Proteomics studies of Alzheimer’s Aβ-oligomers to identify interactions with other proteins in human serum and cerebrospinal fluid.

Rahman Md. Mahafuzur
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Proteomics studier av Alzheimers Aβ-oligomerer för att identifiera interaktioner med andra proteiner i humant serum och cerebrospinalvätska.

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Abstract

Alzheimer’s disease (AD) is the most common neurodegenerative disorder. The pathogenesis of AD is linked to the oligomerization and aggregation of the amyloid-β peptide (Aβ) into protein plaques in the brain. However, the role of these aggregates in the disease pathology remains largely unknown. An important hypothesis today is that neurotoxic oligomeric Aβ aggregates are responsible for cell death. And there might be interaction of neurotoxic oligomeric Aβ with other proteins in human biological fluids that might be involved in amyloid-β neurotoxicity. The project was aimed in identifying which proteins in human biological fluids interact with neurotoxic oligomeric form of Aβ.

A proteomics approach has been developed in which stable oligomers formed by a modified Aβ peptide (Aβ_{42cc}) have been used to capture protein ligands in human serum and cerebrospinal fluid (CSF). The captured proteins have then been separated by gel electrophoresis and characterized by mass spectrometry. Five proteins in human serum have been identified as Apolipoprotein A-I, Apolipoprotein E, Apolipoprotein A-IV, Vitronectin and Antithrombin III. Six CSF samples have been investigated of which three were from AD patients and three from healthy individuals. Four proteins in different CSF samples have been identified as Apolipoprotein A-I, Apolipoprotein E, Clusterin and Gelsolin. Our results show that there are interactions between Aβ_{42cc} oligomers and other proteins in serum and CSF.
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**Abbreviations**

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<tr>
<td>Aβ</td>
<td>Amyloid-β peptide</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>AICD</td>
<td>APP intracellular domain</td>
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<td>Apo A-I</td>
<td>Apolipoprotein A-I</td>
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<tr>
<td>Apo A-IV</td>
<td>Apolipoprotein A-IV</td>
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<tr>
<td>Apo E</td>
<td>Apolipoprotein E</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>Ni-NTA</td>
<td>Nickel-Nitriloacetic acid</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
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<td>TB</td>
<td>Terrific broth</td>
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<tr>
<td>TCEP</td>
<td>Tris-2-carboxyethylphosphine</td>
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<td>TM</td>
<td>Transmembrane</td>
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1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia in the elderly. More than 35 million people worldwide now have AD [1]. If the current numbers hold and no preventative treatments become available, more than 100 million people are expected to have AD worldwide by 2050 [2]. Current treatments of AD are limited and the available cures are not effective.

The disease is characterized by the presence of two abnormal protein deposits: senile plaques and neurofibrillary tangles in the brain. The major component of the senile plaques is the amyloid β-peptide (Aβ) [3], which is the proteolytic cleavage products of the amyloid precursor protein (APP) [4,5]. APP is a transmembrane (TM) polypeptide of 695-770 amino acid residues with three parts: a large extracellular N-terminal region, a TM region and cytoplasmic C-terminal domain [4]. Aβ originates from parts of the TM region of APP (figure 1A) [6]. APP undergoes enzymatic cleavage into two main pathways: nonamyloidogenic and amyloidogenic. In the nonamyloidogenic pathway, α-secretase cuts APP in the middle of Aβ region, which prevents Aβ generation (figure 1B). In the amyloidogenic pathway, β-secretase cleaves APP just before the Aβ domain and produces differently sized Aβ peptides (figure 1C) [6]. The Aβ-peptide is 39-43 amino acid residues in length, among these, Aβ1-40 and Aβ1-42 are most abundant [7,8]. The Aβ1-42 peptide is most hydrophobic, fibrillogenic and more neurotoxic than Aβ1-40 [8]. Aβ peptides are produced both in healthy and AD brains. In healthy brain these peptides are usually
broken down and eliminated. Whereas in AD brain, the peptides undergo conformational changes and accumulate to form hard, insoluble plaques (figure 2).

Figure 1. Amyloid precursor protein (APP) processing with Aβ generation.

(A) The largest isoform (770 residues) of APP with extracellular domain, TM region and intracellular C-terminal domain. Aβ-peptide is partly embedded in TM part. APP undergoes enzymatic cleavage into two pathways, nonamyloidogenic and amyloidogenic.

(B) In the nonamyloidogenic pathway, α-secretase cleaves APP within Aβ-peptide, which results in large soluble APP derivatives called sAPPα and a 83 residues carboxy-terminal fragment called APP-CTFα. The carboxy-terminal fragment is subsequently cleaved by γ-secretase to generate a shortened p3 peptide. The remaining APP intracellular domain (AICD) is released into the cytoplasm. Since α-secretase cuts APP in the middle of the Aβ region this prevents Aβ generation.

(C) In the amyloidogenic pathway, β-secretase cleaves APP just before the Aβ region, which results in soluble sAPPβ peptide. The remaining carboxy-terminal fragment, APP-CTFα or C99 is cleaved by γ-secretase that produces 42-43 residues Aβ-peptide. Similar to nonamyloidogenic pathway, ACID is released to cytoplasm.

Beside AD, amyloidogenic peptides are also involved in several other diseases including Parkinson’s disease (PD), diabetes mellitus and systemic amyloidoses. Aβ peptides can form different conformational products, including soluble Aβ
oligomers and insoluble Aβ fibrils (figure 2) [7]. In AD, the soluble Aβ oligomer aggregartes are more correlated with the pathogenesis than the deposition of insoluble fibrillar Aβ [9].

**Figure 2. Aβ-aggregation in AD brain.**

Aβ-peptide produces in both healthy and Alzheimer’s brain. In Alzheimer's brain, the self-assembly of Aβ-peptide leads to Aβ aggregation and subsequently insoluble plaques formation. A hypothesis suggests Aβ aggregates via two pathways [17]: the β-sheet pathway, involving Aβ aggregation into soluble β-sheet oligomers and then fibril by cross-β packing and the coil pathway, involving aggregation of monomer or disordered low molecular weight oligomers into low molecular weight aggregates (not shown in figure). Neurotoxic Aβ-oligomers are formed via β-sheet pathway.

The amyloid hypothesis and several studies suggest that oligomerization and aggregation of Aβ is linked to the pathogenesis of AD, but the mechanism is not fully understood yet. Extensive research still needs to address many questions including an understanding of the Aβ toxicity mechanism [7].

