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Non-natural protein mimetics for the treatment of Alzheimer's disease

Jennifer Herrera
University of Arkansas, Fayetteville

Amanada Compean
University of Arkansas, Fayetteville

Jasmine Brown
University of Arkansas, Fayetteville

Melissa Moss
University of Arkansas, Fayetteville

Shannon Servoss
University of Arkansas, Fayetteville

Non-Natural Protein Mimetics for the Treatment of Alzheimer's Disease

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Ralph E. Martin Department of Chemical Engineering
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Jennifer Herrera

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Jennifer Herrera

Amanda Compean

Jasmine Brown

Dr. Melissa Moss

Dr. Shannon Servoss

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ABSTRACT:

Alzheimer's disease (AD) is a slow degenerative disease that causes memory loss and eventually leads to death. AD is caused by the aggregation of the amyloid-beta protein, found on the outside of brain cells; once the amyloid-beta protein begins to aggregate it forms plaques on the extracellular part of the neural cells. To date there is no AD medication commercially available that breaks up the amyloid-beta aggregates. Current research has found that certain molecules bind to the amyloid-beta protein and prevent aggregation. The purpose of this research project is to engineer a peptoid-based molecule to stop the aggregation of the amyloid-beta protein. Peptoids have been found to form strong helices, have high bioactivity, and are easy and cost-effective to synthesize. Three peptoids with different chemistries at only the 6th side chain have been designed; the first peptoid is neutral, the second peptoid is positive, and the third peptoid is negative. The binding ability of the peptoids with the amyloid-beta protein will be tested at the University of South Carolina in the laboratory of Dr. Melissa Moss. Currently the peptoids have been synthesized and are in the process of purification and characterization. The peptoids binding ability with the amyloid-beta protein will provide important information regarding their use for the therapeutic treatment of AD.

Introduction and Background:

Alzheimer's disease (AD) was discovered in 1906 by Alois Alzheimer. AD is characterized by memory loss, confusion, and cognitive disabilities and is usually found in people 65 years old and older; the chance of being affected by Alzheimer's increases with age. There are three stages to Alzheimer's disease, with the third stage involving the shutdown of all body functions and causing death. According to the National Center for Health Statistics, AD is one of the top 10 causes of death in the United States. The question many doctors and researchers are asking is what exactly causes AD?

Through experimental data it was discovered that the amyloid-beta protein found in the extracellular part of the brain cells could be related to AD.[1] Investigative research found that all diagnosed AD patients had extracellular formation of amyloid-beta plaques. Further research found that the amyloid-beta protein is harmless in the monomer form, but once it begins to aggregate into protofibrils and mature amyloid-beta fibrils it becomes toxic and begins to form plaques affecting the surrounding neural cells, as shown in figure 1. It is unknown at which stage the aggregates become toxic, but they have clearly been associated with AD. Currently there is no available medication that can stop the aggregation of the amyloid-beta protein; medications such as tacrine (Cognex), donepezil (Aricept), rivastigmine tartrate (Exelon), and galantamine (Reminyl) only relieve or slow down the symptoms. With the hope to find an anti-aggregation medication for AD, this research project targets finding a potential candidate that will prevent the aggregation of amyloid-beta protein.
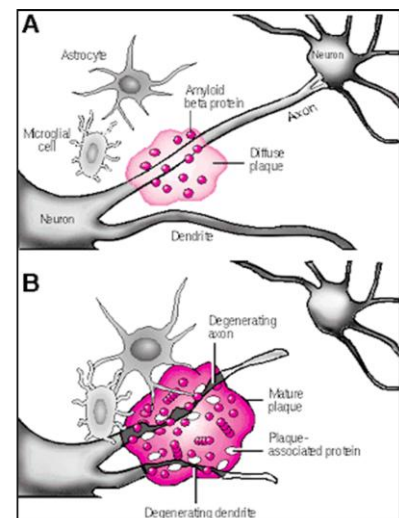

Figure 1. Amyloid-beta aggregation. The amyloid-beta aggregation is shown from the beginning in part A to how its continuous aggregation begins to deteriorate the neural cell in part B.

According to Dr. Melissa Moss at the University of South Carolina, it has been found that endogenous proteins bind to various assembly forms of amyloid-beta, including monomer, protofibril, and fibril, serving as carrier proteins as well as inhibitors for their self association (unpublished data). Current researchers have used nordihydroguaiaretic acid, small heat shock proteins, and sialic acid in search for a solution to the amyloid-beta aggregation.[2,3,4] None of the molecules mentioned above occur naturally in the body, and in consideration of this fact some researchers are analyzing peptides. Peptides are short amino acid chains at about 50 amino acids in length and can be found in insulin, milk, and DNA/RNA processes.[5] The potential versatile use of peptides in the body has made them strong candidates to use for disease therapy including AD.

The use of peptides for disease therapy is also known as peptide therapeutics, and it is currently being considered for an advancement in finding medication that could slow down the progression of AD or stop the disease overall. Peptides are particularly suitable for this research due to their ease of synthesis, their harmless degradation products and the fact that they are already recognized by the body. The key disadvantages to using peptides are that some can aggregate irreversibly, have potential for an immune response, denature easily, and most importantly they have low bioactivity since they are degraded by proteases and so a higher dosage has to be used to counter this effect. Taking into account these disadvantages a peptide mimic, known as a peptoid has been engineered and is believed to surpass the peptide disadvantages therefore, making it more desirable as a therapeutic for AD.

Poly-N-substituted glycines, or peptoids, differ from peptides in their molecular structure as shown in figure 2.[6,7] The peptoid side chain is attached to the amide nitrogen rather than the alpha-carbon, as in peptides. This relatively simple backbone change leads
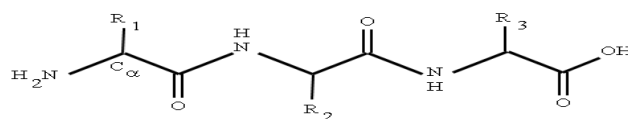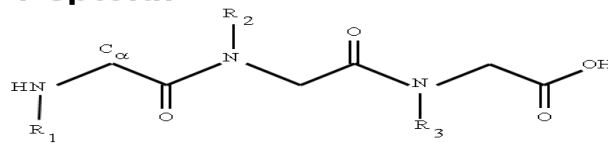

Figure 2. Peptide and Peptoid Sketch. This figure shows a comparison of the peptide and peptoid backbone structures.

to a lack of hydrogen bond donors and chirality in the backbone. Despite these changes, properly designed peptoids are able to exhibit stable helical structures as shown in figure 3.[8]
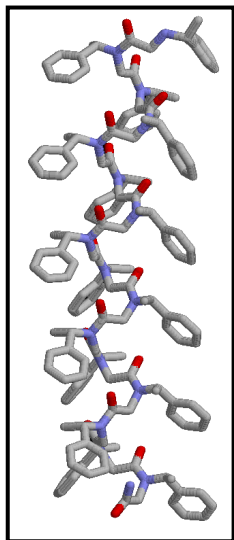
The stable helical structures made by peptoids exhibit no denaturation at 75°C in a 8M Urea solution, most likely because they are stabilized by steric and electronic repulsions.[9] Peptoids are also relatively easy and cost-effective to synthesize and have a higher diversity than peptides.[10,11] They are not degraded by proteases, and therefore have high bioavailability.[12] In addition, they are less likely to trigger an immune response and have been shown to be non-toxic.[13] For all of these reasons, peptoids show great promise for AD therapy.

