Sveriges lantbruksuniversitet
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Abstract

Alzheimer's disease (AD) is the most common type of dementia observed in the elderly. The symptoms of the disease are provoked by neuronal loss caused by neurofibrillary tangles and amyloid plaques. The molecular processes that underlie AD's pathology are at this day not well understood. Amyloid β peptide (Aβ) appears to be one of the causes of neuronal toxicity. 42-residue Aβ (Aβ42) forms insoluble oligomers and fibrils which seem to be one of the leading causes of AD. To study the different molecular processes behind aggregation, toxicity and cellular uptake of Aβ42, monomer purification and labeling with a fluorescent dye are crucial. More successful fluorescent labeling without disrupting the structure and aggregation of Aβ, would increase accuracy and reliability of a wide variety of cellular studies.

This study aims to improve the protocol for monomer purification and labeling. Aβ with an introduced Cys for labeling (Aβ MC1-42) was labeled with ATTO Oxa11 (ATTO-TEC) dye to a degree of labeling between 11 and 13%. Different conditions were explored and the labeling was most successful with 7 M guanidinium hydrochloride buffer, pH 8 and an incubation time of 3 hours. The labeled Aβ (MC1-42), that had been purified under sterile conditions, was added to HEK 293 cells at three different concentrations and studied under the confocal microscope. Aβ is visible in the cells, which suggests that the peptide was internalized and/or interacting with the plasma membrane.

Thioflavin T (ThT) and pentameric formyl thiophene acetic acid (pFTAA) assays were performed to study the aggregation kinetics of labeled Aβ (MC1-42) compared to wild-type Aβ42 and unlabeled Aβ (MC1-42). The assays showed a lower fibrillation rate for Aβ (MC1-42), both labeled and unlabeled. The ThT assay showed that addition of dye seemed to give a slightly lower fibrillation rate, which could mean that the dye interferes with the assay. The pFTAA assay didn't show any signal for Aβ (MC1-42), and needs therefore to be rerun with more controls before any conclusion could be drawn.

A preliminary experiment was carried out, in which labeled Aβ42 at a concentration of 2.1 μM was added to human CHME3 microglial cells and studied under the confocal microscope. Aβ seemed to be internalized by the cells.

These findings provide a promising point of entry for further research on Aβ42 labeling techniques and cellular studies.

Keywords: Alzheimer's disease, Amyloid-beta, fluorescence, cellular uptake, labeling

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Introduction

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder and is the most common type of dementia in the elderly (Purves et al., 2012). At an early stage it causes memory impairment, and with the progression of the disease long term memory and other cognitive functions are affected. The only certain way to diagnose AD is brain histology, which is obviously only possible to carry out post-mortem. The most common histological signs seen in the AD brain are the loss of neurons, the presence of neurofibrillary tangles and the formation of amyloid plaques. These signs are mostly found in the limbic structures, neocortex and basal forebrain nuclei (Purves et al., 2012).
The molecular causes of AD are presently not well understood, and there is a pressure to find better ways to study these molecular processes (Dunning et al., 2016).

Amyloid β peptide (Aβ)

The Aβ peptide is generated from the amyloid β precursor proteins (APP), which is a membrane protein important for membrane transduction (Finder and Glockshuber, 2007). APP can be cleaved by different enzymes, called secretases. By which secretases APP is cleaved decide if the protein will take the non-amyloidogenic or the amyloidogenic pathway. If it is cleaved by α and γ secretases it will follow the non-amyloiddogenic pathway, whereas if it is cleaved by β and γ secretases it will generate Aβ and take the amyloidogenic pathway (Cappai and White, 1999).

Amyloid β (Aβ) peptides accumulate in amyloid plaques, which are the last stage of Aβ aggregation and are formed by insoluble fibrils (Finder and Glockshuber, 2007).
The amyloid cascade hypothesis suggests that the aggregation of Aβ is one of the causes of the pathology of AD (Finder and Glockshuber, 2007).

In the brain Aβ can be found in both soluble and insoluble forms, which have different secondary structures. In fact, in the soluble form it has a structure rich in random coils, while the insoluble form, which is the main cause of fibril formation, has a structure rich in β-sheets (Cappai and White, 1999). Aβ39 keeps its random coil structure at a physiological pH, while Aβ42 rapidly converts to β-sheets (Finder and Glockshuber, 2007).

The aggregates formed by Aβ have a high molecular weight (Cohen et al, 2015) and have a high percentage of β sheets; the tertiary structure is unique for this kind of fibrils because of the β-cross conformation, and this is true for all amyloid forming proteins (Ow and Dunstan, 2014)

Plasmids used

The peptides that were used were wild-type Aβ42 and Aβ (MC1-42) from plasmids provided by Sara Linse. The sequences can be found in Table 1.

Table 1. Amino acid sequences of the peptides used.

Peptide	Sequence
Wild- Type Aβ42	DAEFRHDSG YEVHHQKLVF FAEDVGSNKG AIIGLMVGGV VIA
Aβ (MC1-42)	MCDAEFRHDS GYEVHHQKLV FFAEDVGSNK GAIIGLMVGG VVIA

Fibril Formation and aggregation

The aggregation of Aβ is a catalytic cycle with 3 different steps. It starts with primary nucleation, which is a slow process when monomers form new oligomers; this is followed by elongation step where the fibrils get longer. An additional step is the secondary nucleation, in which oligomer formation is catalyzed by the interactions of fibrillar surfaces and Aβ monomers (Cohen et al,2015).

Cell toxicity

The processes underlying Aβ cell toxicity are not clear, but the peptide can interact with many vital processes of the cells (Cappai and White, 1999), and it can interact with the surface receptors in neurons, such as NMDA, AMPA and nicotinic receptors (Masters and Selkoe, 2012) and interfere with their functions. It has been believed for a long time that the plaques were the main cause of the toxicity, but recent studies indicate that it is the pre-fibrillary oligomers formed during the secondary nucleation that are cause of loss of neurons (Cohen et al, 2015).

Methods

Several different methods were used this project to purify and study the behavior of Aβ42.
Size exclusion chromatography (SEC) was used to separate the oligomers and monomers of the protein, so pure monomers could be used in cellular experiments.
For the labeling of Aβ (MC1-42), ATTO Oxa11 was used because it is a maleimide dye and it reacts with the Cys thiol group.
At the beginning of the project and after successful labeling, aggregation kinetics assays were also used to observe if there were differences in fibrillation rate between different Aβ42 samples. Two different assays were used: Thioflavin-T (ThT) assay and pentameric formyl thiophene acetic acid (pFTAA) assay.

ThT assay

ThT is benthiazole dye that binds to β-sheets. It has a weak fluorescence even when β-sheets are not present but the fluorescence increases when bound to β-sheets as a result of fibril formation, which makes this a good assay to detect fibril formation over time (Nasir et al, 2015).

p-FTAA assay

pFTAA seems to bind to a different structural motif than ThT and it is used to detect other intermediates during fibrillation. The dye interacts and binds to non-thioflavinophilic aggregates during the lag-phase which results in a different curve than in a ThT assay. It also has target-

dependent fluorescence signal because the dye's backbone is rigid and causes very specific interactions with the fibrils (Åslund et al, 2009).

Aim of this study

The aim of this study was to improve Aβ monomer purification and labeling protocols in use. The labeling of Aβ, without changing its structure and aggregation properties, is important for a wide variety of cellular studies, e.g. about cellular uptake, toxicity, how the peptide interacts with the blood-brain-barrier, and how it is internalized and degraded by microglial cells (Jungbauer et al, 2009).

Material and Methods

Protein expression

Medium was prepared by adding ampicillin and chloramphenicol to autoclaved Luria broth (LB) medium to final concentrations of 50 µg/ml and 34 µg/ml, respectively. Start cultures containing 50 ml medium were inoculated with a 500 µl glycerol stock of BL21*pLysS cells and then incubated overnight at 37 °C while shaking.

8 ml of starting cultures were transferred to 800 ml LB medium and incubated with shaking at 31 °C until an OD600 between 0.6 and 0.9 was reached.

Induction of protein expression was achieved by adding 0.5 mM isopropyl β-D1-thiogalactopyranosid (IPTG) and then the cultures were incubated overnight at 21 °C.

The cells were harvested in the next morning by centrifugation at 7200xg for 30 minutes, discarding the supernatant and resuspending the pellet in 30 ml of 100 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH8. The cells were then stored at -20 °C.

