5-2012

Using capillary electrophoresis to detect amyloid protein aggregation

Jennifer Kurtz
University of Arkansas, Fayetteville

An Undergraduate Honors College Thesis

in the

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

by

Jennifer Kurtz

This thesis is approved.

Thesis Advisor:

Chris N. Hu

Thesis Committee:

Jennifer Kurtz

Using Capillary Electrophoresis to Detect Amyloid Protein Aggregation

May 27, 2012

Table of Contents

Abstract

Alzheimer's disease (AD) is the leading cause of dementia accounting for ½ to ¾ of all cases. AD is characterized by a progressive loss of cognitive function over a span of 10 years, ultimately ending in death. The main possible cause of AD being researched is the formation of soluble, oligomer amyloids. Amyloids are proteins that misfold and aggregate in the brain causing potent neurotoxic effects on neurons in the brain. Microchip electrophoresis and capillary electrophoresis are two potential methods of determining the level of amyloid aggregation. In order to detect at physiological concentrations, covalent dyes must be used to allow the protein to be visualized. However, covalent dyes have been shown to inhibit. The dyes may be inhibiting aggregation in any of the following ways: the attachment site of the dye to a particular group interfering with key reactions, the aromatic structure of FAM acting as an inhibitor, or the dye attachment altering a necessary charged group.

In this study, the model protein albumin was labeled with BODIPY-FL dye and run through a column to separate the labeled protein from the excess dye. However, the albumin and BODIPY would not elute from the column in the original experiment. The albumin and BODIPY were run separately through a much smaller column to test if they were getting stuck in the packing. Based on UV absorbance reading from the column elutions, it was determined that albumin was getting stuck in the column. In order to move forward, a better buffer could be used to push the albumin through the column or a different model protein could be used that didn't get stuck in the column.

Introduction

Alzheimer's disease (AD) is the leading cause of dementia accounting for ½ to ¾ of all cases. AD is characterized by a progressive loss of cognitive function over a span of 10 years, ultimately ending in death. There are two types of lesions thought to characterize AD: neurofibrillary tangles (NFT) and neuritic plaque (1). NFT is characterized by paired helical filaments linked together by hyperphosphorylated tau protein and can be found in axons, dendrites, and the extracellular space in neuropil threads. However, an emphasis is placed on the neuritic plaque formed from amyloid deposition in the cerebral neuropil and vasculature because it has the greatest body of evidence to support it (2). Amyloids are 40-42 residue amyloid β-protein that misfold and aggregate. Amyloids are naturally toxic to neurons, and the rampant formation of amyloids forms plaques causing neurodegeneration (3). There are two known types of amyloids: an insoluble, fibrillar form and a soluble, oligomer form. Originally, studies focused on the insoluble form and their potent neurotoxic effects on neurons. Recently, studies have suggested that soluble, oligomeric Aβ causes substantial neuronal dysfunction which possibly occurs before the appearance of amyloid deposits (2). The formation of oligomers could be the immediate cause of neuronal injury and death. Therefore, it's possible that Aβ fibril formation may be the end stages of the process which leads to AD. However, it is known that fibril and oligomeric Aβ assembly exist in equilibrium and the activity of one structure can affect the other. Studies have suggested that neuritic plaque formation may be the end stages of a process that are facilitated by oligomer formation.

Past studies have shown there is a much greater correlation to AD in the area of the plaque deposit as opposed to the amount of fibrillar Aβ present. Levels of Aβ in the etorhinal cortex and in the frontal lobe have been correlated with cognitive impairment (2). Progression from normal to AD correlates to a consistent and significant increase in the soluble and insoluble

forms on Aβ. Wang *et al*. proposed a significantly increase in insoluble and soluble forms of Aβ in AD patients. Oligomers were then determined to be worthy of studying as a critical early cause of AD (2). McLean *et al*. published a study correlating the level of amyloids with AD onset (2). The level of insoluble Aβ discriminated from the control and did not produce a clear correlation. However, the amount of soluble Aβ in confirmed cases of AD was three times as much as the control group. The amount of oligomers was the most significantly discriminatory hypothesis tested (1). According to studies using mice and rats, oligomeric Aβ injected intracerebrally caused significant inhibition of hippocampal long-term potentiation (LTP) where the insoluble Aβ had no effect. The findings of this study suggest that oligomeric assembly may occur before and independently of amyloid deposits (1).