The key factor for a successful outcome is being able to synthesize a peptoid structure that will bind to the amyloid-beta protein and prevent its aggregation. Once this has been accomplished more research would be conducted to determine how the peptoid will be administered to the patient and the dosage that will be needed. If successful, the peptoid designed through this research will lead to a breakthrough in creating an anti-amyloid beta aggregation medication that can potentially slow down or stop Alzheimer's disease.

Figure 3. Peptoid helical structure. This figure demonstrates the type of stable helical structures peptoids can form.

Methods and Materials:

The primary focus is to design a peptoid that can bind to the protofibril or the mature amyloid-beta protein and prevent the continuous aggregation and the formation of the extracellular plaque in the neural cells. For every peptoid synthesis a 12 step procedure is followed which involves the use of characterization and purifying equipment. The procedure, equipment, and peptoid designs are discussed in this section.

The peptoid procedure is shown in the table below:

Table 1. Peptoid Experimental Procedure

Peptoid Procedure
1. Synthesize peptoid.   2. Conduct a test cleavage on peptoid synthesis.   3. Obtain a MALDI analysis on the test cleavage to confirm if the peptoid is present.   4. Run a test cleavage sample through the analytical HPLC to check purity.   5. Perform a large cleavage.   6. Collect fractions using preparative HPLC.   7. Complete individual MALDI analysis on fractions to confirm peptoid presence.   8. Run fractions through analytical HPLC to check their purity.   9. Combine fractions and conduct analytical HPLC analysis.   10. Repeat steps 5-9 if necessary.   11. Characterize secondary structure using circular dichroism.   12. Send purified peptoid to Dr. Moss's lab to check peptoids binding ability with amyloid-beta protein.

The first step is to synthesize the peptoid using an automated peptide synthesizer. Before the peptoid is synthesized a flow test is conducted on the automated peptide synthesizer to verify the flow rate of the chemicals before the chemistry is inputted in the computer and sent to the synthesizer. Once this has been done the desired monomers can be placed on the synthesizer and the peptoid synthesis is conducted. The synthesis is solid-phase which makes it a stable synthesis. The reaction is started with bromoacetic acid and resin as the reactants. The reactants are then placed in a solution with N,N'-Diisopropylcarbodiimide (DIC) as an activator and Dimethylformamide (DMF) as the solvent. This process removes the hydroxide group from the bromoacetic acid and the hydrogen from the resin to create the peptoid backbone. Then a secondary Nucleophylic Substitution (SN2) reaction is conducted

on the peptoid backbone to replace the bromine with the amine side chain where NMP is used as the solvent. Piperidine is used throughout the peptoid synthesis to remove protective groups from peptoid sequences. This process is a submonomer process which means that this procedure can be repeated until the desired length is obtained. The synthesis process is shown in figure 4:
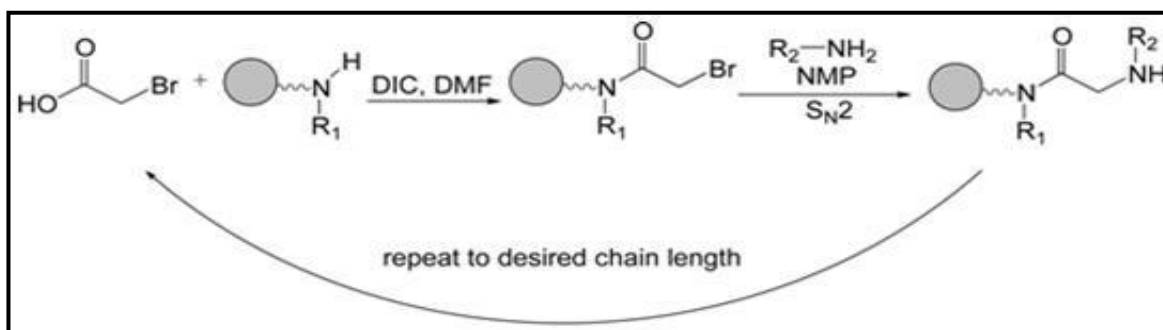

Figure 4. Peptoid submonomer process. The peptoid submonomer process is comprised of two steps.

The synthesized peptoid is attached to the resin and before it is removed from the automated peptide synthesizer it is washed with dichloromethane. The peptoid is placed in a vial where it is labeled and weighed and a one milliliter of trifluoroacetic acid (TFA) mixture composed of 95% TFA, 2.5% of triisopropylsilane (TIS), 2.5% of nano pure water is made to perform a test cleavage. Once the mixture has been made, a test cleavage is performed on about five percent of the total resin collected from the synthesizer. This procedure takes about 5 minutes and then it is drained through a funnel to remove the peptoid from the resin. The cleavage mixture, which now contains the peptoid, is mixed with acetonitrile and water. This solution is frozen, lyophilized, and re-suspended in a 1:1 acetonitrile and water solution.

MALDI and analytical HPLC are then used to determine if the peptoid is present in the sample. MALDI uses a mixture composed of 1 micro liter of the peptoid mixture and 1 micro liter of dihydro benzoic acid (DHB). The mixture is placed onto a plate and ran through MALDI.

Analytical HPLC will determine the purity of the peptoid sample. A gradient of 5-95% acetonitrile in water with 0.01% TFA over 50 minutes is used to separate the sample. For this experiment the analytical HPLC is set to run at two different wavelengths, one is at 254 nm to detect the backbone of the peptoid and the other is set to run at 280 nm to detect the peptoid aromatic side chains. The results from this procedure will help determine what gradient should be used on the preparative HPLC for a fraction collection.

Once the peptoid mass and purity has been confirmed, a large cleavage is done. The large cleavage is conducted in the same manner as the test cleavage; ten milliliters of TFA mixture is mixed with about   to   of the resin. Then the peptoid mixture is drained, mixed with acetonitrile and water, frozen, lyophilized, and re-suspended in a 1:1 acetonitrile/water mixture.

The large cleavage is then used to collect fraction on the preparative HPLC. Using the results obtained from the analytical HPLC a method set is chosen at a 1% per minute rate. Depending on the peptoid molecular weight a certain amount of peptoid mixture is injected into the preparative HPLC (so far 3 milliliters have been used). Individual fractions are collected at 20 second intervals.

Fractions chosen for further analysis are then frozen, lyophilized, and suspended in 1:1 acetonitrile and water solution. The individual fraction mixture is then prepared for a MALDI run in the same manner it was done for the test cleavage. The results of this MALDI run will determine in what fractions the peptoid is located. The fractions where the peptoid is located are then prepared for an analytical HPLC run.

The same analytical HPLC method set, procedure, and gradients are used in this step as the one used for the test cleavage. The individual fraction run will determine how pure the fraction is. The fractions with purity less than 97% will be combined for further purification and fractions with purity greater than 97% will be combined and sent for further analysis.