Purification of wild-type (WT) Aβ42 and Aβ (MC1-42)

Frozen cell pellets from starting culture were thawed and sonicated for 3 minutes (2 seconds on and 2 off at 65% amplitude). The lysed cells were then centrifuged at 24000xg for 10 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 10 mM Tris-HCl, 1 mM EDTA, with 1 mM dithiotreithol (DTT) when Aβ (MC1-42) was purified, pH 8. The suspended pellet was then sonicated and centrifuged again as described above.

The pellet was dissolved with a homogenizer in 8 M Urea in 10 mM Tris-HCl, 1mM EDTA pH 8, and was then diluted to 2 M Urea. DEAE sepharose was washed in a Büchner funnel with water and 10 mM Tris-HCl, 1 mM EDTA, (1 mM DTT, if Aβ (MC1-42) was purified), pH 8 buffer, then added to the 2 M Urea solution and incubated it at 4 ° C for 30 minutes with a magnetic stirrer.

The solution was then filtered through a Büchner funnel with filter paper and washed with 10 mM Tris-HCl, 1 mM EDTA, (1mM DTT for Aβ (MC1-42)) pH 8, and 10 mM Tris-HCl, 1 mM EDTA, (1mM DTT for Aβ (MC1-42)), 25 mM NaCl, pH 8. The protein was then eluted with 10 mM Tris-HCl, 1 mM EDTA, 125 mM NaCl pH 8.

The eluate was run through a 30 kDa Vivaspin concentration tube at 4500xg at 4 °C. The flow through was concentrated using a 5 kDa Vivaspin concentration tube at 4500xg at 4 °C until the desired concentration is reached.

1 ml aliquots of purified Aβ at 20 µM concentration were made and lyophilized in low binding tubes in the Speedvac overnight.

The lyophilized Aβ was then stored at -20 °C until further use.

SDS PAGE

16.5 % Tris-Tricine gels were prepared as specified by the Laemmli SDS PAGE protocol.

The samples were prepared by using a reducing and non-reducing buffer with or without mercaptoethanol and boiled on a heating block at 96 °C.

The samples and the marker (Page Ruler Plus Prest (Thermo) in Figure 1, and SeeBlue Plus2 (Thermo) in Figures 3 and 9 were loaded and the gel was run at 50 V for 20 minutes and then increased to 150 V for 75 minutes. The gel was then stained with Coomassie for 1 hour at room temperature on a shaker and destained in hot water for 1 hour.

Size exclusion chromatography (SEC)

WT Aβ42 and Aβ (MC1-42) were dissolved in 550 µl 7 M guanidinium hydrochloride (Gu-HCl) pH 7 and tris(2-carboxyethyl)phosphine (TCEP) was added to a final concentration of 10 mM or 1 mM (Table 2). The absorbance was measured in a spectrophotometer to calculate the concentration of the sample. A Superdex 75PG column was equilibrated with 20 mM sodium phosphate (NaPi), 1 mM EDTA, 0.2 % sodium azide (NaN3) and the sample was then injected via a 500 µl loop. The separation was carried out at a flow rate of 0.7 ml/min. The protein monomers were collected in fractions of 0.4 ml. The fractions from the monomer peak, that elutes at a volume of 17 ml, were mixed in a low binding tube; the concentration was calculated by peak integration. 500 µl aliquots were made in low binding tubes and stored at -20 °C.

Aggregation kinetics

ThT assay

Samples for the ThT assay were prepared in low binding tubes on ice to prevent fibril formation. The total sample volume was 350 µl for 4 replicates. The amount of protein needed to get 3 µM concentration was calculated and added in the respective tubes. ThT, from a 1 mM stock solution, was added to the protein to a final concentration of 10 µM (3.5 µl). 20 mM NaPi pH 8 buffer was added to get to 350 µl volume. The tubes were then vortexed shortly and centrifuged. 80 µl were used for each replicate per well of a black half-width 96 wells plate with transparent bottom (Cornig 3881).

For the ThT assay shown in Figure 9, a stock solution of ATTO Oxa11 (ATTO-TEC) was added to the samples. The stock solution was made by dissolving 36 µg dried ATTO Oxa11 (ATTO-TEC) in 5 µl acetonitrile, adding 6.5 µl of 76.47 mM mercaptoethylamine (MEA) and adjusting the final volume to 3 ml with 20 mM NaPi buffer. 12.8 µl of stock dye solution were added to the control samples.

The fibrillation was observed by following the increase in fluorescence over time at 37 ° C without shaking, bottom optic and 300 seconds between each time point for at least 16 hours.

pFTAA assay

A master mix for 4 replicates for the pFTAA assay were prepared in low binding tubes on ice to prevent fibrillation. The amount of Aβ needed to get a final concentration of 3 µM and the amount of pFTAA, froma 15 µM stock solution, to get a concentration of 0.3 µM (7 µl) was added to the respective master mix. 20 mM NaPi pH 8 buffer was added to the master mix to the final volume of 350 µl. 80 µl of the master mix was used for each well in a black half-width 96 wells plate with transparent bottom (Cornig 3881).

The pFTAA assay was run at 37 ° C with no shaking, bottom optics and 300 seconds between each time point.

Labeling

A Superdex 75PG column was equilibrated with 20 mM NaPi, 1 mM EDTA, 0.2 % NaN3.

3 tubes or more, depending on the desired concentration, of lyophilized Aβ (MC1-42) were dissolved in 550 µl 7 M Gu-HCl in a low binding tube by pipetting and vortexing shortly. TCEP was added from a 0.625 M stock to the desired concentration, either 10 mM or 1 mM in our case.

Previously 1 mg ATTO Oxa11 (ATTO-TEC) was dissolved in 500 µl acetonitrile and aliquots of 18 µl containing 50 nmol dye were lyophilized in the Speedvac.

The amount of ATTO Oxa11 (ATTO-TEC) to be used was calculated based on the amount of protein used and the desired excess of dye (Table 2). The lyophilized dye was dissolved in 20 µl acetonitrile.

The 20 µl dye were added to the dissolved Aβ (MC1-42), the low binding tube was covered with aluminum foil to keep it in the dark and the mixture was incubated under different conditions for each attempt (Table 2), with 3 hours at room temperature giving the best results.

Table 2. Different conditions used for labeling attempts.

Attempt nr	Buffer	Incubation	Protein amount	Dye amount	TCEP
1 failed	7M Gu-HCl pH 7	2.5 h at room temperature	20 nmol	100 nmol	10mM
2 failed	7M Gu-HCl pH 7	o.n. at 4 degrees C	25 nmol	150 nmol	10 mM
3	7M Gu-HCl pH 8	3 h 20 m at room temp	25 nmol	150 nmol	-
4	7M Gu-HCl pH 8	4 h 35 m at room temp	34 nmol	150 nmol	1 mM
5	7M Gu-HCl pH 8	3 h shaking at room temp	60 nmol	300 nmol	1 mM
6 (sterile)	7M Gu-HCl pH 8	3 h at room temperature	50 nmol	200 nmol	-

The labeled monomers were collected at the highest peak for ATTO Oxa11 (ATTO-TEC) (Abs 658, elution volume circa 18-19 ml), fractions of 0.4 ml were taken. The labeled monomers were pooled in one low binding tube and 100 µl aliquots prepared and stored at -20 ° C.

The specific absorbances of the conjugated dye and the protein was calculated from their respective monomer peak by peak integration, which gave us the absorbance; this was used to calculate the degree of labeling (DOL) by using Equation 1.

Equation 1. Equation for calculating the degree of labeling. Abs658 is the absorbance at the maximum for ATTO Oxa11 dye (ATTO-TEC), Abs280 is the absorbance at the maximum of Aβ, ε658 and ε280 are the extinction coefficients of the dye and Aβ, and CF280 is the correction factor of the dye absorbance at 280 nm.

$$\mathrm{DOL} = \frac{Abs658 \times \varepsilon 280}{\left(Abs280 - (Abs658 \times CF280)\right) \times \varepsilon 658}$$

Sterile monomer purification

A Superdex 75PG column was washed with 70 % ethanol overnight to get sterile conditions. Afterwards it was equilibrated with 20 mM NaPi for 1 hour. The pipette tips, low binding tubes, 96 well plate and buffers used were autoclaved.

The preparation of the labeled Aβ (MC1-42) was the same as for the non-sterile labeling, as described above. In Table 2 the conditions used for attempt 6 (sterile labeling) can be seen. The monomer collection and calculations are also described above.