Aβ oligomers might interact with other biomolecules in human biological fluids and such interactions might be involved in Aβ neurotoxicity. The presence of certain proteins, including immunoglobulins, antitrypsin, serum amyloid-P, acute-phase proteins, cell adhesion molecules and cytokines in the senile plaques of AD brain suggests that these proteins may be involved in the pathogenesis of AD [10,11]. These interactions can be specific or less specific. There might certain epitopes on Aβ oligomers that are capable of specific interactions with other cellular
components. Beside the specific interaction, unspecific interaction can also be directed by surface properties of Aβ oligomers, the hydrophobic surface of Aβ oligomers can be involved in non-specific interactions [12]. Human blood is the most valuable body fluid as blood proteins including albumin, immunoglobulin G and α1-antitrypsin have been purified and used in different therapies [13]. Along with blood, cerebrospinal fluid (CSF) is another important biological fluid. Blood and CSF components could be used as biomarkers of AD [14,15,16].

Figure 3. Engineering of Aβ peptide to Aβcc.
(A) The wild type Aβ40 peptide with a β-hairpin conformation that is observed in complex with an affibody binding protein (left). Engineered Aβcc where the two alanine residues have been mutated to cysteines (Cys21/Cys30) that from a disulfide bond, and this disulfide bond locks the β-hairpin conformation therefore confirm Aβ oligomer stabilization (right).
(B) Transmission electron microscopy images of oligomers of Aβ40cc (left) and of amyloid fibrils formed in presence of TCEP that reduce the cysteine residues and break the disulfide linkage (right). Figures from Sandberg et al. 2010 (figures used with permission) [17].

To study the interactions of Aβ oligomers with other components of blood serum and CSF, Aβ oligomers with a stable structure are required. Neurotoxic Aβ oligomers have been stabilized via protein engineering [17] and double cysteine mutated Aβ (Aβcc) can be produced in Escherichia coli (E. coli) by co-expression with Zab3 affibody [7]. Histidine tagged Zab3 affibody binds to a hairpin conformation of Aβcc and produces Aβcc with an intramolecular disulfide bond so as to avoid its aggregation and remain as a stable complex with affibody (figure 3A) [17]. Different proteomics approaches have been using to study protein-protein interactions.
Currently most of the proteomics technologies are based on mass spectrometry (MS) in combination with liquid chromatography and gel electrophoresis [18,19].

The work plan of this project has been divided into two parts where one is to produce pure Aβ42cc oligomers. An engineered Aβ-affibody complex (Aβ42cc:ZAβ3, constructed in Prof. Torleif Härd’s group at department of molecular biology, SLU) has been overexpressed, purified and separated using existing protocols [8]. Aβ42cc oligomers were produced by dialysis of peptide in denaturing solution against sodium phosphate buffer at pH 7.2. The other part of the project has been to study the interactions of Aβ42cc oligomers with other proteins in human serum and CSF. The main focus of this second part was to develop a capturing method to capture protein ligands in human serum and CSF. After successful developing and capturing, the captured proteins have been separated by gel electrophoresis and analyzed by MS. We have identified five proteins in serum that are fished out with Aβ42cc oligomers. For the identification and comparison of binding proteins in CSF, we used six different samples of which three are from different AD patients and the other three are from non-AD individuals. Four proteins that were fished out with Aβ42cc oligomers from different CSF samples have been identified.
2. Materials and Methods

2.1 Protein expression

A mutant Aβ protein (Aβ_{42}cc) was used in this project. The double cysteine mutated Aβ (Aβ_{42}cc) was produced in *E. coli* by co-expression with Z_{Aβ3} affibody. The Aβ_{42}cc:Z_{Aβ3} complex was constructed in a bacterial plasmid called pACYCDuet-1 where genes for Aβ_{42}cc and affibody Z_{Aβ3} have been introduced under separate promoters and the construct has been transformed into *E. coli* Orgami B cells that are stored as glycerol stock. The affibody Z_{Aβ3} protein is tagged with six histidine residues [8]. Cell culture for overexpression was started from a glycerol stock. A pre-culture was grown overnight in Terrific Broth (TB) medium (appendix 1) at 37 °C with continuous shaking at 120 rpm. In addition to the TB medium, the pre-culture also contained 110 ml/L of 1 M (10X) potassium phosphate buffer (appendix 1), 34 mg/L chloramphenicol, 12.5 mg/L tetracyclin and 15 mg/L kanamycin. The pre-culture was transferred to the main culture, as 75 ml pre-culture/liter main culture. The main culture contained TB medium, potassium phosphate and all three antibiotics in the same concentrations used in pre-culture. The culture was grown at 37 °C with shaking at 120 rpm. The optical density (OD) at 600 nm was monitored and when the OD reached approximately 0.8 AU, the expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested by centrifuging at 4500 rpm, 4 °C for 20 minutes following 4 hours of incubation after IPTG induction. The cell pellets were resuspended in 100 mM tris-hydrochloride pH 8.0, 500 mM sodium chloride with protease inhibitors tablet (Roche).
2.2 Preparing cell pellets

Cell pellets were completely thawed and sonicated (Ultrasonic processor, Sonics Inc.) at 75% amplitude in pulses (5 sec on and 9.9 sec off) with a total sonication time of 2.5 minutes. After that the cell lysates were clarified by centrifugation at 18000 rpm (Sorvall centrifuge, SS-34 rotor) at 4 °C for 30 minutes. The supernatant was filtered through 0.45 µm and then 0.2 µm filter (Sarstedt).

2.3 Protein purification
2.3.1 Immobilized metal ion affinity chromatography (IMAC)

In the first purification step, IMAC technique was applied where the nickel-nitriloacetic acid (Ni-NTA) column was employed on an Äkta purifier (column and Äkta from GE healthcare). After charging the column with 0.1 M nickel sulfate (NiSO₄), about 35 ml of filtered cell lysate sample was loaded onto the column. IMAC purification was done in 20 mM tris-hydrochloride, pH 8.0; 500 mM sodium chloride (buffer A) and 20 mM tris-hydrochloride, pH 8.0; 500 mM sodium chloride; 300 mM imidazole (buffer B). After capturing the proteins onto the column, elution was done by washing the column with 30 mM imidazole and then a 30-300 mM imidazole gradient. The fractions corresponding to the target protein were collected, pooled and concentrated using Vivaspin-20 spin column with a MW cutoff of 5 kDa (sartorius stedim biotech) at 4 °C. A schematic presentation of techniques used in protein purification to oligomers preparation has been stated in figure 4.