Capillary electrophoresis (CE) and microchip electrophoresis are two methods to detect the level of Aβ oligomers when making the process as close to physiological conditions as possible. Capillary electrophoresis is an inexpensive and efficient way to separate oligomers based on their charge, shape, and size, known as electrophoretic mobility. CE can be used to monitor the appearance of oligomers over time when aggregation is occurring (4). A basic model of capillary electrophoresis is shown in Figure 1. The source vial, capillary, and the destination vial are filled with an aqueous buffer solution. The capillary is placed into the sample vial containing the protein then returned to the source vial. The protein movement into the destination vial is initiated by the electric field supplied by the high voltage power supply. The protein separates during migration due to their electrophoretic mobility and is detected near the destination vial. The computer reports the responses as a function of time where compounds will show up as peaks with different migration times.

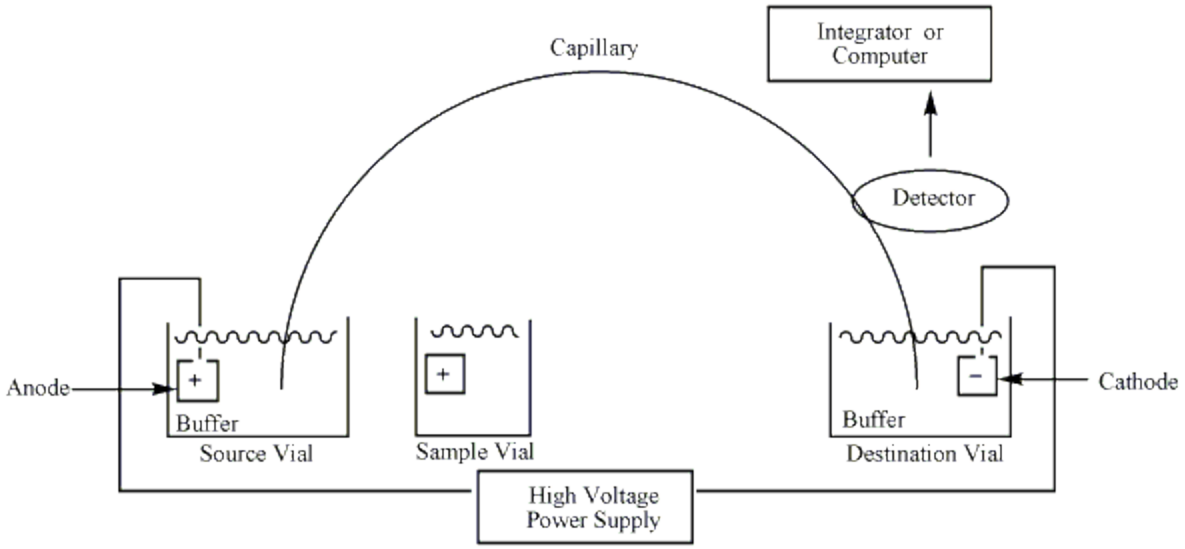

Figure 1. Diagram of the capillary electrophoresis process (8).

Microchip electrophoresis is a newer process than capillary electrophoresis. Microchip electrophoresis is less time consuming because it provides automation capabilities and rapid separations due to higher electric fields and shorter column lengths (5). The microchip system is shown in Figure 2. The laser detects the oligomers on the chip and the information is signaled to the spectrometer. Specific sensors are required in both methods to detect oligomer formation. These fluorescent dyes bond covalently to the oligomers and allow for the oligomers to be visualized by computer/laser.