Cell cultures

HEK293 cells were thawed in a 37 ° C water bath. Sterile medium was prepared by adding fetal calf serum to DMEM/F12 media + Glutamax (Gibco). Thawed cells were added to 10 ml of medium in a T75 culture flask. The medium was changed to fresh, temperated medium after one day. The cells were washed with PBS, and 2 ml 1x trypsin was added and incubated for a few minutes until the cells detached. Fresh medium was added and the cells were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 3 ml fresh medium. 10 ml of fresh medium and 500 µl of cells were transferred to a new T75 culture flask and incubated.

Chamber slides (Nunc^{TM}) were coated with poly-D-lysine and incubated at 37 °C for 5 hours. The poly-D-lysine was removed and the chambers were washed with PBS. 500 µl of fresh medium were added to each of the 8 chambers and 5 µl of cells, and incubated at 37 °C overnight. The medium was gently removed and 100 µl of serum-free medium was added to the chambers and in 3 wells labeled Aβ was added (Table 3) at different concentrations.

Table 3. Different volumes of serum free buffer and labeled Aβ (MC1-42), and corresponding Aβ (MC1-42) concentrations used for the incubation of the cells are shown**.**

Samples	Serum free buffer	Labeled Aβ (MC1-42)	Aβ (MC1-42) Concentration
a	50 µl	100 µl	4.8 µM
b	100 µl	50 µl	2.4 µM
c	140 µl	10 µl	0.5 µM

After 5 hours of incubation at 37 °C, cells were washed twice with PBS, and fixed with 4% PFA for 15 minutes, followed by additional washing in PBS and the cells were stored in PBS overnight at 4 ° C.

40 µl of DAPI, used to stain the cell nucleus, were added to each chamber, and glass slides were put on top and sealed with nail polish.

Microglia experiment

Human CHME3 microglia cells were provided by Erik Hjorth, NVS department KI, and labeled Aβ (MC 1-42) was added to the medium (DMEM/high glucose + glutamax serum free) to a concentration of 2.1 µM and incubated for 3 hours at 37 °C. The cells were stained with carboxyfluoresceindiacetate (CFSE), which stains cytosolic components and is used to study live cells, and scanned in LSM510 confocal microscope.

Results

SDS –PAGE analyses

Figure 1 shows an SDS PAGE gel analysis of the different purification steps for WT Aβ (Fig 1A) and Aβ (MC1-42) (Fig 1B). Aβ42 has a molecular weight of circa 4 kDa and a band at 4 kDa can be seen at all relevant purification steps.

In the final elution only the band at 4 kDa should be visible. Figure 1.B shows a few bands around 28 kDa, which could mean that there are contaminants still present.

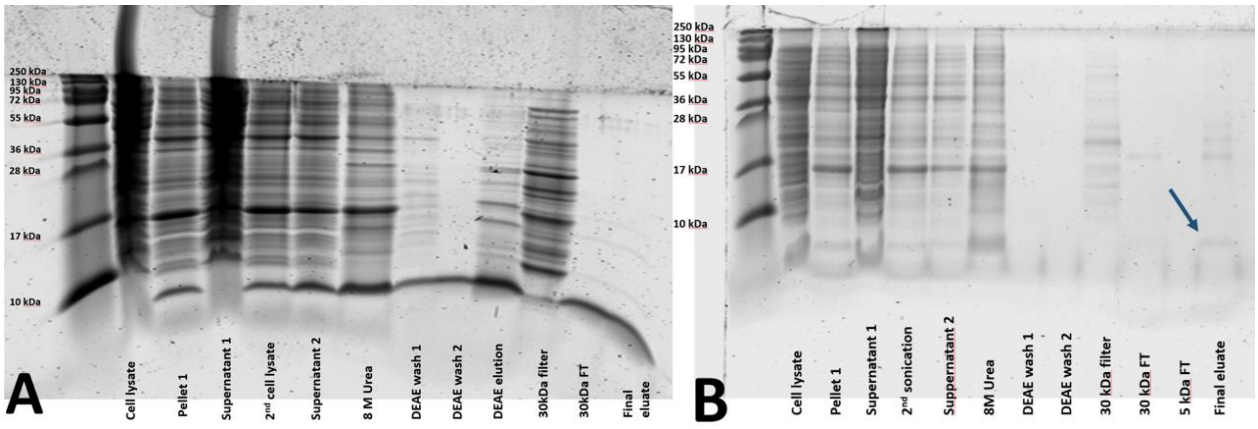

Figure 1 16.5% Tris Tricine gels for the different steps of WT Aβ (A) and Aβ (MC1-42) (B) purification.

In Figure 1.B the bands are very faint because too little protein was loaded on the gel.

Size exclusion chromatography of WT and MC

SEC was run for WT Aβ, WT Aβ with TCEP, Aβ (MC1-42) and Aβ (MC1-42) with TCEP to see if there was a significant difference in the amount of monomers and oligomers. From earlier studies we know that the oligomer peak starts around 9 ml and the monomer peak starts at 16 ml. Figure 2 shows both peaks where we expected them. There is no significant difference in the size of the monomer peak. The oligomer peak shows a slight difference between Aβ WT and Aβ (MC1-42), with the peaks for WT Aβ and WT Aβ with TCEP being slightly higher than the peaks for Aβ (MC1-42).

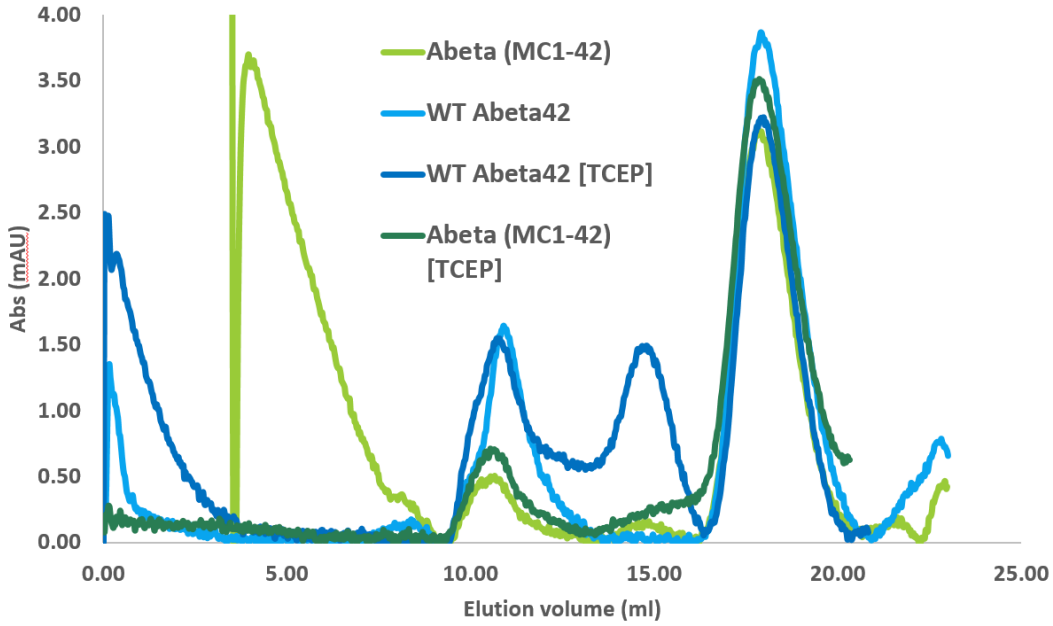

Figure 2 SEC curves for WT Aβ42, WT Aβ42 with TCEP, Aβ (MC1-42), and Aβ (MC1-42) with TCEP. The monomer peak is seen between 16 and 20 ml, and the oligomer peak is seen between 10 and 13 ml for all samples.

SDS gel for different fractions

Monomers and oligomers taken from the fractions during SEC separation (Figure 2) were prepared under reducing and non-reducing conditions and loaded on a 16.5 % Tris Tricine gel (Figure 3).

The samples from the oligomer peak show a clear band under reducing conditions (labeled in Fig. 3 as WT Aβ42 O R) and a very faint band under non reducing conditions (labeled in Fig. 3 as WT Aβ42 O NR) at 14-17 kDa. No band can be seen at 4 kDa, which would be where we expect the monomer band, which means that no monomers are present.

In all monomer samples a band at 4 kDa is present under both reducing and non-reducing conditions. Under the non-reducing conditions, we would expect bands for dimers because Aβ (MC1-42) can form disulphide dependent dimers. Bands at 17 and 14 kDa were seen for all the monomer samples, thet are more pronounced under reducing conditions.