2.3.2 Size exclusion chromatography (SEC)

Further purification was achieved through SEC. The concentrated fractions from IMAC were loaded onto a Superdex™ 200 PG 16/60 column (GE Healthcare) equilibrated with 20 mM tris-hydrochloride, pH8.0; 200 mM sodium chloride at 0.7-0.9 ml/minute flow rate. The eluted fractions were collected and concentrated on 5 kDa cut-off Vivaspain-20 column (sartorius stedim biotech).
2.4 Separation of Aβ_{42}cc from affibody

To separate the affibody from Aβ_{42}cc, the eluted fractions from SEC were run on another IMAC under denaturing condition. For denaturation, the concentrated complex was diluted in guanidinium buffer in a final volume of 25 ml. The guanidinium buffer contains, 7 M guanidinium hydrochloride, 0.5 M sodium chloride, 0.1 M tris-hydrochloride pH 8.0. After that, the sample was kept at room temperature for 30 minutes on tilting table to allow the guanidinium hydrochloride to dissolve and complete separation of the complex. The sample was filtered using 0.2 µm filter (Sarstedt).

2.4.1 IMAC under denaturing condition

25 mL sample (dissolved guanidinium buffer) was loaded onto a Ni-NTA column equilibrated with 20 mM tris-hydrochloride pH 8.0; 7 M guanidinium hydrochloride at 0.5 ml/minute flow rate. Flow-through was collected and then the column was washed with slow imidazole gradient (0-40 mM, 20 min at 1.5 ml/min) followed by quick gradient up to 400 mM imidazole.

2.4.2 Confirmation of separation by gel electrophoresis

Gel electrophoresis (SDS-PAGE) was run to confirm the separation of Aβ_{42}cc from the affibody. Denatured IMAC flow through, wash and elution fractions were separately run on SDS-PAGE. Before loading on SDS-PAGE, samples were dialyzed overnight against 7 M urea. The SDS-PAGE was carried out using the Criterion™ Gel system and precast 16.5% tris-tricine gels (Bio-Rad) with 100 mM tris, 100 mM tricine and 0.1% SDS (pH 8.25) as cathode buffer and 100 mM tris-hydrochloride (pH 8.9) as anode buffer. The gel system was operated at a constant voltage of 120 V. 10 µl of loading buffer (3X) was added with 20 µl of sample. The loading buffer contains 50 mM tris-hydrochloride, 1% SDS, 20% glycerol, 0.23% bromophenol blue, and 15 mM heat stable tris-2-carboxyethyl-phosphine (TCEP) reducing agent. Samples were then heated at 95 °C for 5 min and approximately 15 µL were loaded in each well.
Figure 4: Schematic presentation of techniques involved in oligomeric Aβ42 formation including a short description of the most important steps.

2.4.2.1 Staining of the gel
The gel bands were visualized by silver staining [20]. Initially, the gel was fixed with 50% ethanol, 12% acetic acid and 0.05% formalin for 1.5 hours. After washing with 20% ethanol for 20 minutes, the gel was sensitized by 2 minutes incubation in 0.2% sodium thiosulphate. After sensitizing, the gel was washed with deionized water and then incubated in 0.2% silver nitrate along with 0.076% formalin solution for 20 minutes. The gel was developed in 6% sodium carbonate, 0.0004% sodium thiosulphate and finally the reaction was terminated with 12% acetic acid. A table describing all solutions used in silver staining is found in appendix 3.
2.5 Aβ_{42}cc oligomers preparation

After separation on denatured IMAC, Aβ_{42}cc containing fractions were pooled and concentrated on 3 kDa cut-off Vivaspin-20 column (sartorius stedim biotech). The sample was dialyzed overnight against 20 mM sodium phosphate pH 7.2, 50 mM sodium chloride with 5 mM ethylenediaminetetraacetic acid (EDTA) and then another dialysis against 20 mM sodium phosphate pH 7.2, 50 mM sodium chloride. The sample was then removed from the dialysis bag and treated on water bath at 60 °C for 10 minutes.

2.5.1 Confirmation of oligomers

After dialysis, a 200 µl sample was loaded on a Superdex 200™ column (GE Healthcare) equilibrated with 20 mM sodium phosphate pH 7.2, 50 mM sodium chloride at 0.5-0.7 ml/minute flow rate. The corresponding fractions were collected. This analysis was done to confirm the formation of oligomers.

2.6 Capturing of proteins in human serum and CSF

The main part of this project has been to develop a method for capturing proteins in serum and CSF. The serum sample was purchased from 3H Biomedical; Uppsala and CSF samples were provided by Prof. Henrik Zetterberg at Department of Psychiatry and Neurochemistry at the University of Gothenburg. The protein capturing was first started with only serum sample not CSF. Initially, immunoprecipitation (IP) was used to capture proteins using Aβ_{42}cc oligomers.

2.6.1 IP with Dynabeads® protein G and 6E10 antibody

In the first IP setup, we used Dynabeads® protein G (Invitrogen) and Aβ specific 6E10 antibody (Covance). The experiment was carried out using Invitrogen™ protocol and kits (Cat. no. 100.07D). Briefly, 50 µl (a concentration of 30 mg/ml) dynabeads was added to 5 µl (concentration, 1 mg/ml) antibody and incubated for 10 minutes at room temperature to allow antibody binding to dynabeads. After washing with 200 µl antibody binding and washing buffer (supplied with dynabeads), 100 µl serum and 35 µl Aβ_{42}cc oligomers (The final Aβ_{42}cc concentration of the oligomer sample was 0.45 mM) were added and the sample was incubated 10 minutes at room temperature with rotation. Following
incubation, the complex was washed three times using 200 µl washing buffer each time. Finally, the proteins were eluted in SDS-PAGE buffer by heating 10 minutes at 70 °C. After the first experiment, we run this protocol several times with altered protein-ligand concentrations, incubation times and temperatures.