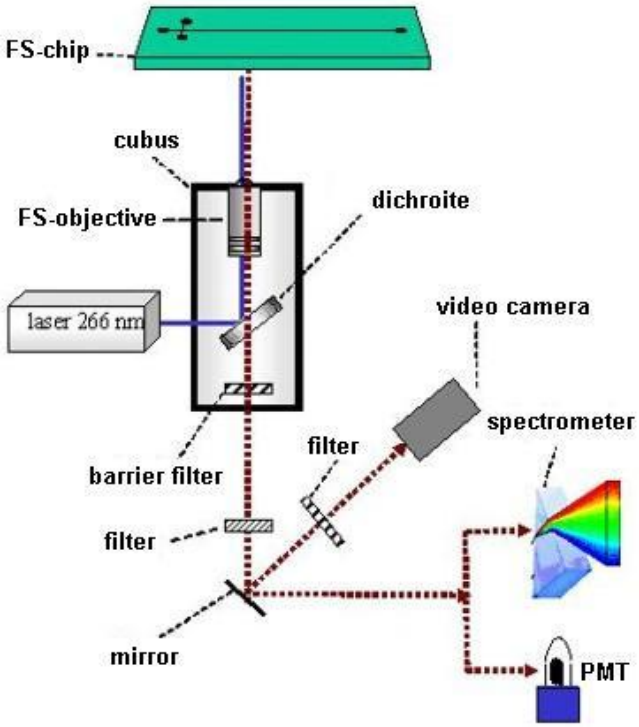

Figure 2. Diagram of the microchip electrophoresis process (9).

However, covalent dyes have been shown by Pryor *et al.* to inhibit aggregation due to no changed observed in the migration time when using FITC to label insulin and FAM to label Aβ (4). Possible reasons for the aggregation of inhibition could be due to: the attachment site of the dye to a particular group interfering with key reactions, the aromatic structure of FAM acting as an inhibitor, or the dye attachment altering a necessary charged group.

FAM is known to attach to amide groups, which is a common attachment site for proteins also. Previous work suggests a long aggregation lag time after methylation of amino groups in Aβ (6). The attachment site hypothesis can be tested by labeling Aβ using AMCA hydrazide with EDC which attach carboxyl groups (4).

Fluorescent dyes are largely aromatic so, it is possible that the size of the polyaromatic compounds is inhibiting Aβ aggregation. Previously, small polyaromatic compounds have been

proposed to inhibit β-sheet formation by Walsh *et al*. (7). It is likely that the same concept is inhibiting the oligomer formation and that less bulky fluorescent dyes may reduce the inhibition of Aβ. The ATTO series of dyes range from three rings to six rings and can be used to test the aromaticity inhibition hypothesis (4).

The covalent dyes typically attach to a charged group, altering the native charge of the protein. If this charged group is necessary for aggregation, it could be concluded to be the reason for aggregation inhibition. The dye CE 503 can be used to test the native charge of the protein since it attaches to an amide group, but maintains the native positive charge (4).

Dye attachment sites are the main focus of this paper. A model protein, albumin, was used to determine how to perform dye labeling reactions. FAM-Aβ used as a negative control and unlabeled Aβ used as a positive control, while testing the effects of BODIPY-Aβ and AMCA Hydrazide Aβ. Understanding the relationship between dye attachment sites to Aβ, dye aromaticity, and native Aβ charge will allow for better design of dyes to label Aβ without inhibiting aggregation. In this work, we used a model protein (albumin) to determine if the dye reaction worked properly and if the protein with the dye attached could be purified from excess due in a packed column.

Methods

The method detailed below includes information on the reaction between the dye and the albumin protein as well as the purification protocol for the packed column.

1. Prepare the stop reagent for the BODIPY dye protocol fresh before each use. Dissolve 210 mg of hydroxylamine in 1mL de-ionized water.
2. Adjust the pH to 8.5 using 5M NaOH.

3. Dilute the solution to 1.5 M by adding 250 µL of the hydroxylamine solution to 250 µL of de-ionized water.
4. Dissolve ~1 mg of protein (albumin) in 133 µL of 200 mM sodium bicarbonate buffer (or 7.5 mg/mL). The protein concentration should be 5-20 mg/mL.
5. Dissolve BODIPY-FL dye in DMSO to 10 mg/mL immediately before starting the reaction. BODIPY-FL stocks are stored at -20ºC.
6. While stirring the protein solution from step 4, slowly add the solution from step 5 in the following amounts:

   1:17 (protein:dye)= 130µL 7.5 mg/mL albumin +12.38 µL 10 mg/mL dye stock + 57.62 µL sodium bicarbonate

7. Incubate for 1 hour at room temperature with continuous stirring.
8. Make 1.5 M hydroxylamine by dissolving hydroxylamine hydrochloride in distilled water at 210 mg/mL and adjusting the pH to 8.5 with 5 M NaOH. Dilute the resulting 3M concentration to 1.5 M by adding an equal volume of distilled water.
9. Stop the reaction by adding 18.2 µL of 1.5M hydroxylamine, pH 8.5, to a final concentration of .125 M and incubate for one hour at room temperature.
10. Weigh out 4 g of dry sephadex for a 6mL swelled/g dry resin ratio for a 24 mL bed volume.
11. Add enough buffer to equal total volume of the column plus 30%. Allow the sephadex to swell for 3 hours then decant the supernatant.
12. Add buffer to make a 75% suspension.