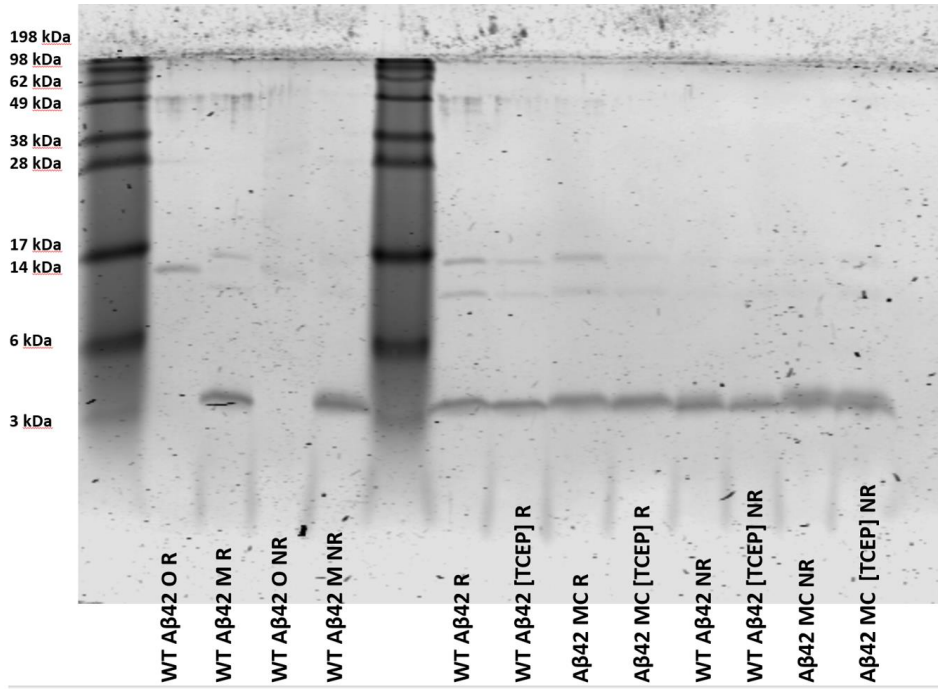

Figure 3 16.5 % Tris Tricine gel with samples from the SEC purification shown in Figure 2. From the left: SeeBlue Plus2 marker, WT Aβ42 reduced oligomer, WT Aβ42 reduced monomer, WT Aβ42 non-reduced oligomer, WT Aβ42 non-reduced monomer, SeeBlue Plus2 marker, WT Aβ42 reduced monomer, WT Aβ42 with TCEP reduced monomer, Aβ (MC1-42) reduced monomer, Aβ (MC1-42) with TCEP reduced monomer, WT Aβ42 non-reduced monomer, WT Aβ42 with TCEP non-reduced monomer, Aβ (MC1-42) non-reduced monomer, Aβ (MC1-42) with TCEP non-reduced monomer. A monomer band at 4 kDa is visible in all samples except the oligomer samples.

Thioflavin-T curve for WT Aβ and Aβ (MC1-42)

A ThT test was run with the purified monomers shown in Figure 2 to see if TCEP would affect the fibril formation and if there is a difference in fibrillation between WT Aβ42 and Aβ (MC1-42).

Figure 4 shows how the samples fibrillate during 25 hours. The lag phase is quite similar between Aβ (MC1-42) with and without TCEP and WT Aβ42. WT Aβ with TCEP has a longer lag phase which means that the fibrillation is delayed. Such a big difference was not expected. TCEP is a reducing agent and breaks disulphide bonds. It should however not affect WT Aβ42 because there are no cysteines in the peptide that could form disulphide bonds.

Aβ (MC1-42) shows a slightly shorter lag phase which means that it fibrillated faster than WT Aβ42 and Aβ (MC1-42) with TCEP. This could be due to the presence of a Cys in Aβ (MC1-42), which can form disulphide bonds and thereby create aggregates faster. TCEP seems to affect Aβ (MC1-42) and slow down the fibrillation.

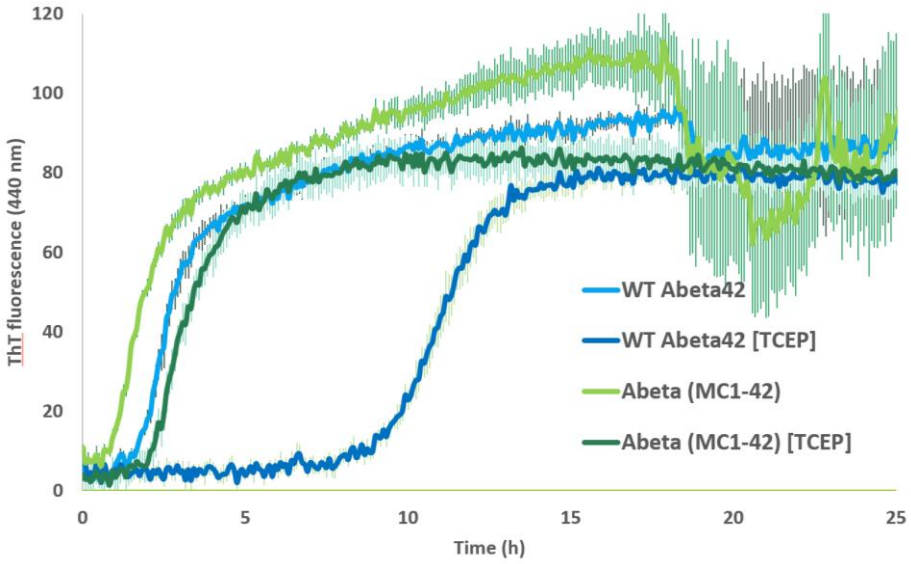

Figure 4 ThT curves for Aβ (MC1-42). Aβ (MC1-42) with TCEP, WT Aβ42, and WT Aβ42 with TCEP. Longer lag phase, suggesting slow fibrillation, is visible for WT Aβ42 with TCEP.

Labeling attempts

The labeling attempts 1 and 2 in Table 2 did not work and no monomers got labeled. When the conditions were changed to the ones seen in attempt 3, we could see that the labeling was successful. The absorbance for the dye, measured at 658 nm, showed an increase to circa 89 mAU, as can be seen in Figure 5.

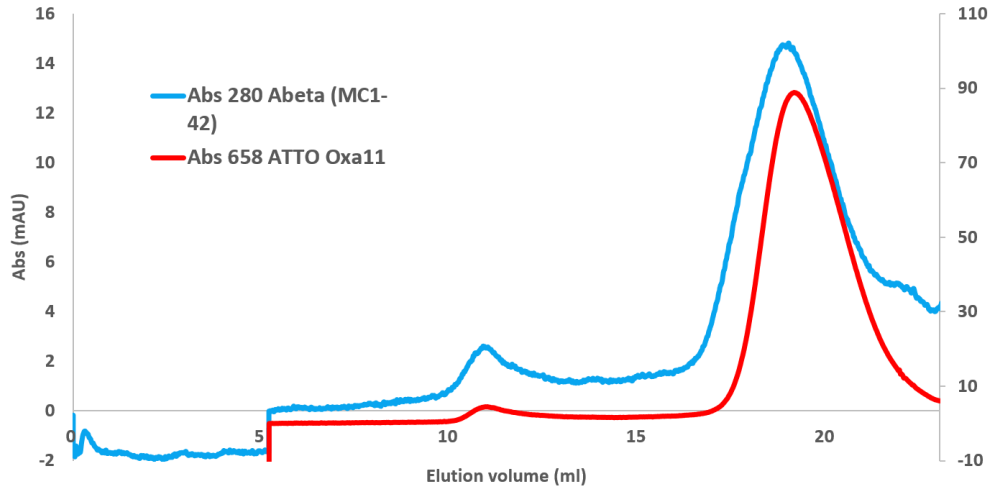

Figure 5 SEC curve for labeling attempt nr 3 (Table 2). Absorbance curves for both Aβ (MC1-42) at 280 nm and ATTO Oxa11 (ATTO-TEC) at 658 nm are presented. The monomer peak can be seen for both absorbances between 17 ml and 21 ml.

The protein concentration (4.4 μM) and the degree of labeling (13 %) were calculated by using Equation 1.