We also tried the same beads and antibody with an IP protocol from Covance. This protocol was the reversed compared to the Invitrogen IP protocol. In the Invitrogen protocol, antibody was first added to beads and then the antibody-beads complex was incubated with Aβ42cc and serum. But in the Covance protocol, Aβ42cc and serum was first added to antibody and then Aβ42cc-serum-antibody complex was incubated with beads. 35 µl Aβ42cc and 100 µl serum was added to 5 µl of antibody and incubated for 1 hour at 4 °C. After addition of 50 µl dynabeads Protein G, the incubation was continued for additional 1 hour. The complex was washed three times with washing buffer and for each wash the complex was incubated for 20 minutes at 4 °C. Elution was done with 10 µl gel loading buffer by heating at 70 °C for 3 minutes.

2.6.2 IP with Dynabeads® M-280 Tosylactivated and 6E10 antibody
We thought to change the beads and started to use Dynabeads® M-280 Tosylactivated (Invitrogen, Cat. no. 142.03). The 6E10 antibody was coupled with dynabeads® M-280 according to manufacturer instructions. After coupling 25 µl (concentration, 20 mg/ml) coupled beads were incubated with 150 µl serum and 35 µl Aβ42cc for 1 hour at room temperature and then washed three times with PBS, 0.1% tween (washing buffer, appendix 2). The target proteins were eluted in 10 µl SDS-PAGE sample buffer by boiling at 70 °C for 10 minutes.

2.6.3 The developed method
At this point we excluded the antibody. The tosylactivated beads bind to amino groups of ligands by forming amine bond (appendix 2). Therefore, we could couple Aβ42cc oligomers directly to the beads instead of the antibody (see section 2.6.2). At the same time we also decided to block the beads (to use as control) with another protein and used bovine serum albumin (BSA). Later, we instead used glycine to block the bead, to remove any interference between blocking agent and the target protein. A schematic presentation of the optimized protocol is described in figure 5.
Briefly, dynabeads® M-280 tosylactivated was coupled with Aβ_{42}cc oligomers, and then the coupled-beads were incubated with serum/CSF.

**Figure 5.** A schematic presentation of the developed capturing method

Initially the beads were prepared according to manufacturer’s instructions. 5 mg of beads were added to 100 µg Aβ_{42}cc oligomers along with 0.1 M sodium phosphate pH 7.4 (coupling buffer, appendix 2) buffer and incubated overnight at 37 °C on a rocking platform to allow covalent binding of Aβ_{42}cc to the beads. The beads-Aβ_{42}cc complex was pelleted by using a magnet and supernatant was removed. After
adding phosphate buffer seline (PBS) with 0.5% tween (blocking buffer, appendix 2) the beads-Aβ_{42}cc complex was incubated for additional 1 hour. After incubation the supernatant was removed and the complex was washed with PBS-0.1% tween (washing buffer, appendix 2). The coupled beads were stored in same buffer at a final concentration of 20 mg/ml. Besides coupling, the beads were blocked with glycine to use as control sample. The protocol was the same except that glycine was added to bead instead Aβ_{42}cc in the same concentration. After coupling/blocking, 25 µl and 40 µl coupled/blacked beads (at a final concentration of 20 mg/ml) were added to 150 µl serum and 200 µl CSF, respectively. The samples were incubated at 37 °C for 1 hour on a rocking platform. Following the incubation, the beads were pelleted by using a magnet and washed three times with PBS-0.1% tween. After the final wash the target proteins were eluted in 10 µl SDS-PAGE sample buffer by heating to 70 °C for 10 minutes. The sample buffer contains 62.5 mM tris-hydrochloride, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 15 mM heat stable TCEP.

2.6.4 Proteins analysis by gel electrophoresis
SDS-PAGE gel electrophoresis was run to analyze the proteins captured by the different capturing methods. After elution by SDS-PAGE sample buffer, 10 µl samples were loaded in each well. The operation was carried out with Criterion™ Gel system and 4-20% gradient gels (Bio-Rad). The gel system was operated at a constant 200 V voltage with 1X SDS running buffer for around 45 minutes. SDS running buffer contains, 25 mM tris, 192 mM glycine and 0.1% SDS, pH 8.3.

2.6.4.1 Staining of the gel
A major focus of this project was to develop a capturing method to capture enough quantity of protein from serum and CSF. When we first started to develop the method, we stained the gel in silver staining. Silver staining procedure is already described in section 2.4.2.1. Since the silver staining is not compatible with mass spectrometry, later the gels were stained in Acquastain. In acquastain method, the gel was put in a plastic box and aquastain solution (Bulldog Bio, Inc) was poured upon the gel. The gel was kept in solution until the bands were clearly visualized.
2.7 Sampling for MS
The MS analysis of the captured proteins was carried out at the SciLifeLab MS-platform, Uppsala University. We have prepared the samples for MS in our lab and handled them to the SciLifeLab. Briefly, selected bands were cut and placed into separate eppendorf tubes. The gelbands were washed three times with 30% ethanol for 30 minutes. Then the gelbands were incubated in 10 mM dithiothreitol for 1 hour at 50 °C and then again incubated in 55 mM iodoacetamide solution for 1 hour in darkness. The gelbands were washed in 25 mM ammonium hydrogen carbonate for 5 minutes, followed by 5 minutes incubation in acetonitrile. After drying the gelbands in a SpeedVac® for 15 minutes, the proteins in the gelbands were digested by incubation in 12 ng/µl trypsin in 25 mM ammonium hydrogen carbonate for 1 hour at room temperature. The solution was discarded and the gelbands were incubated in 25 mM ammonium hydrogen carbonate at 37 °C, overnight in darkness. Solvents from each gelbands were collected in new tubes. After sonicatation of the gelbands in 60% acetonitrile, 5% formic acid for 5 minutes, the solutions from the sonicated gelbands were pooled with the previous corresponding fractions. Finally, the samples were dried in SpeedVac®.

2.8 MS analysis and database search
The samples were loaded onto a MTP 384 ground steel target using the dried droplet technique and the matrix used was α-cyano-4-hydroxycinnamate. Mass spectra were recorded using an Ultraflex II MALDI TOF MS (Bruker Daltonics, Bremen, Germany) in positive mode. Peptide mass mapping was performed using the Mascot search engine (experimental information of this section was provided by the SciLifeLab MS-platform, Uppsala).
3. Results

3.1 Protein expression and purification

The expression vector pACYCDuet-1 had already been constructed with genes for 
Aβ<sub>42</sub>cc and Z<sub>Aβ3</sub> and transformed into bacterial cells. The Aβ<sub>42</sub>cc:Z<sub>Aβ3</sub> complex was overexpressed using existing protocols [8]. The cell culture was allowed to grow 4 hours after IPTG induction and the protein was obtained in the soluble fractions of cell lysates.