After the desired amount of peptoid is obtained, the peptoid will be run through circular dichroism to characterize its secondary structure. Once this step is completed the final step of the peptoid procedure can be done, which is the shipment of the peptoid to Dr. Melissa Moss's lab at the University of South Carolina for further analysis on the peptoids binding ability with the various forms of the amyloid-beta protein. Depending on the results obtained from Dr. Moss's lab the peptoids will be redesigned and the peptoid procedure will be followed until a suitable peptoid structure is found. Currently there are 3 peptoid designs created by Dr. Shannon Servoss and Dr. Melissa Moss based on peptides currently being investigated for use as aggregation inhibitors (unpublished data). The peptoid designs are discussed in the results and discussion section.

The procedure presented in this section varies from peptoid to peptoid especially if step 10 applies. Troubleshooting procedures for the equipment used in this project are not presented in this report.

Peptoid Design and Result Discussion:

The 3 peptoid structures, sequences, and molecular weights are presented below. The results obtained from the peptoids syntheses are also discussed in this section.

-Peptoid Design:

The 3 potential peptoid candidates are shown in Figure 5 and they are presented in the order in which they were synthesized, their structure, molecular weight, and sequence. Peptoid 1 is abeta-1, peptoid 2 is abeta-2, and peptoid 3 is abeta-3. The 3 peptoid designs contain the following side chains; K represents N-lysine its chemical name is tert-Butyl N-(4-aminobutyl)carbamate, F represents N-phenylalanine its chemical name is methyl-benzyl amine, and L represents N-leusine its chemical name is sec-butyl amine.
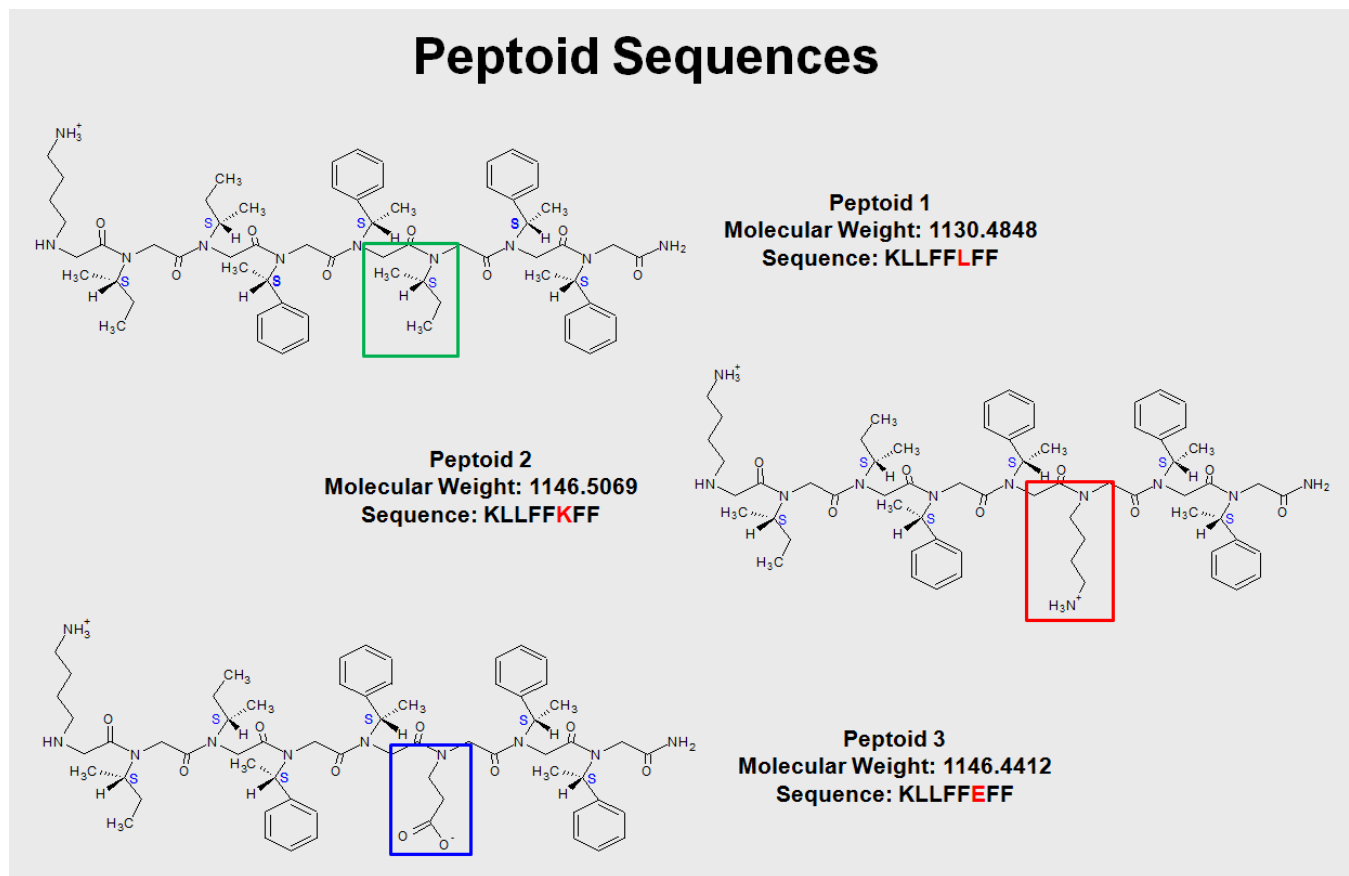

Figure 5. Peptoid Sequences. These are the potential peptoid amyloid-beta aggregation inhibitors.

