The Alzheimer Aβ Peptide:
Identification of Properties Distinctive for Toxic Prefibrillar Species

Anna-Lena Göransson
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To Patrick,
Holger and Helga
Abstract

Proteins must have specific conformations to function correctly inside cells. However, sometimes they adopt the wrong conformation, causing dysfunction and disease. A number of amyloid diseases are caused by misfolded proteins that form amyloid fibrils. One such disease is Alzheimer’s disease (AD). The protein involved in this deadly disease is the amyloid β (Aβ) peptide. The formation of soluble prefibrillar oligomeric Aβ species has been recognized as an important factor in the development of AD. The aim of work described in this thesis was to investigate which properties of these oligomeric species can be linked to toxicity. We approached this task by comparing the aggregation behavior and biophysical properties of aggregates formed by variants of the Aβ peptide that have been shown to differ in neurotoxicity when expressed in the central nervous system (CNS) of Drosophila melanogaster. A combined set involving different fluorescent probes was used in parallel with transmission electron microscopy. The toxicity of species formed during the aggregation process was examined by exposing human SH-SY5Y neuroblastoma cells to Aβ aggregates. We deduced that there is a correlation between cell toxicity and the propensity of the Aβ peptide to form small prefibrillar assemblies at an early stage of aggregation in vitro. Moreover, these prefibrillar species were characterized by their ability to be recognized by pentamer formyl thiophene acetic acid (p-FTAA) and the presence of exposed hydrophobic patches. We also found that larger aggregates did not induce cell death.
Included papers

This thesis is based on the following papers which are included in the last section

Paper I

Identification of distinct physiochemical properties of toxic prefibrillar species formed by Aβ peptide variants
Anna-Lena Göransson, K. Peter R. Nilsson, Katarina Kågedal and Ann-Christin Brorsson
Accepted for publication in BCBR: doi 10.1016/j.bbr.2012.03.097

Paper II

Dissecting the aggregation events of Alzheimer's disease associated Aβ peptide variants by the combined use of different fluorescent probes
Anna-Lena Göransson, Mildred Otieno, K. Peter R. Nilsson and Ann-Christin Brorsson
Manuscript
### Abbreviations

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<th>Description</th>
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<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilino-1-napthalenesulfonate</td>
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<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>HFIP</td>
<td>1, 1, 1, 3, 3, 3-hexafluoro-2-propanol</td>
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<tr>
<td>LCO</td>
<td>luminescent conjugated oligothiophene</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazoliumbromide</td>
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<tr>
<td>n-FTAA</td>
<td>nonamer formyl thiophene acetic acid</td>
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<tr>
<td>p-FTAA</td>
<td>pentamer formyl thiophene acetic acid</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>wt</td>
<td>wild type</td>
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Preface

Proteins are essential molecules for life but can sometimes malfunction, causing devastating diseases. This thesis focuses on one such malfunctioning peptide, Amyloid β, which has been implicated in Alzheimer’s disease.

It has been a privilege to spend three years researching the mysterious and fascinating world of proteins. During my work, which was mainly conducted at the Department of Physics, Chemistry and Biology, Linköping University, I have deepened my theoretical knowledge in protein science and gained valuable insights into how protein research is carried out.

During the course of research, I was also enrolled in the Swedish National Graduate School in Science, Technology and Mathematics Education Research (FontD). Within this program, I've had the opportunity to reflect on current science education and consider how it should best be implemented to maintain and improve a student's interest in scientific studies.
Protein structure
Proteins perform a variety of vital functions in our cells and bodies. Apart from being the main building blocks of cells, they also store and transport different molecules, are central for enabling movement, protect our bodies from invading microorganisms via the immune response and function as enzymes, catalyzing all kinds of chemical reactions required in the cells. Proteins comprise chains of amino acids with a backbone constructed by linkages between amino and carboxylic acid groups. Each amino acid has a different side chain attached to the α-carbon; there are 20 naturally occurring amino acids with different properties according to their side chain. A peptide bond with partially delocalized electrons connects the amino group of one amino acid to the carboxyl group of an adjacent amino acid (Figure 1).