Two liquid chromatography techniques were applied to purify Aβ<sub>42</sub>cc:Z<sub>Aβ3</sub> complex. The protein complex was first purified by using IMAC purification and then polished by SEC. In the Aβ<sub>42</sub>cc:Z<sub>Aβ3</sub> complex, Z<sub>Aβ3</sub> was tagged with six-histidines. During loading of the sample, Aβ<sub>42</sub>cc:Z<sub>Aβ3</sub> complex was bound onto the nickel column while other cellular proteins pass through it. The first absorbance peak in figure 6A corresponds to those proteins that pass through the column while histidine-tagged proteins are capturing onto the matrix. The second and third peaks are wash fractions using 30 mM and 300 mM imidazole respectively. The third peak (in the dotted frame in figure 6A) is the elution of the Aβ<sub>42</sub>cc:Z<sub>Aβ3</sub> complex. Whereas the second peak indicates that proteins that bind weakly to the column are eluted. After the IMAC purification, the fractions corresponding to the elution of the Aβ<sub>42</sub>cc:Z<sub>Aβ3</sub> complex were pooled and concentrated. After concentration the absorbance value was OD<sub>280</sub> = 3.92 AU.
Figure 6. Elution profiles of $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ complex on IMAC and SEC

(A) An IMAC purification of $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ with Ni-NTA column in 20 mM tris-hydrochloride, pH 8.0; 500 mM sodium chloride buffer and eluted with a gradient of 0-300 mM imidazole. Chromatogram showing protein absorbance at 280nm (blue) and 0-300 mM imidazole gradient (green). The third peak corresponds to $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ (uses around 90% of final imidazole concentration, in the dotted frame).

(B) Polishing of $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ with Superdex™ 200 PG 16/60 column in 20 mM tris-hydrochloride, pH 8.0; 200 mM sodium chloride. The second peak (in dotted frame) corresponds to $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$.

Following IMAC, SEC was run to obtain increased purity of the $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ complex and to remove imidazole from the sample. Proteins with higher molecular weight are expected to elute first (first peak in figure 6B). The second peak in figure 6B corresponds to $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ complex. All fractions that correspond to the $\text{A}\beta_{42}$,$\text{cc}:Z_{\text{A}\beta_{3}}$ complex were pooled for further characterization.
complex were collected and concentrated up to \( \text{OD}_{280} = 4.57 \text{ AU} \).

### 3.2 Separation of the protein complex

Protein samples from SEC were diluted in guanidinium buffer (see section 2.4) and then subjected to another IMAC step. Guanidinium hydrochloride is a common chemical denaturant used in for protein denaturation [21,22]. From the IMAC run, flow through, wash and elution fractions were collected separately. Since \( \text{A}\beta_{42}{\text{cc}} \) is not his-tagged and separated from the his-tagged \( ZA\beta_{3} \) protein, it passes through the column during sample loading and elutes with the flow-through (the first two peaks in figure 7A, in the dotted frame). IMAC wash fractions using 40 mM and 400 mM imidazole (third and fourth peaks in figure 7A respectively) contain \( ZA\beta_{3} \) affibody, as it binds to the column and elutes by the imidazole gradient.

#### 3.2.1 Analysis of the separation

The fractions containing \( \text{A}\beta_{42}{\text{cc}} \) were identified by SDS-PAGE (16.5% tris-tricine gel described in section 2.4.2) analysis. The flow-through fractions give band at 6 kDa (figure 7B, lane: 2-5; in the dotted frame), indicating pure \( \text{A}\beta_{42}{\text{cc}} \). IMAC wash fractions using 40 mM imidazole give weak bands at approximately 8 kDa (figure 7B, lane: 6-7), indicating the presence of affibody. Eluted fractions using 400 mM imidazole also contains affibody, as indicated by a relatively clear band around 8 kDa (figure 7B, lane: 8-9). The bands at 15 kDa are most likely affibody dimer.
Figure 7. Separation of Aβ_{42}cc from affibody

(A) Elution profile of Aβ_{42}cc:Z_{Aβ} under denaturing condition. Ni-NTA column was used with 20 mM tris-hydrochloride pH 8.0; 7 M guanidinium hydrochloride buffer and eluted with a gradient of 0-400 mM imidazole. Chromatogram showing protein absorbance at 280 nm (blue) and 0-400 mM imidazole gradient (green). The first two peaks correspond to Aβ_{42}cc (in the dotted frame), whereas gradient eluted fractions corresponds to Z_{Aβ}.

(B) SDS-PAGE analysis of Aβ_{42}cc IMAC fractions with 16.5% tris-tricine gel. Lane 1 and 10: molecular size marker. Lane 2-5: flow through fractions (fractions from first two peaks in figure 7A). Lane 6 and 7: IMAC wash fractions (40 mM imidazole, fractions from third small peak in figure 7A). Lane 8 and 9: IMAC wash fractions (400 mM imidazole, fractions from fourth peak in figure 7A).
3.3 Aβ₄₂cc oligomers preparation

Aβ₄₂cc containing fractions from denatured IMAC (first and second peak in figure 7A, lane 2-5 in figure 7B) were collected for oligomer formation via renaturation. Fractions were pooled and dialyzed against sodium phosphate buffer at pH 7.2 (see section 2.5). Aβ₄₂cc oligomers formed spontaneously during the dialysis [17].

3.3.1 Confirmation of oligomers

After dialyzes, samples were loaded on a Superdex 200™ column and the chromatogram was compared with the expected elution volume for monomer, dimer, trimer (figure not shown). The comparison confirms the formation of Aβ₄₂cc oligomers. Moreover, there was one peak (figure 8, in dotted frame) in the elution profile, which indicates homogenous sample. The final Aβ₄₂cc concentration of the oligomer sample was 0.45 mM.

![Figure 8. Elution profile of Aβ₄₂cc oligomers on SEC](image)

3.4 Development of the capturing method

The main goal of the project was to develop an effective capturing method to capture proteins from human serum and CSF that interact with neurotoxic Aβ₄₂cc oligomers. At the beginning of method development, two IP protocols: Dynabeads® protein G and Dynabeads® M-280 Tosylactivated were used. Many changes
including dynabeads type, antibody, ligand-proteins concentrations, incubation times, temperatures, washing steps and elution steps have been done during capture method developing.