13. Degas the suspension before packing
14. Pour the slurry into the column in one portion, being careful not to trap air bubbles.
15. Add the BODIPY- conjugated protein solution dropwise into the column. Collect a fraction for every 5 drops (~ 250 µL).
16. Take a Nanodrop reading at A_{280}(protein absorbance) and A_{504} (dye absorbance) to determine which fractions contain protein and which contain unconjugated dye. Determine the degree of labeling (DOL). A DOL between 3-8 is desirable.
17. Store the protein conjugates dessicated at -20ºC.

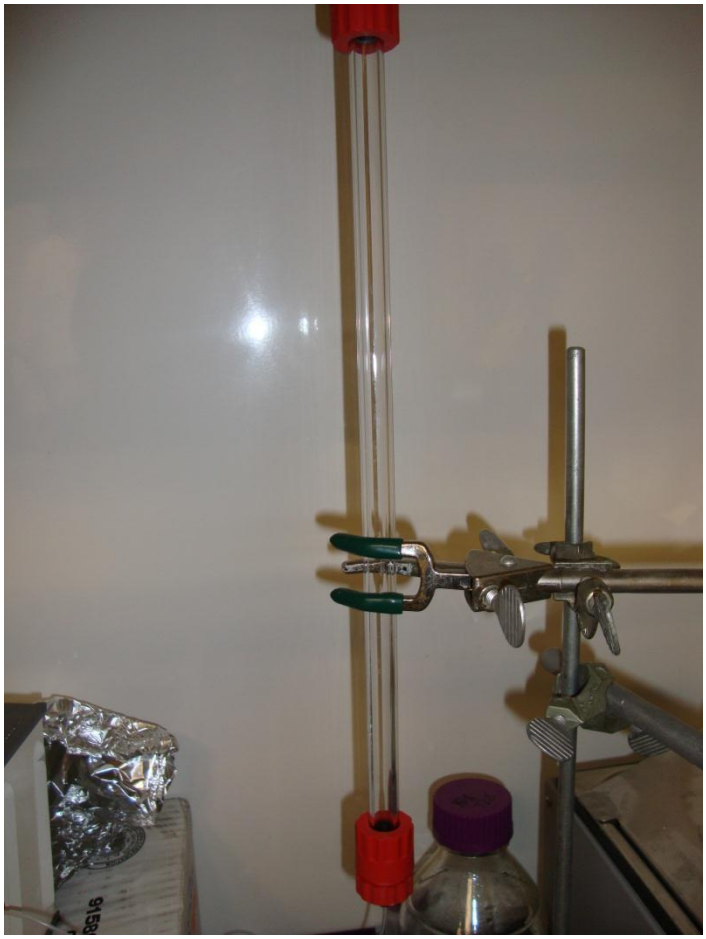

Figure3. Column used to separate excess and labeled protein.

Results

After running the fractions on the UV spectrometer (Nanodrop), none of the readings absorbed at a wavelength of 280 or 504 as expected. To determine if the solution was getting stuck somewhere in the column, a protein solution and a dye solution were run separately through smaller (~8 cm) columns. Fifteen fractions were collected for the dye solution. The buffer to push the solution through the column for the first 7 fractions was sodium bicarbonate and the final 8 was sodium hydroxide. A peak at a wavelength of 504 started to form at the 6th fraction and the absorbance increased for each remaining fraction. The buffer didn't seem to have an effect on the ability for the dye to elute through the column. The protein solution also had 7 sodium bicarbonate fractions followed by 8 sodium hydroxide fractions after being added to the column. However, no peak was noticed at a 208 wavelength. So, it is determined that the albumin was getting caught in the column.

