranes into half inch wide strips
3. Spot 2 µL of sample onto each membrane for each time point
4. After all spots have been applied to membrane, allow last applied spot to dry, approx 10 min to 1 hour
5. Block membranes in 5% nonfat milk/TBS-T solution (15 ml) at RT for 1 hour with gentle shaking
6. Wash membranes 3 times in 1X TBS – T
7. Cover each membrane with the following amounts of primary antibody AB18018 5 mL 0.1% BSA/TBS-T + 1.0 µL 6E10 primary antibody (1:5000 dilution)
8. Incubate overnight at 4 degrees C
9. Decant antibody solution and rinse 3 times in 1X TBS – T
10. Cover membrane with 5 ml 0.1% BSA/TBST + 10.0 ul secondary antibody (goat-anti-mouse conj. Alkaline phosphatase) at RT for 1 hour with gentle shaking
11. Decant antibody solution and rinse 3 times with 1X TBS – T
12. Apply 5 mL of visualization solution and shake gently
13. Decant solution and use water to stop reaction when dots develop
14. Dry in a kim wipe

A11 primary antibody

1. Use only tweezers when handling nitrocellulose membranes
2. Cut nitrocellulose membranes into half inch wide strips

3. Spot 2 µL of sample onto each membrane for each time point
4. After all spots have been applied to membrane, allow last applied spot to dry, approx 10 min to 1 hour
5. Block membranes in 5% nonfat milk/TBS-T solution (15 ml) at RT for 1 hour with gentle shaking
6. Wash membranes 3 times in 1X TBS – T
7. Cover each membrane with the following amounts of primary antibody AB18018 5 mL 0.1% BSA/TBS-T + 1.0 µL 6E10 primary antibody (1:5000 dilution)
8. Incubate overnight at 4 degrees C
9. Decant antibody solution and rinse 3 times in 1X TBS – T
10. Cover membrane with 5 ml 0.1% BSA/TBST + 10.0 ul secondary antibody (goat-anti-mouse conj. Alkaline phosphatase) at RT for 1 hour with gentle shaking
11. Decant antibody solution and rinse 3 times with 1X TBS – T
12. Apply 5 mL of visualization solution and shake gently
13. Decant solution and use water to stop reaction when dots develop
14. Dry in a kim wipe

OC primary antibody

1. Use only tweezers when handling nitrocellulose membranes
2. Cut nitrocellulose membranes into half inch wide strips
3. Spot 2 µL of sample onto each membrane for each time point
4. After all spots have been applied to membrane, allow last applied spot to dry, approx 10 min to 1 hour
5. Block membranes in 5% nonfat milk/TBS-T solution (15 ml) at RT for 1 hour with gentle shaking
6. Wash membranes 3 times in 1X TBS – T
7. Cover each membrane with the following amounts of primary antibody AB18018 5 mL 0.1% BSA/TBS-T + 1.0 µL 6E10 primary antibody (1:5000 dilution)
8. Incubate overnight at 4 degrees C
9. Decant antibody solution and rinse 3 times in 1X TBS – T
10. Cover membrane with 5 ml 0.1% BSA/TBST + 10.0 ul secondary antibody (goat-anti-mouse conj. Alkaline phosphatase) at RT for 1 hour with gentle shaking
11. Decant antibody solution and rinse 3 times with 1X TBS – T
12. Apply 5 mL of visualization solution and shake gently
13. Decant solution and use water to stop reaction when dots develop
14. Dry in a kim wipe

Th-T Protocol

Procedure: 1 mM Th-T Stock Solution

1. Weigh out 9.566 mg of Th-T powder
2. Mix with 30 mL deionized water

Procedure: Th-T solution 5mM NaCl and pH 8 for use at 25^{o}C

Th-T should be in a concentration of 35.7 µM

1. Take a 15 mL tube and fill with 2407.87 µL Tris H-Cl pH 8 for use at 25^{o}C
2. Add 2.27 µL 4400 mM NaCl
3. Add 89.29 µL 1 mM Th-T stock solution

Total volume should be 2500 µL

Procedure: Th-T solution 5mM NaCl and pH 8 for use at 37^{o}C

Th-T should be in a concentration of 35.7 µM

1. Take a 15 mL tube and fill with 2407.87 µL Tris H-Cl pH 8 for use at 37^{o}C
2. Add 2.27 µL 4400 mM NaCl
3. Add 89.29 µL 1 mM Th-T stock solution

Total volume should be 2500 µL

Procedure: Th-T solution 140mM NaCl and pH 8 for use at 37^{o}C

Th-T should be in a concentration of 35.7 µM

1. Take a 15 mL tube and fill with 2331.16 µL Tris H-Cl pH 8 for use at 37^{o}C
2. Add 79.55 µL 4400 mM NaCl
3. Add 89.29 µL 1 mM Th-T stock solution

Total volume should be 2500 µL

Procedure: Th-T solution 5mM NaCl and pH 7 for use at 25^{o}C

Th-T should be in a concentration of 35.7 µM

1. Take a 15 mL tube and fill with 2407.87 µL Tris H-Cl pH 7 for use at 25^{o}C
2. Add 2.27 µL 4400 mM NaCl
3. Add 89.29 µL 1 mM Th-T stock solution

Total volume should be 2500 µL

CE Protocol

Capillary length

Cut capillary to appropriate length (31 cm) using jig

Separation Sequence

1. Rinse capillary with DI water for 10 minutes at 50 psi
2. Rinse capillary with 100 mM Tris for 2 minutes at 20 psi
3. Inject sample into capillary at 0.7 psi for 8 seconds
4. Separate at 7 kV for 60 minutes

*When programming the CE all steps should be done reverse so that separation occurs in the short length of the capillary.

A new capillary should be used before each time course.