By changing the labeling conditions, we could see a decrease in the protein concentration and degree of labeling (Appendix), so when we prepared the sterile labeled Aβ (MC1-42) we used the same conditions as in attempt 3.

Figure 6 shows the SEC curve for the sterile labeling attempt. The monomer peak can be seen at both 280 nm and 658 nm between 17 and 21 ml. After calculating the peak area, we calculated both protein concentration (7.1 μM) and degree of labeling (12%).

A peak can be seen after the monomer peak at 21 ml. To be sure that it was not a contaminant a sample from it was run on a Tris Tricine gel (Figure 9).

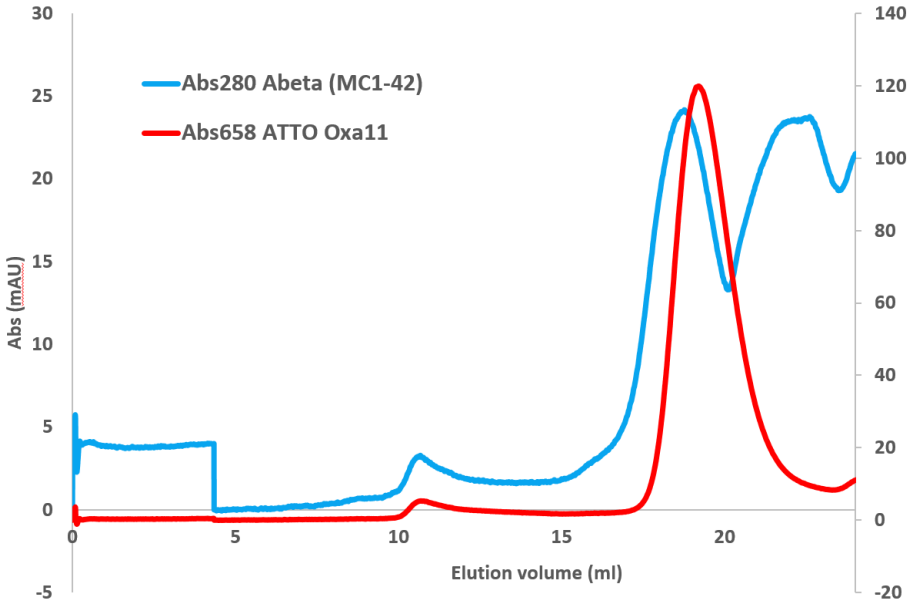

Figure 6 SEC curve for sterile labeling (attempt 6 in Table 2). Curves for both Aβ (MC1-42)'s absorbance at 280 nm and ATTO Oxa11 (ATTO-TEC)'s absorbance at 658 nm are present. The monomer peak can be seen for both absorbances between 17 ml and 21 ml.

ThT curves for WT Aβ42, Aβ (MC1-42), and labeled Aβ (MC1-42)

ThT assays were run for WT Aβ 42, Aβ (MC1-42) and labeled Aβ (MC1-42) to see if ATTO Oxa11 (ATTO-TEC) affects the fibrillation of the protein.

Figure 7 shows that labeled Aβ (MC1-42) apparently does not form fibrils. This could mean that the dye inhibits fibrillation. The unlabeled Aβ (MC1-42) also seems to have a lower fibrillation rate.

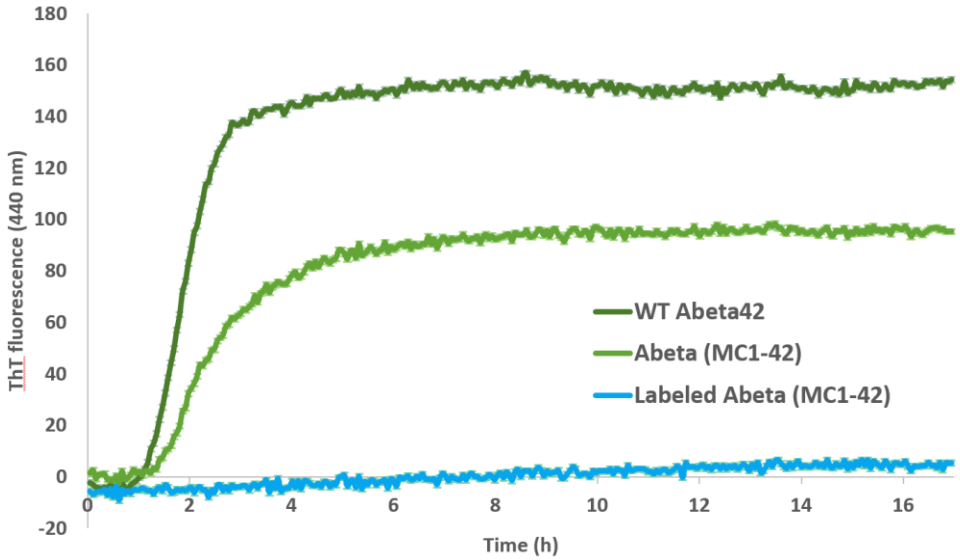

Figure 7. ThT curves for WT Aβ42, Aβ (MC1-42), and labeled Aβ (MC1-42). Fibrillation is visible for WT Aβ42, and Aβ42 (MC1-42), no fibrilation is seen for labeled Aβ (MC1-42).

Because of the results seen in Figure 7, we decided to redo the ThT test and include more controls. In order to see if the dye has effects on the fibrillation, we added the free dye (12.8 µl) to the WT Aβ42 and unlabeled Aβ (MC1-42) ThT master mixes and to the blank.

The curves for WT Aβ42 with and without dye are as high as we expected and similar to the one shown in Figure 8, with the one with the dye being slightly lower. The pure unlabeled Aβ (MC1-42) also shows a curve that is similar to the one that we saw in the previous ThT test, and when the dye solution was added the curve was again slightly lower.

In the second ThT run we see a curve for the labeled Aβ (MC1-42) even if it is lower than the other samples. This means that there is fibril formation, although at a lower rate. To be sure that it is not the dye that affects the fibrillation we analysed a sample were the labeled Aβ (MC1-42) and WT Aβ42 were mixed at a ratio of 1:200 (Nasir et al, 2015). This sample behaves very similar to WT Aβ42 with dye present, which means that the dye does not inhibit fibrillation.

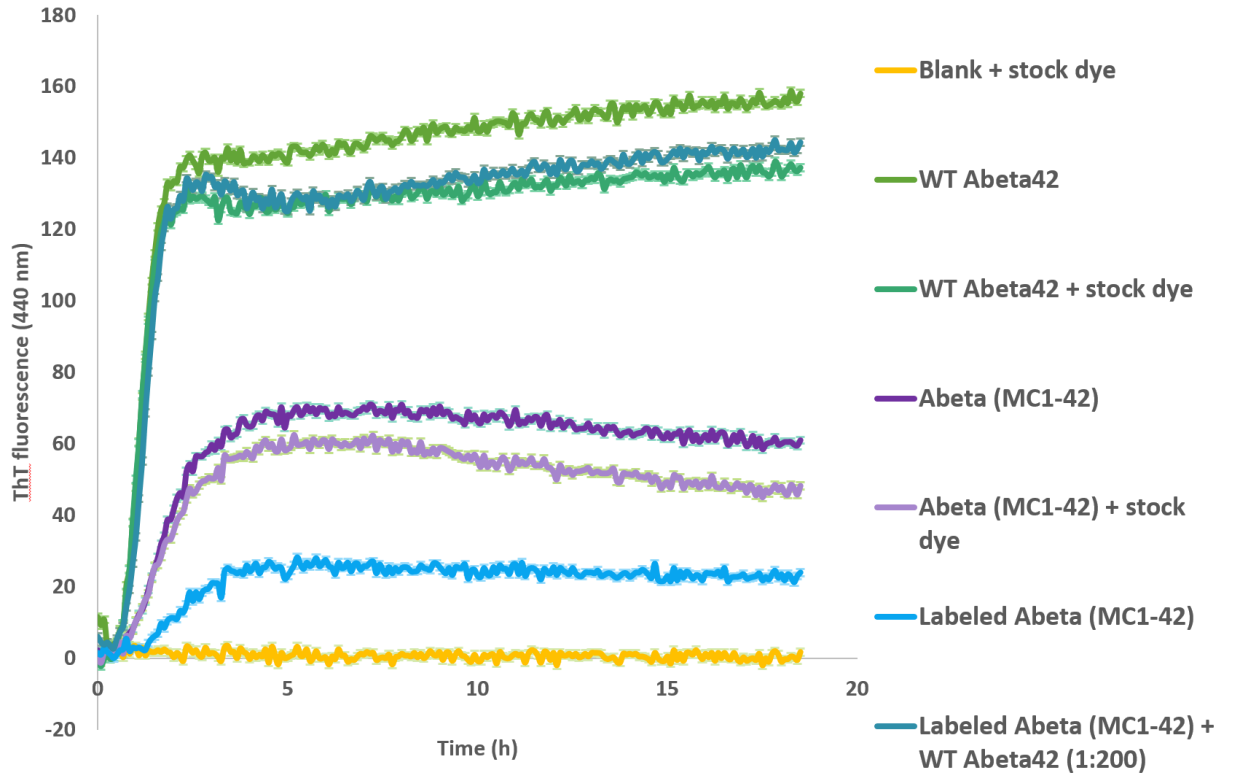

Figure 8 ThT curves for blank with stock solution of ATTO Oxa11, WT Aβ42, WT Aβ42 with free dye, Aβ (MC1-42), Aβ (MC1-42) with free dye, labeled Aβ (MC1-42), and a mixture of labeled Aβ (MC1-42) and WT Aβ42 at a ration of 1 :200. Fibrillation is visible for all the samples.