As it can be noted the peptoids have been designed to have sequences that differ only at the 6th side chain: the first peptoid will have a neutral side chain, the second peptoid will have a positive side chain, and the third will have a negative side chain. The red highlighted letter in the sequence corresponds to the peptoid's 6th side chain. These peptoid structures are further analyzed into faces which are presented in figure 6. Each peptoid contains three faces which are grouped from left to right; each phase is composed of a red box and a blue box. Molecules containing benzene rings have been shown to interact with amyloid-beta aggregates and that is why the peptoids were designed to contain two faces that contain two benzene rings each. Phenylalanine side chains have been added to each peptoid to form the helical structure and each peptoid contains at least six (S) stereogenic centers to increase the chances of the peptoid interacting with amyloid-beta aggreagates.
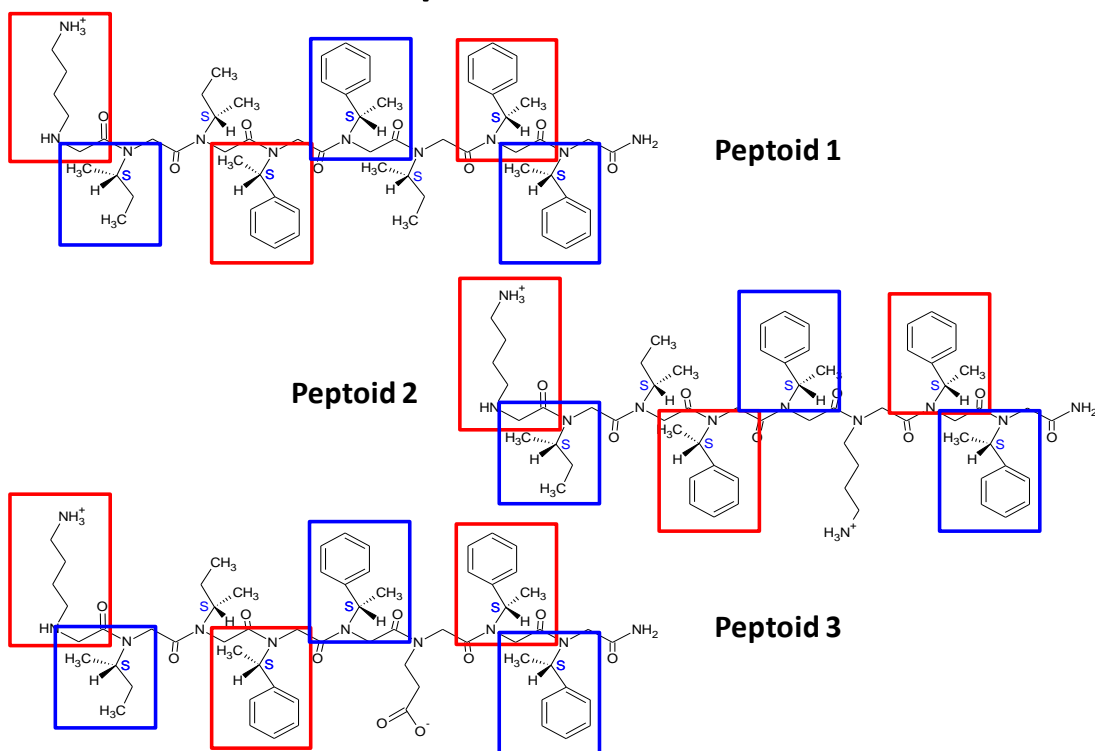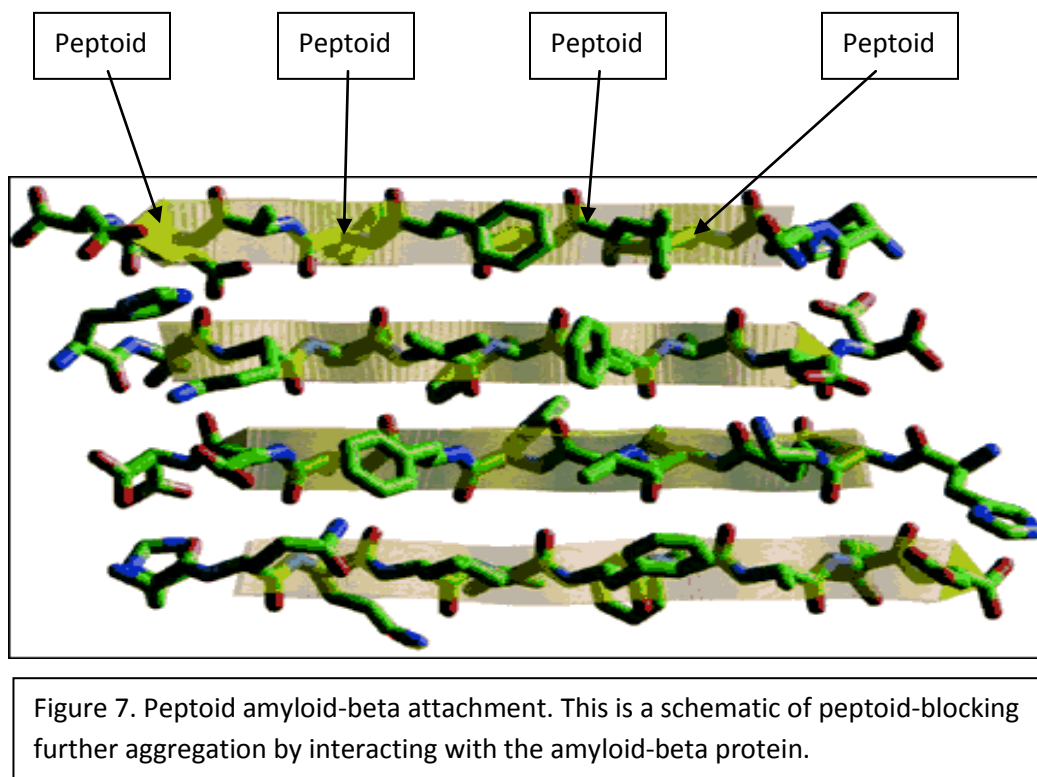
Peptoid Phases

Figure 6. Peptoid faces. Each peptoid face is composed of a red and blue box.

The desired peptoid would attach itself to the amyloid-beta fibril and inhibit its continuous aggregation; a schematic is presented in figure 7 depicting the sites where the peptoid would attach itself in the amyloid-beta fibril. At this point of the research it is unknown whether the peptoid will break up aggregates previously formed, or just prevent further aggregation. If the peptoid can break up aggregates previously formed it could possibly reverse some of the damages. If it just prevents further aggregation it will help slow down or even stop the disease from causing more damage.

Figure 7. Peptoid amyloid-beta attachment. This is a schematic of peptoid-blocking further aggregation by interacting with the amyloid-beta protein.

The peptoid designs presented are subject to modification once they have been analyzed in Dr. Moss's lab. So far all 3 peptoid sequences have been synthesized and the data collected for every individual peptoid is presented below.

-Result Discussion:

The first peptoid, abeta-1, was synthesized 3 times. A test cleavage was done all three times and MALDI was performed on those test cleavages. The most recent MALDI analysis for abeta-1 is presented

in the figure below; as it can be noted the molecular weight for abeta-1 was not found in the MALDI

sample, the same result was obtained for the other two syntheses. For the first two abeta-1 synthesis it

was confirmed that there was something wrong with the automated synthesizer. A possible explanation

for abeta-1 not showing up on the third MALDI run is that maybe some of the sequences did not attach

to the peptoid, other compounds could have attached themselves to the peptoid, or the automated

peptide synthesizer may have had some problems. The abeta-1 test cleavages were run through the

analytical HPLC, and the analysis showed that the sample contained other compounds that were not

abeta-1. For the first abeta-1 test cleavage fractions were collected on the preparative HPLC to test how

well the machine was working but no further analysis was done on the fractions since the previous tests