3.4.1 Proteins capturing in serum
Initially, the development was carried out using serum samples. In the very first step of the development, dynabeads® protein G and 6E10 antibody were used and the Aβ specific 6E10 antibody was allowed to bind with dynabeads protein G. The antibody-bead complexes were incubated with serum and Aβ_{42}cc and then eluted by SDS-PAGE sample buffer. We did not find any unique bands in Aβ_{42}cc samples (lane 1 and 2 in figure 9A) compared to the control (lane 3 in figure 9A). After the first experiment, we set-up several additional experiments with same type of beads but with different ligand-protein concentrations, temperatures, and incubation times (figure not shown) but the results were not promising; because no proteins were fished out with Aβ_{42}cc. We also used a reverse protocol from Covance where Aβ_{42}cc and serum were first incubated with the 6E10 antibody and then dynabeads® protein G was added to the complex. But the results were disappointing us again, as no proteins were fished out with Aβ_{42}cc.

Then we decided to change binding strategy i.e. use of different type of beads to bind the antibody. Dynabeads® M-280 tosylactivated beads were chosen. For the first time we noticed some bands that were unique for the Aβ_{42}cc samples (lane 2, 3, and 4 in figure 9B, marked in dotted frame) and not found in the control (lane 1 and 5 in figure 9B). After this experiment we decided to exclude the antibody, because the tosylactivated bead can bind to amino group of any protein by forming amine bond. So it is possible to couple the Aβ_{42}cc oligomers directly to the beads. The beads have activated tosyl groups that are cleaved in the presence of amino groups while the other part on the bead binds to amino groups of ligand by forming amine bond (appendix 2). At the same time we also blocked the beads (lane 4 in figure 9C) with bovine serum albumin (BSA) for using as control. In this experiment we found several unique bands for Aβ_{42}cc sample (lane 1 in figure 9C) that are not present in control sample (lane 4 in figure 9C).
Figure 9. SDS-PAGE analysis of proteins captured in serum

(A) Dynabeads® protein G and 6E10 antibody (gel stained with silver staining). Lane 1 and 2: dynabeads-antibody complex, Aβ_{42}cc and serum. Lane 3: dynabeads-antibody complex and serum (control). Lane 4-6: Supernatants from last washing step of 1, 2, and 3 respectively.

(B) Dynabeads® M-280 Tosylactivated beads and 6E10 antibody (gel stained with silver staining). Lane 1: dynabeads-antibody complex and serum (control, without Aβ_{42}cc). Lane 2-4: dynabeads-antibody, Aβ_{42}cc and serum (different concentrations). Lane 5: dynabeads-antibody and Aβ_{42}cc (control, without serum). Lane 6-10: Supernatants from last washing step of 1, 2, 3, 4 and 5 respectively.

(C) Dynabeads® M-280 Tosylactivated beads conjugated with Aβ_{42}cc (gel stained with acquastain). Lane 1: Aβ_{42}cc conjugated beads and serum. Lane 2: Aβ_{42}cc conjugated beads (without serum). Lane 3: BSA blocked beads. Lane 4: BSA blocked beads, Aβ_{42}cc and serum.

(D) Dynabeads® M-280 Tosylactivated beads conjugated with Aβ_{42}cc (gel stained in acquastain). Lane 1 and 2: Aβ_{42}cc conjugated beads and serum. Lane 3: Glycine blocked beads and serum. Gel bands numbered as 1-7 in lane 1 means those bands were cut for MS analysis.
As the silver staining method is not compatible to MS, we decided to stain the gel with acquastain, which is more suitable for MS analysis. However, the lower sensitivity of this detection method posed a challenge to visualize proteins on the gel. To overcome this problem we paid attention to elute more proteins and we used different volume of gel loading buffer. Another challenge for us was to figure out whether or not the proteins are binding specifically to Aβ_{42} ligand or to the bead surface. We tried mild elution at low pH buffer (figure not shown), that showed no protein elutes at low pH buffer.

Now the protocol seemed to be working. At this point we decided to use glycine to block the beads instead BSA to avoid BSA interference with Aβ_{42} or serum or CSF, if there are any. It seems glycine blocked beads (comparing lane 4 in figure 9C with lane 3 in figure 9D) is quite better than BSA blocked beads, as no binding proteins was found in the samples with glycine blocked bead. For the final experiment, we again made some changes in temperatures and incubation times. We found several unique bands in the Aβ_{42} samples (lane 1 and 2 in figure 9D) that were not present in the control sample with glycine blocked beads (lane 3 in figure 9D). The bands around 15 kDa and other bands around 6 kDa most likely originates from the Aβ_{42} itself, confirming its attachment to the beads and these two bands are present in all Aβ_{42} samples in all experiment. The oligomers are coupled to bead and then contact to target protein, in the elution step this oligomer are denatured and dissociates as we have bands for Aβ_{42} in the gel. The unique bands for Aβ_{42} sample (numbered as 1-7 in lane 1 in figure 9D) were cut and submitted for MS analysis.

### 3.4.2 Proteins capturing in CSF

For capturing proteins in CSF the same developed method was used. Six CSF samples have been investigated of which three from AD patients and other three from healthy individuals. Besides the identification of binding proteins we also wanted to figure out the differences of captured proteins among all six samples and also differences compared to proteins captured in serum. We found several bands in the Aβ_{42} samples (all odd numbers lane in figure 10) and there are almost no bands in control samples (all even numbers lane in figure 10). From this gel, five protein bands (numbered as 1-5 in different lanes in figure 10) were submitted for
MS analysis.

3.4.3 Comparisons of proteins captured in CSF and serum samples
All samples display bands around 6-7 kDa and 15 kDa, generated from Aβ-itself indicating its attachment to the beads. All three non-AD (lane 1, 3 and 5 in figure 10) and two of AD samples (lane 7 and 9 in figure 10) gives clear bands around 25 kDa whereas the third AD sample (lane 11 in figure 10) gives a week band at the corresponding size. Bands with the same MW were also present in serum samples (figure 9D). Protein bands at 35 kDa is present in all samples (all odd number lanes in figure 10) and that was also found in the serum sample, but in the serum sample there were two bands in this region. Potentially, there are also two bands around 35-37 kDa in CSF samples but not visible due to low protein concentration.