SDS gel for Aβ monomers

Figure 9 shows different labeled and unlabeled monomer samples from Aβ under reducing and non-reducing conditions.

Samples 1 and 2 in Figure 9 are samples from the protein expression part, before and after induction, respectively. Before induction we can see bands for a wide range of proteins, but no band can be seen at 4 kDa, but it can be seen after induction, which means that the induction was successful and Aβ is expressed.

Sample 3 is the WT Aβ42 monomer and it shows a clear monomer band at 4 kDa under reducing and non-reducing conditions. In the reduced sample a faint band can be seen at 14 kDa, which could mean that there was contamination or that the protein has aggregated. Under non-reducing conditions we can see clear bands at 14 and 17 kDa, which is expected and shows that the protein has aggregated.

Sample 4 is the collected Aβ (MC1-42) monomer peak. Under reducing and non-reducing conditions only the monomer band can be seen at 4 kDa. Under non reducing conditions the band is faint, this could be caused by the low amount that was loaded on the gel. No higher mass bands are visible.

Sample 5 is the labeled monomer peak of Aβ (MC1-42) (Figure 6). As in sample 4 only the monomer band at 4 kDa is visible under reducing and non-reducing conditions. The band is more faint, and as in sample 4 it could be caused by a low amount of protein that was loaded on the gel.

Sample 6 is a side fraction of the labeled monomer peak in sample 5. Clear monomer bands can be seen under reducing and non-reducing conditions. Under non-reducing conditions faint bands at 14-17 kDa can be seen, suggesting protein aggregation.

Sample 5 and 6, with the labeled Aβ (MC1-42), seems to migrate slower than the wild-type peptide, suggesting that the labeled monomers are bigger.

Sample 7 is from the peak eluting after the labeled monomer peak at 21 ml in Figure 6. No bands are visible under reducing or non-reducing conditions which strongly suggests that it was salt eluting after the monomer.

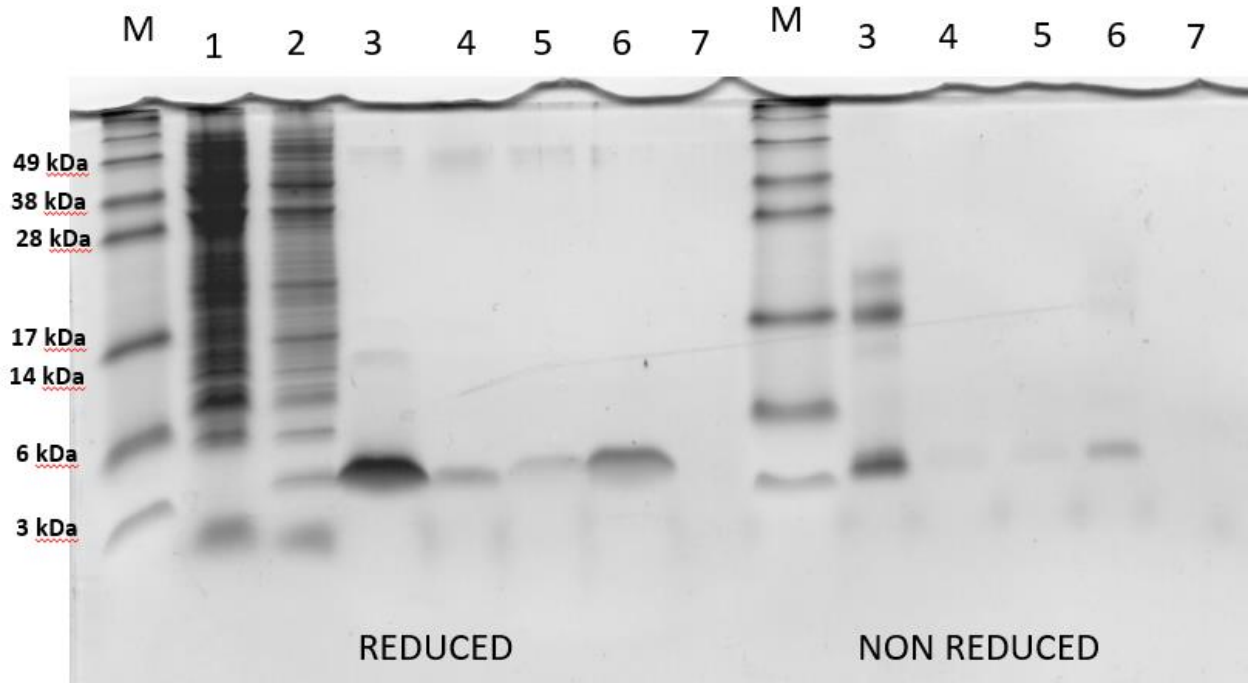

Figure 9 Samples 1 and 2 are samples from before and after induction, a monomer band at 4 kDa is visible only after induction. Sample 3 is WT Aβ42, sample 4 is Aβ (MC1-42), sample 5 is labeled Aβ (MC1-42), sample 6 is the side peak of the labeled Aβ (MC1-42), and sample 7 is salt eluting after the labeled monomer. All samples were run under both reduced and non-reduced conditions and monomer bands at 4 kDa for all the samples, except sample 7, are visible.

pFTAA curves for WT Aβ42, Aβ (MC1-42), and labeled Aβ (MC1-42)

pFTAA assays were run to see if it would show different curves for WT Aβ42, pure unlabeled Aβ (MC1-42) and labeled Aβ (MC1-42). pFTAA should be more sensitive than ThT, so we would expect to be able to detect also intermediates that are low in abundance.

Figure 10 shows that the fluorescence increase measured over time at 510 nm and 540 nm are almost identical. No fibrillation can be detected for unlabeled and labeled Aβ (MC1-42), this could mean that the ATTO Oxa11 (ATTO-TEC) dye interacts with pFTAA. To test this, we would need to run a new pFTAA test with the same controls as in Figure 8.