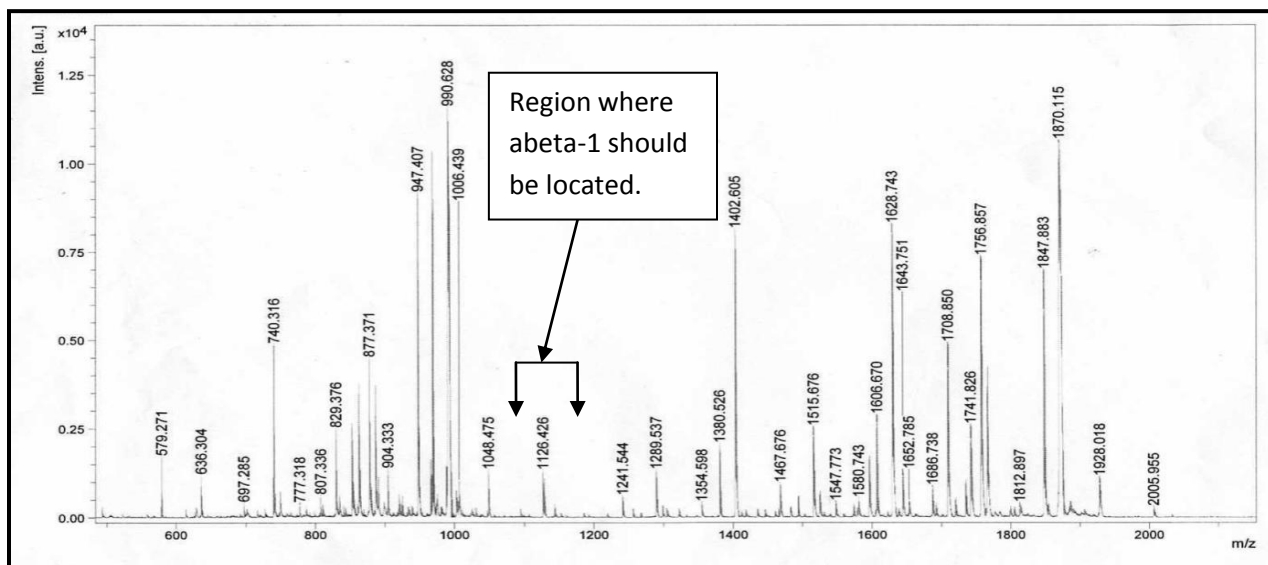did not show presence of abeta-1.

Figure 8. Abeta-1 test cleavage MALDI analysis.

Abeta-1 was synthesized for the fourth time and currently there was a test cleavage performed.

MALDI will be run soon to confirm presence of abeta-1. For this synthesis the automated peptide

synthesizer was having issues with the needle, but the synthesis was carefully observed so there is still a

chance that abeta-1 will be found in this synthesis.

Abeta-2 has been synthesized two times so far. On the first MALDI analysis it was observed that there were compounds present that had a molecular weight close to abeta-2's molecular weight and it was speculated that some small molecule could have attached itself to abeta-2 so it was decided that abeta-2 would be further analyzed. Figure 9 shows the analytical HPLC run conducted on the first abeta-2 synthesis and based on the 254 nm and the 280 nm wavelength contrast it was speculated that abeta-2 would be found in the green region.
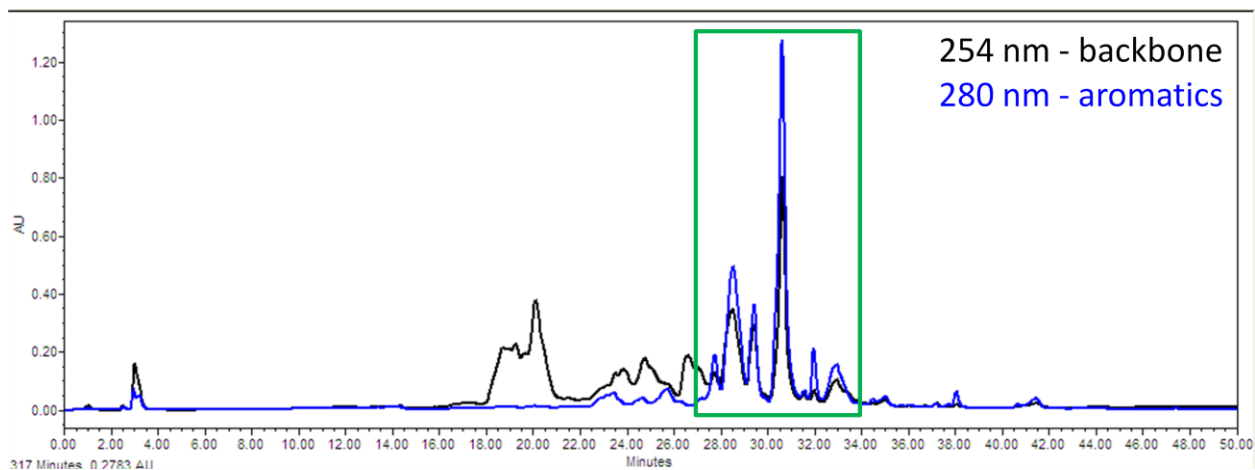

Figure 9. Abeta-2 analytical HPLC run.

After the analytical HPLC result, a large abeta-2 cleavage was done and fractions were collected on the preparative HPLC. About 70 fractions were collected, but only fractions 53-61 were further analyzed since those were the fractions that collected material from the desired region shown in figure 9. Fractions 53-61 were ran through the analytical HPLC to check their purity. The figure below shows the analytical HPLC run for fractions 53-56, in this figure fraction 56 shows the highest purity since it has the highest peak and the smallest amount of surrounding peaks. MALDI was run on these fractions and it was found that abeta-2 was not present or any molecular weight close to abeta-2.
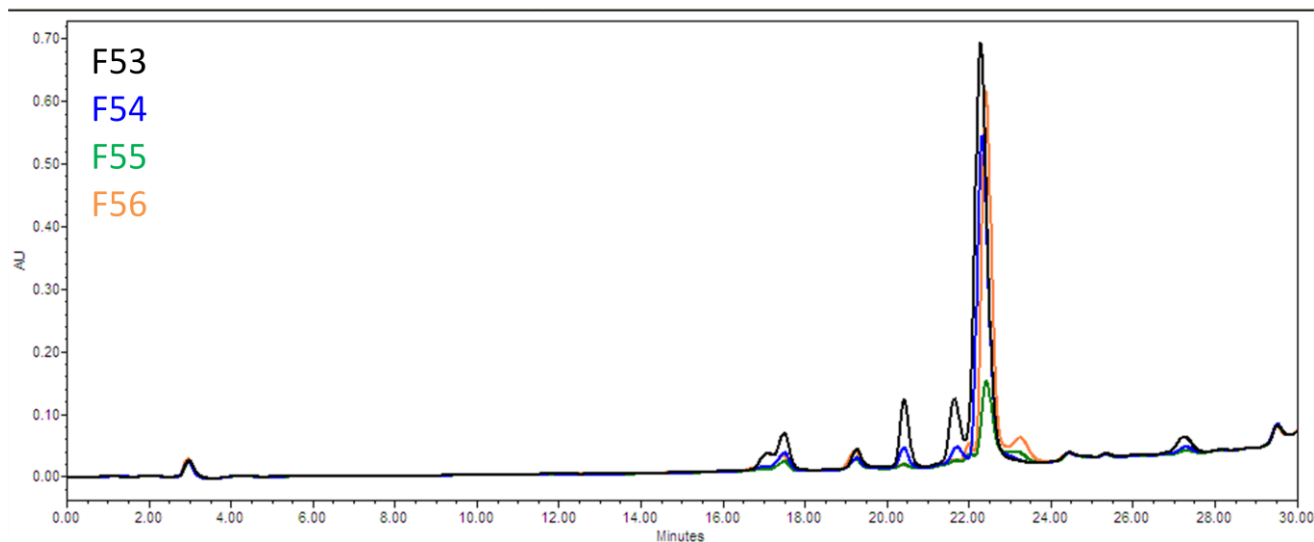
Figure 10. Abeta-2 fractions 53-56 analytical HPLC run.