![Figure 10. SDS-PAGE analysis of proteins captured in CSF samples](image)

Lane 1,3 and 5: Aβ conjugated beads and CSF samples from three non-AD individuals. Lane 7, 9 and 11: Aβ conjugated beads and CSF samples from three AD patients. Even number lanes are controls (glycine blocked beads) for each of the samples.

Two non-AD (lane 1 and 3 in figure 10) and two AD (lane 7 and 9 in figure 10) samples give some week protein bands around 60-70 kDa and some bands were also found in the serum sample in the same region but their MW were quite higher.
(in between 65-80 kDa). Apparently, two non-AD (lane 3 and 5) and one AD (lane 9 in figure 10) give bands about 80 kDa, comparable to serum sample as there was also band about 80 kDa. It is interesting that several protein bands in CSF samples are observed at approximately the same MW as in the serum sample.

3.5 MS analysis of proteins captured in serum

From the SDS-PAGE of serum, seven samples (selected bands from lane 1 in figure 9D) were extracted for MS analysis and proteins from six of these samples were successfully identified. The proteins were identified according to table 1.

<table>
<thead>
<tr>
<th>Sample Numbers(^a)</th>
<th>Identified protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Apolipoprotein A-I</td>
</tr>
<tr>
<td>2</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>3</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>4</td>
<td>Apolipoprotein A-VI</td>
</tr>
<tr>
<td>5</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>6</td>
<td>No protein Identified</td>
</tr>
<tr>
<td>7</td>
<td>Antithrombin III</td>
</tr>
</tbody>
</table>

\(^a\) corresponds to the number in lane 1 in figure 9D.

No protein was identified for sample 6 (gelbands numbered as 6 in lane 1 in figure 9D), although the gel band was clearly visible and the protein should be there. Potentially something went wrong at the sample preparation step.

3.6 MS analysis of proteins captured in CSF

Five samples (bands numbered as 1-5 in figure 10) from the SDS-PAGE of the CSF samples were submitted for MS analysis. Proteins from four of the five samples

<table>
<thead>
<tr>
<th>Sample Numbers(^a)</th>
<th>Identified protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Apolipoprotein A-I</td>
</tr>
<tr>
<td>2</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>3</td>
<td>No protein Identified</td>
</tr>
<tr>
<td>4</td>
<td>Clusterin</td>
</tr>
<tr>
<td>5</td>
<td>Gelsolin</td>
</tr>
</tbody>
</table>

\(^a\) corresponds to gel band number in figure 10.
were successfully identified (table 2). No protein was found in sample 3 (gel band numbered as 3 in lane 1 in figure 10), possibly due to error at the preparation step.
4. Discussions

4.1 Proteins captured in serum
Among the six identified proteins that were fished out with Aβ₄₂cc from serum, four protein bands (bands at approximately 25 kDa, 30 kDa, 35 kDa and 45 kDa in figure 9D) have been identified as apolipoproteins (apolipoprotein A-I, A-IV and E) and two of them are identified as same protein (Apolipoprotein E). The other two proteins are Vitronectin (at 64 kDa, lane 1 in figure 9D) and Antithrombin III (at 80 kDa, lane 1 in figure 9D). Associations of these proteins in AD have not been extensively reported but apolipoproteins including apolipoprotein E (Apo E) and apolipoprotein A-IV (Apo A-IV) have been reported to play role in amyloid fibril formation in atherosclerosis [23]. Apolipoprotein A-I (Apo A-I) acts as cholesterol transporter and inflammation regulator, has been reported at decreased level in serum and CSF with a variety of neurodegenerative diseases including AD, PD and down syndrome [24]. Apo A-I is also found in arterial plaques of atherosclerosis [25,26,27].

Among all apolipoproteins, Apo E with its E4 isoform has frequently been reported as risk factor for late-onset AD [28,29]. The E4 isoform is a cholesterol-carrying protein [30] linked to sleep apnea, a frequent condition in AD [31] and may underlie in disease pathology. But the exact role of Apo E in AD has not been extensively reported [32,33].
Apo A-IV, another typical apolipoprotein in plasma and CSF [34,35], has been reported to play role in amyloidosis [36] but the pathologic role in AD still unknown [37]. The next protein on the identified proteins list is vitronectin, a glycoprotein that has been found in amyloid deposits in senile plaques [38,39]. And the last one on the list, Antithrombin III, has been identified in AD serum but interaction with Aβ oligomer has not been reported as for other identified proteins [40].

4.2 Proteins captured in CSF

Four proteins in different CSF samples have been identified (table 2). Two of the four identified proteins are reported as Apo A-I (band about 25 kDa in lane 5 in figure 10) and Apo E (band about 35 kDa in lane 7 in figure 10). These two proteins were also indentified in serum (see section 3.5). Apo A-I and Apo E proteins are abundant in CSF [37]. The possible role/interaction of these proteins in/with AD is already discussed in section 4.1.

Clusterin is an enigmatic glycoprotein [41]. The protein is also known as apolipoprotein-J [42]. Clusterin is capable of interacting with broad spectrum of molecules and is found in amyloid plaques and cerebrovascular deposits [43]. Moreover, the protein is involved in apoptotic cell death [41], so it is not unexpected to find an interaction of clusterin with Aβ_{42}cc oligomer and that happened in this study. In the pathological condition, clusterin is considered as amyloid-associated protein [44].

The next protein that was fished out with Aβ_{42}cc from CSF is Gelsolin. It has been reported to act in protein misfolding including amyloid fibril formation of AD [45], but the pathological role is not clear [46] and further studies are required.

4.3 Concluding remarks

To date, no interactions between Aβ_{42}cc oligomers and other proteins have been reported. This project has reported such interactions for the first time. The developed capturing method worked nicely for capturing proteins in both serum and CSF but still it might need some modifications for capturing proteins in CSF, since the protein concentration in CSF is lower than in serum and that caused a problem to visualize protein bands in CSF experimental gel.
In the future, additional techniques could be used for better understanding of the Aβ_{42}cc oligomers-serum/CSF protein interactions. Surface Plasmon Resonance (SPR) could be applied, that will give information about binding specificity, affinity, kinetics as well. For SPR study the protein that are fished out with Aβ_{42}cc can be recovered from SDS-PAGE gel or the proteins can be recombinantly express as we have the protein details now, including sequence and MW. Circular dichroism (CD) spectroscopy technique could be employed to study protein stability. This experiment will provide information while the identified proteins are interacting with Aβ_{42}cc oligomers, what type of conformational change occurs there, and interaction patterns as well. The identified proteins could be individually express to study their structures.