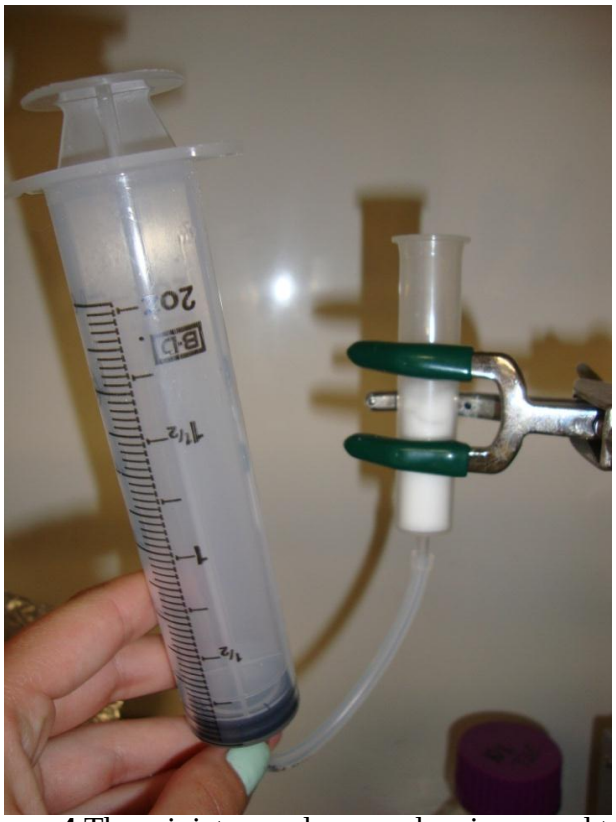

Figure4. The miniature column and syringe used to collect fractions.

Discussion

Since the readings didn't absorb at the expected wavelengths, this suggests that the protein and dye are not eluting from the column. It is possible the protein and/or the dye are getting stuck in the sephadex, or the wrong buffer is being used to push the solution through the column. Since the albumin is not eluting from the column, a different protein could be used to bind with the dye. More fractions could be taken with the albumin and BODIPY solution, but when scaling this process up to about a 30 cm column, pulling the solution through the column could take a considerable amount of time.

Conclusions

Consequently, the albumin is getting stuck in the larger column and must elute before being able to determine the effect of dye attachment sites to Aβ. In order for the protein to elute from the column, the protein can be changed to something other than albumin or different buffers can be tested to see if they could better push the albumin through the column. Once the excess dye and labeled protein are separated using the sephadex column, then they can be run through the capillary electrophoresis to determine the effect of dye attachment sites.

Recommendations

It is recommended to try both, different dyes and different proteins, to determine if they get stuck in the sephadex column. This will allow for the optimum combination to run through the capillary electrophoresis.

Acknowledgements

I would like to acknowledge Dr. Christa Hestekin and Elizabeth Pryor from the Ralph E. Martin Department of Chemical Engineering at the University of Arkansas for their help with this project.

References

1. Walsh, D. M. (1999). Amyloid B-Protein Fibrillogenesis. *The Journal of Biological Chemistry*, *274*(36), 25945-25952.
2. Kirkitadze, M. D., Bitan, G., Teplow, D.B. (2002). Paradigm Shifts in Alzheimer's Disease and Other Neurodegenerative Disorders: The Emerging Role of Oligomeric Assemblies. *Journal of Neuroscience Research*, *69*, 567-577.
3. Klein, W. L. (2001). Targeting small ab oligomers: the solution to an alzheimer's disease conundrum?. *TRENDS in Neurosciences*, *24*(4), 219-224.
4. Pryor, E., Kotarek, J., Moss, M., & Hestekin, C. (2011). Monitoring insulin aggregation via capillary electrophoresis. *International Journal of Molecular Sciences*, (12), 9369-9388.
5. Osiri, J., Shadpour, H., & Soper, S. (2010). Ultra-fast two-dimensional microchip electrophoresis using sds m-cge and microemulsion electrokinetic chromatography for protein separations.
6. Lee, Y.-H.; Maus, R.G.; Smith, B.W.; Winefordner, J.D. Laser-induced fluorescence detection of a single molecule in a capillary. *Anal. Chem.* **1994**, *66*, 4142–4149.
7. Walsh, D.; Hartley, D.; Condron, M.; Selkoe, D.; Teplow, D. *In vitro* studies of amyloid β-protein fibril assembly and toxicity provide clues to the aetiology of flemish variant (Ala692 → Gly) Alzheimer's disease. *Biochem. J.* **2001**, *355*, 869–877.
8. *Capillary electrophoresis*. (2012, March 12). Retrieved from http://en.wikipedia.org/wiki/Capillary_electrophoresis
9. *Deep uv fluorescence detection*. (n.d.). Retrieved from http://www.uni-leipzig.de/~belder/en/research/fldet.html