Figure 11 shows Abeta-2's fractions 59-61 analytical HPLC run, as it can be seen fraction 61 is barely noticeable as for fraction 59 and 61 it can be noticed that there are two peaks present for both fractions. Before fractions 59-61 were further analyzed a MALDI analysis was done and the same conclusion was made as for fractions 53-56, abeta-2 was not present. Abeta-2 was synthesized once more and a MALDI analysis will be done on a test cleavage to confirm its presence in the sample.
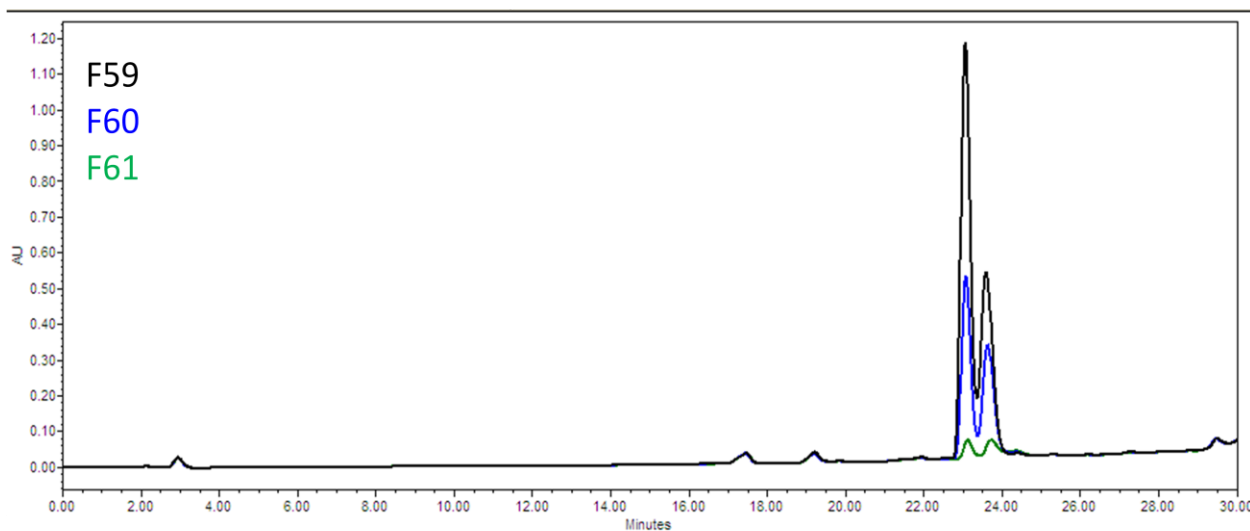

Figure 11. Abeta-2 fractions 59-61 analytical HPLC run.

The problems encountered with the synthesis of abeta-1 and abeta-2 led to an investigation to determine if something was not working properly and/or human error was involved. The notes taken during the peptoid procedure were revised and it was found that in 2 occasions 7 side chains were attached to the peptoid instead of 8 as shown in figure 5, and in another instance the wrong method set was used on the automated peptide synthesizer, which led to the machine not delivering enough bromoacetic acid for the reaction to take place. It was also discovered that the wrong wavelength (254 nm) has been used on the analytical HPLC to detect the peptoid backbone but it has been changed to the correct wavelength which is 214 nm. Once these errors were discovered a higher emphasis was put in making sure everything was done properly and the chosen method set was checked before running a synthesis. More time was allowed to observe each synthesis, meaning that if a synthesis would take 18 hours it would have to be started in the morning to be able to keep the synthesis under observation. Another error that was discovered was that chemicals with the wrong stereochemistry were being used and reminders were put in place to make sure the right chemicals were used. Other precautions taken included; checking the expiration date of chemicals and checking for precipitates, making sure bromoacetic acid was kept fresh for every synthesis, storing chemicals properly to avoid their deterioration, and making sure vials were labeled correctly to prevent more errors. After this was done abeta-3 was synthesized.