Another approach could be co-expression of Aβ_{42}cc peptide with the identified proteins and purification of the complex. Following the expression and purification, structural studies could be conducted by x-ray crystallography or NMR. This approach will give opportunity to study the interaction mechanism in more detail. The findings will improve the knowledge about Aβ neurotoxicity and therefore mechanisms behind AD.
Acknowledgements

First and foremost I would like to show my gratitude towards my supervisor Dr. Christofer Lendel for giving me the opportunity to do this project, for his adroit supervision, inspiring ideas, enthusiastic encouragement and guidance throughout the project work.

My sincere thanks to Prof. Torleif Härd for allowing me to use his engineered protein in the project. Special thanks to Anatoly Dubnovitsky and Elisabeth Wahlberg for guiding me and for fruitful discussions that stirred me at capturing method developing step in the project.

I would also like to thank Prof. Henrik Zetterberg at Department of Psychiatry and Neurochemistry at the University of Gothenburg for ensuring the availability of the CSF samples. Thanks to Jerry Ståhlberg and Mats Sandgren group’s for allowing me several times to use their aquastain solution and gel running buffer.

Surely a special thank goes to Margareta Ramström Jonsson at the SciLifeLab MS-platform, Uppsala University, for analyzing my protein gel samples with mass spectrometry.

Finally I would like to thank all the people at the department of molecular biology, SLU, for their unlimited help and warm behavior.
References


42. Stephanie Materia, Michael A. Cater, Leo W. J. Klomp, Julian F. B. Mercer, and Sharon La Fontaine (2011) Clusterin (Apolipoprotein J), a


Alzheimer’s disease (AD) is the most common neurodegenerative disorder. The prevalence of AD is rapidly increasing and more than 100 million elderly are expected to have AD by 2050. Despite the extensive research of the last few decades, we do not have the mechanism details of AD in our hands. The amyloid hypothesis guides us that the oligomerization and aggregation of Aβ peptides have been linked to the pathogenesis of AD. An important hypothesis today is that neurotoxic oligomeric Aβ aggregates are responsible for cell death and there might be interactions between Aβ oligomers and other critical cellular components that lead Aβ neurotoxicity. The project was aimed at identifying which proteins that interact with Aβ oligomers in serum and cerebrospinal fluid (CSF).

The work plan of this project has been divided into two parts where one has been to produce pure neurotoxic Aβ42CC oligomers. An engineered Aβ (Aβ42CC) was used in this project. The double cysteine mutated Aβ (Aβ42CC) was produced in E. coli by co-expression with ZAβ3 affibody. The Aβ42CC:ZAβ3 protein complex has been overexpressed and then purified using immobilized metal ion affinity chromatography (IMAC) and size exclusion chromatography (SEC). After purification, the complex was separated by another IMAC under denaturing condition [8]. Aβ42CC oligomer has been produced by dialysis of peptide in denaturing solution against sodium phosphate buffer at pH 7.2. The other part has been to study the interaction of Aβ42CC oligomers with other proteins in human serum and CSF. The main focus of this second part has been to develop a protocol to capture protein ligands in human serum and CSF. An effective capturing method has been developed and applied to capture proteins in serum and CSF. Then the captured proteins have been separated by gel electrophoresis and analyzed by mass spectrometry (MS). Five proteins in serum have been reported as Apolipoprotein A-I, Apolipoprotein E, Apolipoprotein A-IV, Vitronectin and Antithrombin III. In the case of identifying and comparing binding proteins in CSF, six different samples have been used of which three are from AD patients and another three are from non-AD individuals. From different CSF samples four proteins have been identified as Apolipoprotein A-I, Apolipoprotein E, Clusterin and Gelsolin.
Many studies suggest the presence of other proteins in amyloid plaque of AD but the pathologic role is still unknown. From this study, we now know that there are also interactions between certain proteins and Aβ oligomers. The findings of this project will improve the knowledge about the properties of Aβ aggregates and the related toxicity mechanism and therefore improve the understanding of the mechanisms behind the onset and progress of AD. Moreover, the developed capturing technique could be helpful to study other neurodegenerative diseases where protein aggregation is a fact, including Parkinson’s disease, Huntington’s disease and frontotemporal dementia.
Appendices

Appendix 1

Table 1: Preparation of 1 liter of TB medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
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<td>Tryptone</td>
<td>12 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>24 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4 ml</td>
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</table>

Table 2: 1 M (10x) potassium phosphate buffer: 0.17 M KH$_2$PO$_4$ and 0.72 M K$_2$HPO$_4$

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>23.13 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>125.40 g</td>
</tr>
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Appendix 2

Table 1: Buffers used in developed captured method

<table>
<thead>
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<th>Buffers name</th>
<th>Buffers compositions</th>
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</thead>
<tbody>
<tr>
<td>Coupling buffer</td>
<td>0.1 M Na-phosphate buffer pH 7.4</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>PBS pH 7.4 with 0.5% Tween</td>
</tr>
<tr>
<td>Washing buffer</td>
<td>PBS pH 7.4 with 0.1% Tween</td>
</tr>
</tbody>
</table>

Figure 1. The Tosylactivated beads have activated a tosyl group that cleaves in the presence of amion group while the other part on the bead binds to amino groups of ligand by forming amine bond.
## Appendix 3

Table 1: Silver staining solutions [20]

<table>
<thead>
<tr>
<th>Solutions Name</th>
<th>Ingredients</th>
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<tbody>
<tr>
<td>Fixation solution</td>
<td>50% ethanol, 12% acetic acid, 0.05% formalin</td>
</tr>
<tr>
<td>Wash solution</td>
<td>20% ethanol</td>
</tr>
<tr>
<td>Sensitizing solution</td>
<td>0.02% sodium thiosulphate</td>
</tr>
<tr>
<td>Staining solution</td>
<td>0.2% silver nitrate, 0.076% formalin</td>
</tr>
<tr>
<td>Development solution</td>
<td>6% sodium carbonate, 0.0004% sodium thiosulphate</td>
</tr>
<tr>
<td>Termination solution</td>
<td>12% acetic acid</td>
</tr>
</tbody>
</table>