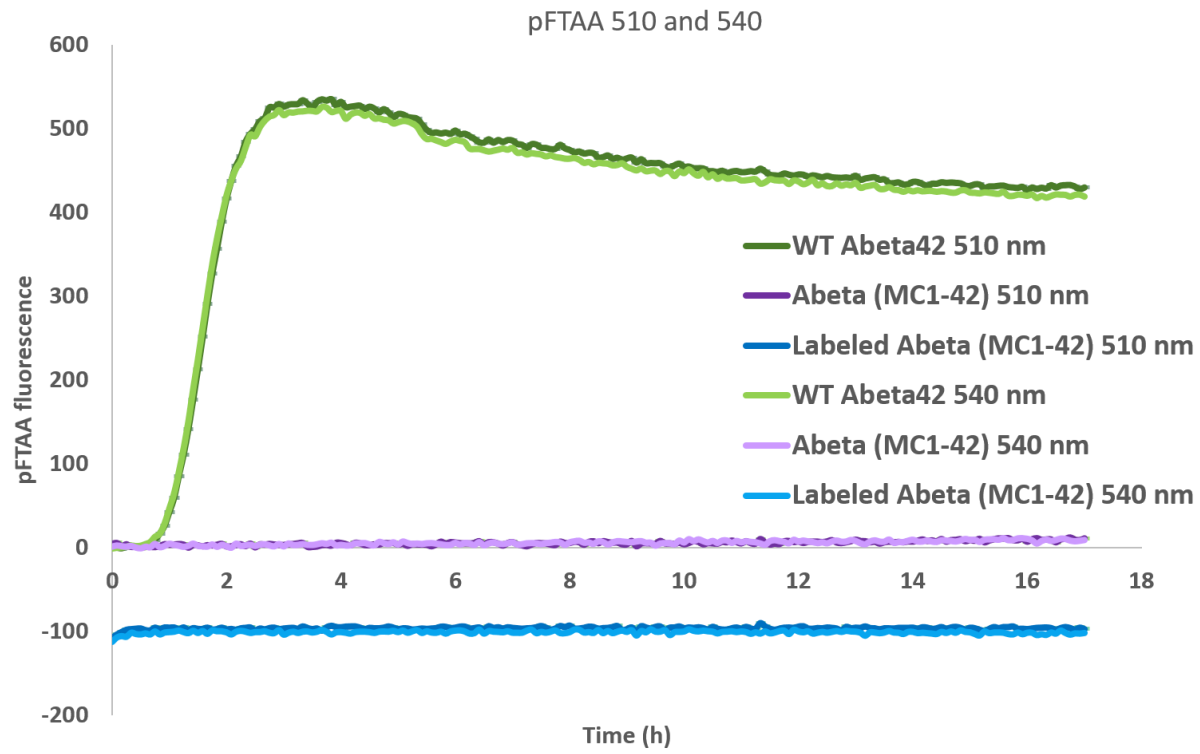

Figure 10 pFTAA fluorescence curves for WT Aβ42, Aβ (MC1-42) and labeled Aβ (MC1-42) for both emission wavelengths 510 nm and 540 nm. The curves look almost identical at both wavelengths and only show fibrillation for WT Aβ42.

Cell incubation with labeled Aβ (MC1-42)

Figure 11 shows the nuclei of HEK293 stained with DAPI (blue) and Aβ (MC1-42) labeled with ATTO Oxa11 (ATTO-TEC) (red) at concentrations of 4.8 µM (Fig. 11A and B), 2.4 µM (Fig. 12 C and D), and 0.5 µM (Fig. 11E and F). The labled monomerhad a DOL of 11.56%.

Figure 11A and B show colocalization of Aβ (MC1-42) (red) and the cell nuclei (blue) at protein concentration of 4.8 µM. In Figure 11 B, a red signal is visible around most of the cell nuclei. In Figure 11 A the red signal is clearly visible around one nucleus, which could mean the peptide was taken up by the cell or that it interacts with the cell surface.

At protein concentrations of 2.4 µM and 0.5 µM (respectively Figures 11C and D, and 11E and F), the red signal is a lot weaker, but single spots where the signal is higher can still be found, for example in Fig. 11C and Fig. 11F.

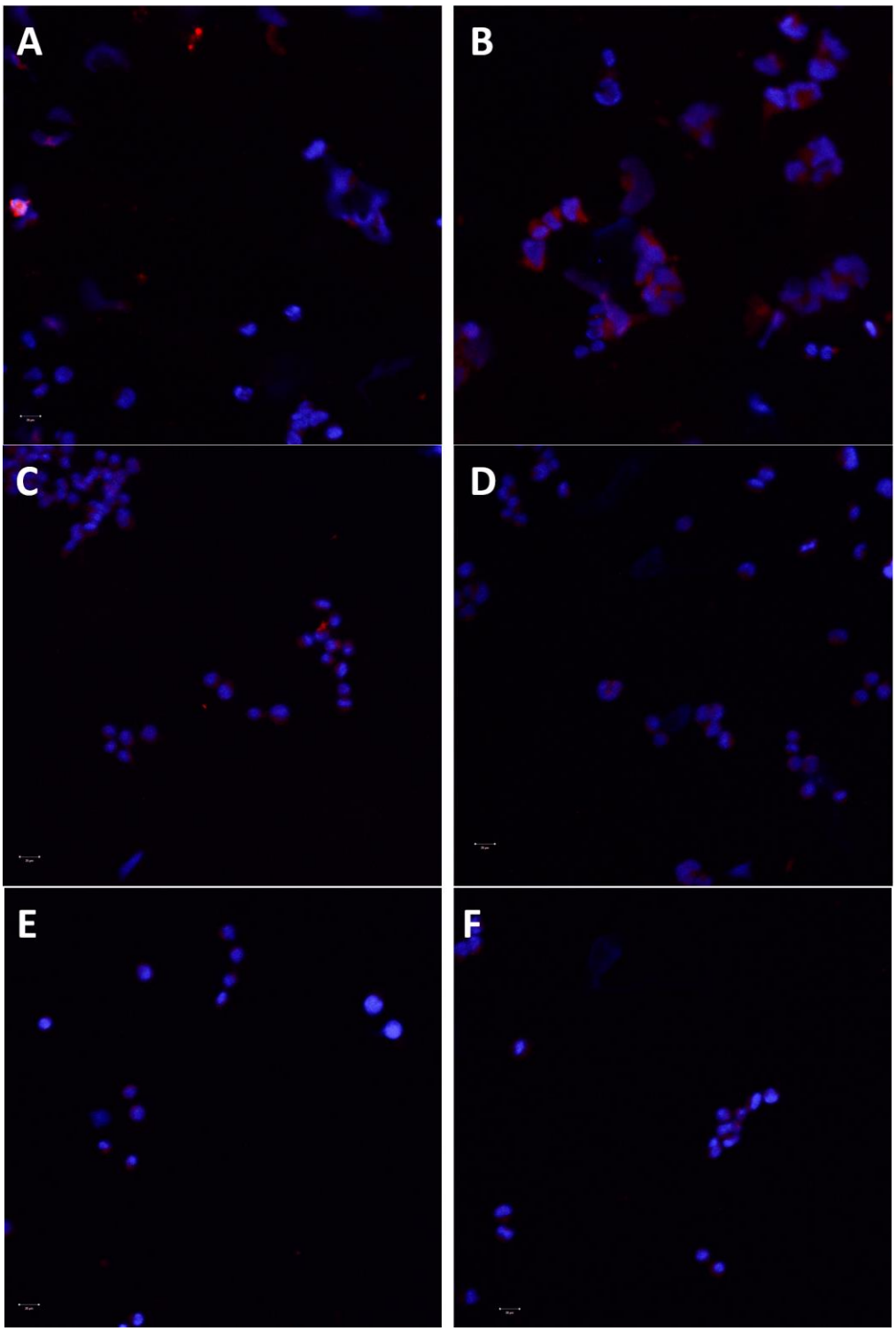

Figure 11 Microscopic images of HEK293 cells incubated with labeled Aβ (MC1-42) with different concentrations: 4.8 μM (A and B), 2.4 μM (C and D) and 0.5 μM (E and F). The nuclei of the HEK 293 cells were stained with DAPI, shown in blue. The Aβ (MC1-42) labeled with ATTO Oxa11 (ATTO-TEC) is shown in red.

Microglia interactions of Aβ

Figure 12 shows labeled Aβ (MC1-42) and human CHME3 microglia cells. The cells were incubated with a 2.1 µM concentration of Aβ (MC1-42) for 3 hours. Figure 12C could indicate that the peptides have been phagocytosed by the cells.