Abeta-3 was synthesized and MALDI was performed on a test cleavage. The MALDI analysis shown in figure 12 shows that abeta-3's molecular weight was found. Once abeta-3's molecular weight was found the sample was run through the analytical HPLC and a preliminary assumption was made on the location where abeta-3 would be found. As shown on figure 13, abeta-3 is hypothesized to be located near the highest peak in the figure.
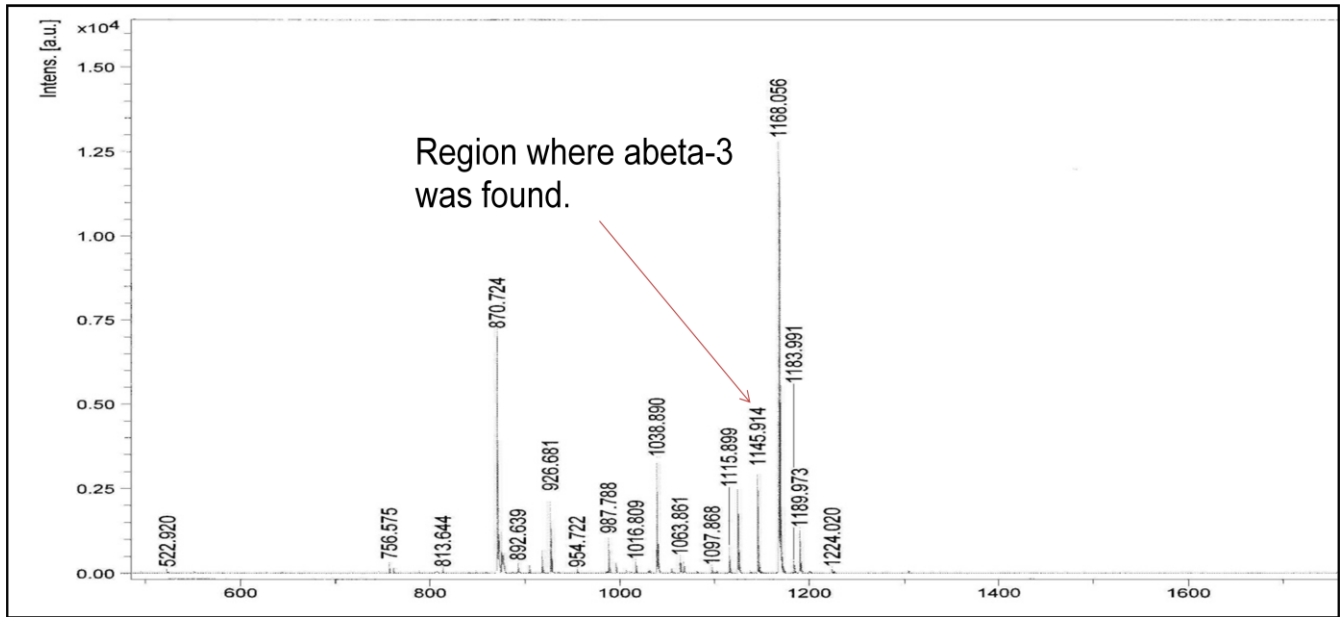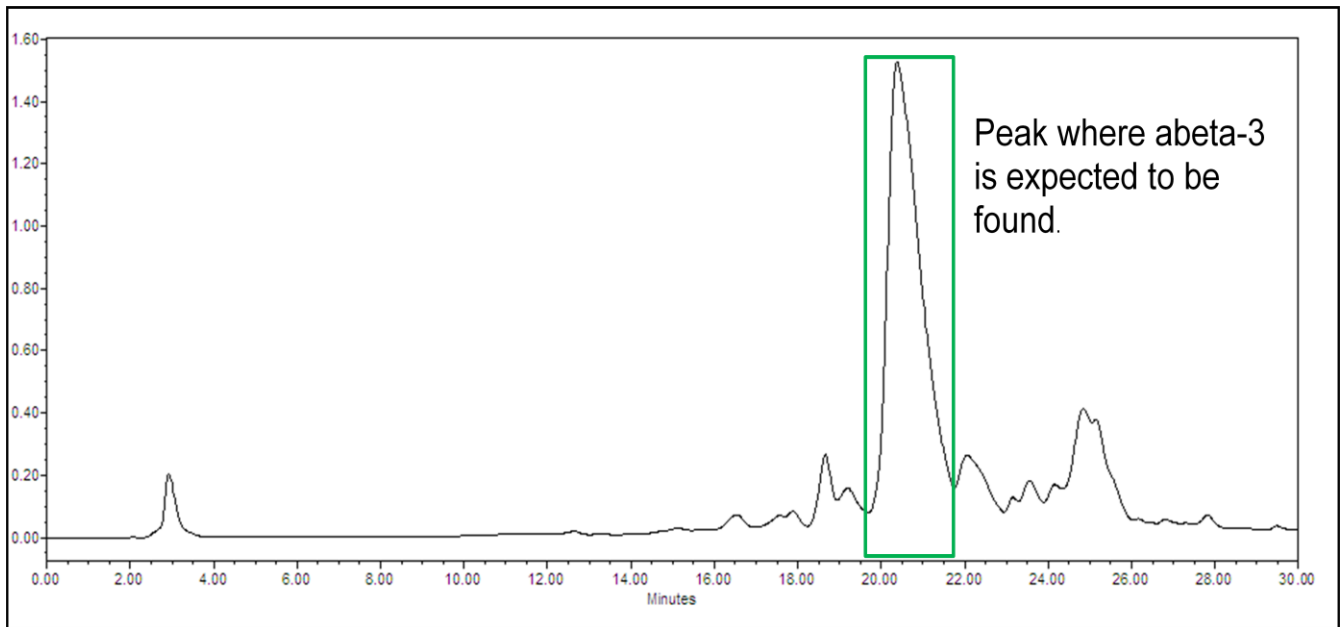
Figure 12. Abeta-3 MALDI analysis.

Figure 13. Abeta-3 analytical HPLC run.

A large abeta-3 cleavage was done and about 50 fractions were collected on the preparative HPLC. The fractions collected around the highest peak in figure 13 were further analyzed through MALDI and six fractions 15, 22-26 showed presence of abeta-3 which are shown in the figures below. These figures were provided by Amanda Compean who is now currently working on the project as well. The purity of the fractions will be checked on the analytical HPLC and then they will be combined and further purified to get a higher abeta-3 concentration. After this is done the purified abeta-3 can be run through circular dichroism and sent to Dr. Moss to check its binding ability.
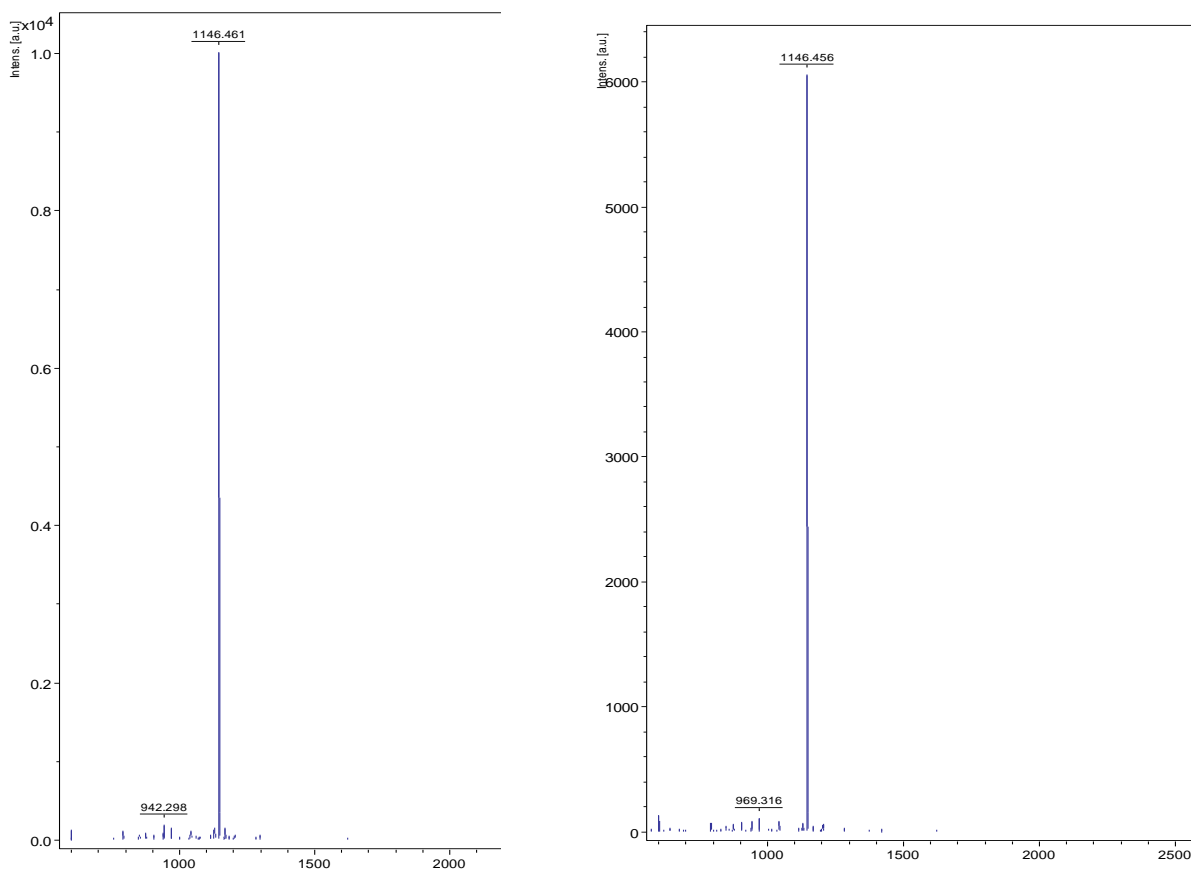

Figure 14 and Figure 15 show the presence of abeta-3 in fractions 15 and 22. Provided by Amanda Compean.
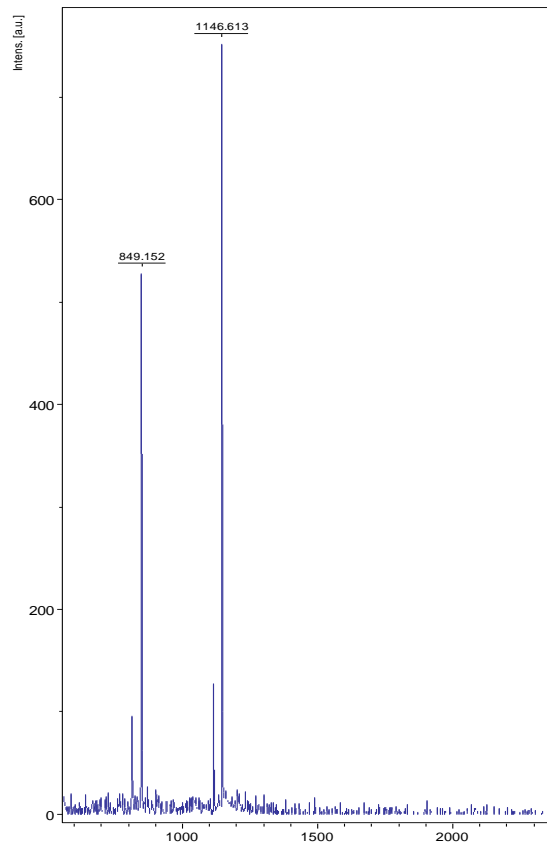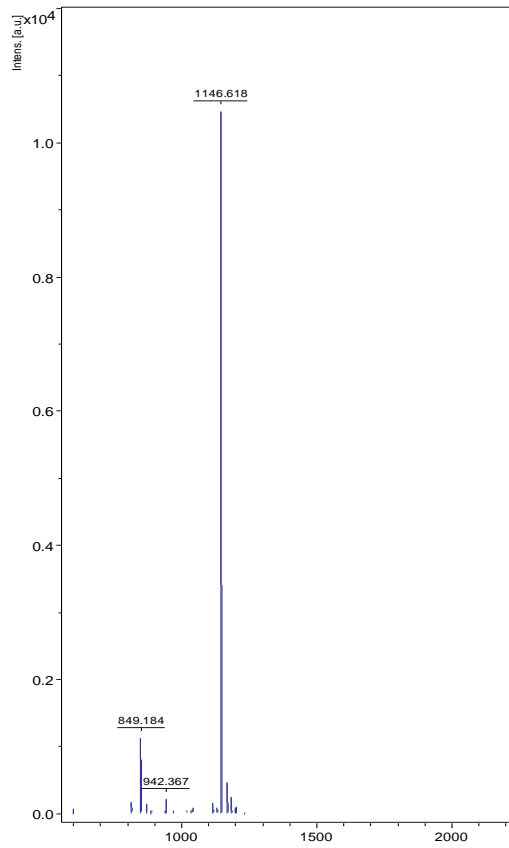
Figure 16 and Figure 17 show the presence of abeta-3 in fractions 23 and 24. Provided by Amanda Compean.
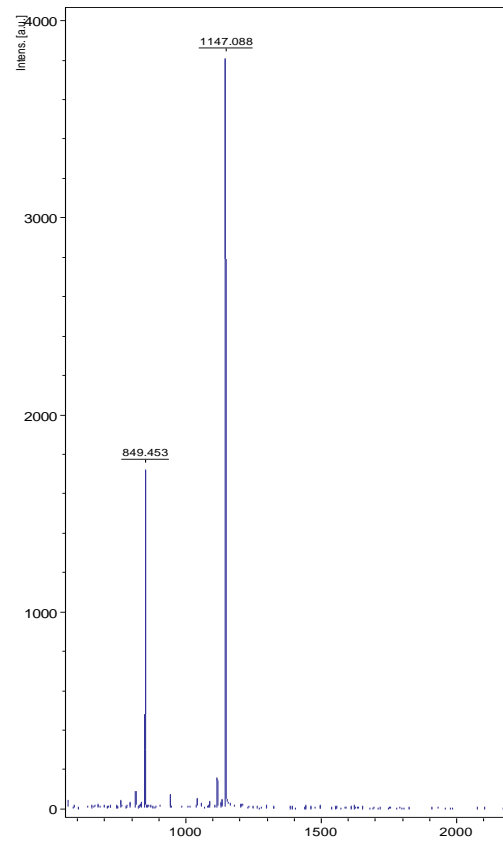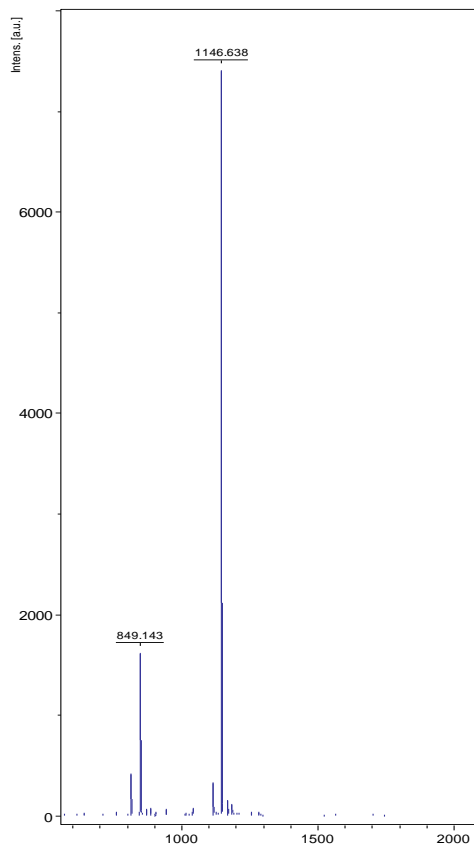

Figure 18 and Figure 19 show the presence of abeta-3 in fractions 25 and 26. Provided by Amanda Compean.

Conclusions:

The number of patients that die every year from AD is increasing which makes this research important and the information obtained vital to find a solution. This research project will test the peptoids therapeutic potential for AD. The treatment found through this research would be applied to AD patients and could possibly be applied to other amyloid-beta linked diseases such as; Parkinson's, Huntington's, and other muscular diseases.

The first abeta-1 and abeta-2 synthesis was not successful but the improvements made in the peptoid procedure through trial and error can assure that the re-syntheses of abeta-1 and abeta-2 will be successful, and that abeta-3 will be the first purified peptoid sent to Dr. Moss. The current goal is to have all 3 peptoids sent to Dr. Moss by summer of 2011, and results can be expected by August 2011. The peptoids will be redesigned/modified based on the results from Dr. Moss. The information obtained on peptoid and amyloid-beta interaction will be published in a scientific paper and can potentially lead to an anti-amyloid-beta aggregate medication that AD patients have been seeking for.

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