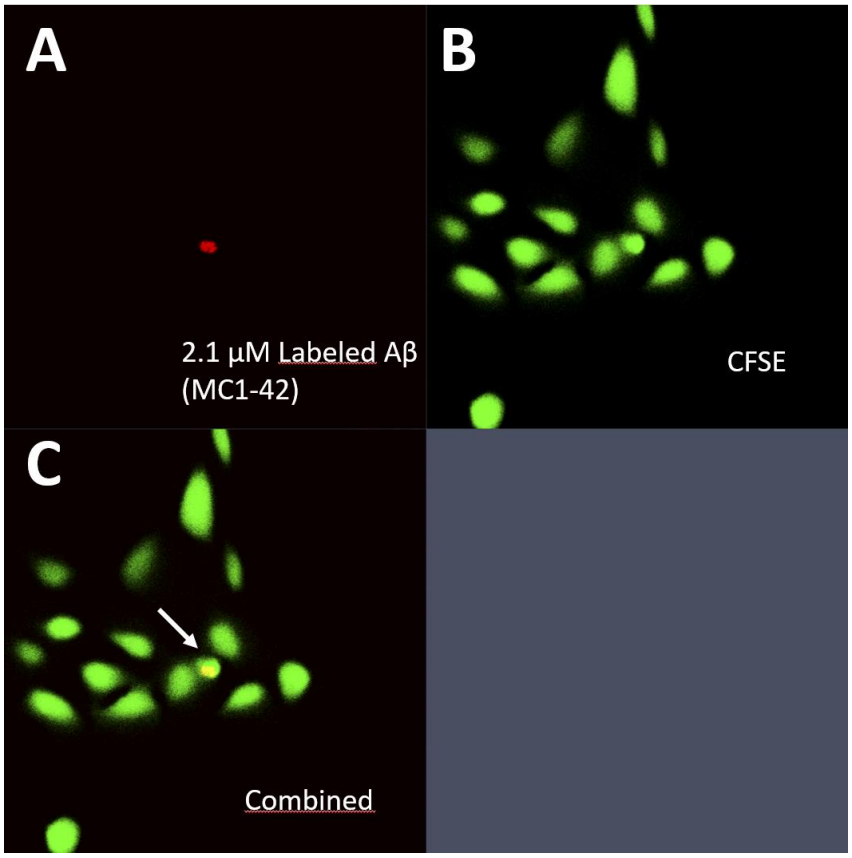

Figure 12 In Fig A, the fluorescence from ATTO Oxa11 (ATTO-TEC) labeled Aβ (MC1-42) is pictured. CSFE stained human CHME3 microglia are shown in Fig. B. In Fig C the previous pictures are superimposed and seem to colocalize.

Discussion

The aim of this study was to improve the labeling and monomer purification protocol for the Aβ (MC1-42) peptide. The amount of labeled Aβ monomers is important for the detection during cell experiments, which could facilitate the studies of Aβ toxicity and cellular uptake.

Labeling

The dye used to label the peptide in this study was ATTO Oxa11 (ATTO-TEC), which reacts with the Cys thiol groups.

To get a good label degree, the pH of the Gu-HCl was important, in fact when the pH was 7 the labeling failed, while when the pH of the buffer was adjusted to 8 we could see an improved labeling, with an increased degree of labeling from 4 % in previous studies to 11-13%.

The amount of dye and TCEP were also changed, but we tried to always have a 5 or 6-fold excess of dye compared to the amount of protein. Even when we used a doubled amount of dye the labeling results were not better than the ones we got for attempt 3 in Table 2.

In the first to attempts we used 10 mM concentration of TCEP, which we then calculated was a 200-fold excess, which could be the reason the first two attempts in Table 2 failed. When we lowered the TCEP concentration to 1 mM, the labeling seemed to be more successful.

In all the SEC curves for the successful labeling attempts we can see that the two curves at 280nm and 658 nm are not overlapping. This could be caused by the dye interacting with the sepharose in the column.

Aggregation kinetics

ThT and pFTAA assays were run to make sure that the dye does not affect the fibrillation of the peptide. The ThT curves in Figures 4, 7 and 8 show that there may be an interference or that the dye could inhibit ThT binding to the peptide. In Figure 4 the curves for WT Aβ42 seem to be lower than in Figure 7 and 8. The assays need to be rerun to find possible explanations to this. A way to see if the dye affects the ThT binding to the protein would be to look at the fluorescence of the labeled fibrils with added ThT to see if there is a superimposition of the dye and ThT.

The pFTAA assay should also be rerun to see if an increase of fibrillation could be detected for labeled and unlabeled Aβ (MC1-42) and more controls similar to the ones used in Figure 8 should be used to see if the dye interacts with pFTAA and inhibits the detection of fibril formation.

Cell experiments

Usually for the cell experiments, astrocytes are used; but because no astrocytes were available, we used HEK 293 cells. These cells are easy to cultivate and grow fast, but they are kidney cells which means that they do not behave as astrocytes and microglia. We did not expect to see colocalization with the labeled Aβ and HEK 293 cells, but in Fig 11 it is visible. Some factors have to been taken into account: e.g. when a lot of cells cluster together, the labeled protein can get caught and give an unspecific signal, which could be the case for some cluster of cells in Figure 11, but there are single DAPI stained nuclei, the most obvious one in Fig 11.A, where it seems to be a specific signal. Also we cannot deduce from Figure 11 whether it is labeled

monomer or free dye that is detected, or whether the labeled peptide has been internalized or if it is just unspecifically bound to the plasma membrane.

When we looked at the cells, we could see that they were dead, probably due to drying during fixation and washing, but it could be due to Aβ toxicity. To know what the cause of death was, we should have looked at the cells in the microscope after incubation with Aβ (MC1-42) and during the fixing steps. The next step in the study after the confocal experiment would have been a toxicity assay.

Microglia experiments

The microglia experiment was a pilot study. Colocalization of labeled Aβ with cells could be detected, but the signals were far too few to be trustworthy without more data. Because these results showed that ATTO Oxa11 (ATTO-TEC) can be detected, and that colocalization is visible, further studies should be done with higher concentrations of Aβ and longer incubation times.

Future perspectives

More studies are required to improve the reliability of these findings. There is a need of better labeling protocols of Aβ42 to facilitate cellular studies, that can help to understand how the molecular processes of the protein affects toxicity, how it is taken up and degraded by microglial cells, how the peptide activates macrophages and how it interacts with the blood-brain-barrier, for example (Jungbauer et al, 2009). With more knowledge about the molecular processes and interactions of Aβ, the hunt for effective therapies against AD may be facilitated.

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References

Purves, Dale, George J. Augustine, David Fitzpatrick, William C. Hall, Anthony-Samuel LaMantia, and Leonartd E. White, "Neuroscience" 5th edition, Sinauer, (2012): 713-714.

Dunning, Christopher J., Gavin McGuaran, Katarina Willén, Gunnar K. Gouras, David J. O'Connel, and Sara Lise. "Direct High Affinity Interaction between Aβ42 and GSK3α Stimulates Hyperphosphorylation of Tau. A New Molecular Link in Alzheimer's Disease?"ACS Chemical Neuroscience (2016), 7: 161-170.

Finder, Verena H., and Rudi Glockshuber. "Amyloid-β Aggregation" Neurodegenerative diseases (2007), 4:13-27

Cappai, Roberto and Anthony R. White. "Amyloid β" The International Journal of Biochemistry and Cell Biology (1999)31: 885-889.

Cohen, Samuel I A, Paolo Arosio, Jenny Presto, Firoz Roshan Kurudenkandy, Henrik Biverstål, Lisa Dolfe, Christopher Dunning, Xiaoting Yang, Birgitta Frohm, Michele Vendruscolo, Jan Johansson, Christopher M Dobson, André Fisahn, Tuomas P J Knowles, and Sara Linse. "A molecular chaperone breaks the catalytic cycle that generates toxic Aβ oligomers" Nature Structural and Molecular Biology (2015) 22: 207-213

Ow, Sian-Yang, and Dave E Dunstan. "A Brief Overview of Amyloids and Alzheimer's Disease." *Protein Science : A Publication of the Protein Society*23.10 (2014): 1315–1331. *PMC*. Web. 30 Mar. 2016.

Masters, Colin L., and Dennis J. Selkoe. "Biochemistry of Amyloid b-Protein and Amyloid Deposits in Alzheimer Disease" Cold Spring Harb Perspect Med 2012;2:a006262

Åslund, Andreas, Christine J. Sigurdson, Therése Klingstedt, Stefan Grawthwohl, Tristan Bolmont, Dara L. Dickstein, Eirik Glimsdal, Stefan Prokop, Mikael Lindgren, Peter Konradsson, David M. Holtzman, Patrick R.Hof, Frank L. Heppner, Samuel Gandy, Mathias Jucker, Adrian Aguzzi, Per Hammarström, and K. Peter R. Nilsson. "Novel pentameric thiophene derivates for *in vitro* and *in vivo* optical imaging of a plethora of protein aggregates in cerebral amyloidosis" ACS Chemical Biology (2009) 4 (8): 673-684.

Jungbauer, L. M., C Yu, K. J. Laxton, and M. J. LaDu. “Preparation of fluorescently-labeled amyloid-beta peptide assemblies: the effect of fluorophore conjugation on structure and function” Journal of Molecular Recognition (2009) 22(5): 403-413

Nasir, Irem, Sara Linse, and Celia Cabaleiro-Lago. “Fluorescent Filter-Trap Assay for Amyloid Fibril Formation Kinetics in Complex Solutions” ACS Chemical Neuroscience (2015)6 :1436-1444