![Figure 1: The general formula of an amino acid (left) and two amino acids linked by a peptide bond (right) where R marks the side chain.](image-url)
Each protein has a unique sequence of amino acids, which determines the shape and thereby the function of the protein. The specific order of amino acids is regarded as the first structural level of a protein and is referred to as the primary structure (Figure 2).

![Amino acid sequence, governing the primary structure of a protein.](image)

Figure 2: Amino acid sequence, governing the primary structure of a protein.

The highly polar backbone gives rise to the next structural level: hydrogen bonds are formed between amino hydrogen and carboxyl oxygen atoms, stabilizing the secondary structure. Depending on the side chains, two main types of secondary structure are formed in regions of the amino-acid chain: α-helices or β-sheets (Figure 3).

![α helix and β sheet structures determine the tertiary structure.](image)

Figure 3: α and β structures determine the tertiary structure.

These structures were first discovered in studies of hair and silk more than 50 years ago (Marsh et al., 1955; Pauling et al., 1951). The first structure to be discovered was the α-helix located in α-keratin, a constitutive protein of skin, hair, and nails, whereas β-sheets were found in the protein fibroin, the main component of silk. In an α-helix, the amino-acid chain is twisted around itself and forms a rigid cylinder with hydrogen bonds between every fourth peptide bond, giving rise to a regular helix with 3.6 amino acids residues per turn. β-sheets are formed by neighboring β-strands that run in either the same (parallel) or opposite (anti-parallel) directions. In both types of
β-sheets, the β-strands are held together with hydrogen bonds, resulting in a very rigid structure. The β-strands in a β-sheet are not necessarily close to each other in the amino acid chain. The final three-dimensional structure of a protein when it is properly folded is referred to as the tertiary structure. This final conformation is stabilized by hydrophobic interactions, hydrogen bonds, electrostatic attractions, van der Waals attractions and covalent disulphide bridges between formed between different parts of the chain. Both atoms in the backbone and atoms in the side chains are involved in these interactions. When a protein is properly folded, its conformation is referred to as the native form of the protein.

**Protein aggregation**

The tertiary structure of a protein is governed by the primary structure, but folding is also influenced by environmental factors which affect interactions with the amino-acid chain. Until a critical number of tertiary interactions are established, the protein is very sensitive to factors that can interfere with formation of the structure. Thus, partly folded peptides may establish different interactions compared to when they are correctly folded. Hence, proteins may misfold and form aggregates with a predominance of β-sheet conformation, in contrast to native proteins, which commonly contain a mixture of α-helices and β-sheets. Misfolding can result from mutations causing changes in the primary structure which disturb normal folding. Misfolded proteins can cause disease, either because of the loss of a functional protein (Cystic fibrosis), the toxicity of aggregated species (Alzheimer’s disease) or accumulation of insoluble fibrils and plaques (Lysozyme amyloidosis) (Dobson, 1999; Soto, 2001). Such insoluble fibrils are often referred to as amyloid fibrils.

**Amyloid**

The definition of amyloid, according to the Nomenclature Committee of the International Society of Amyloidosis, is a deposit of fibrils composed of proteins in a tissue that is detectable by transmission electron microscopy (TEM) and gives a characteristic X-ray diffraction pattern. The amyloid must have an affinity for Congo red and cause green birefringence when viewed under polarized light (Westermark et al., 2005). ThT binding is also a typical property of
amyloid fibrils and is a well-established method for following amyloid fibril formation in vitro (Westermark et al., 1999). A mature amyloid fibril often consists of protofilaments entwined in a rope-like structure with a diameter of 5-10 nm and length up to a few micrometers (Jimenez et al., 1999; Jimenez et al., 2002). Amyloid fibrils are often unbranched, very stable and resistant to protease degradation and denaturants (Jimenez et al., 2002; Serpell et al., 2000). Because degradation of these fibrils is difficult in cells, they can accumulate and form plaques in different organs or systems in the body. These abnormal deposits can eventually harm the body and cause a condition known as amyloidosis. In Table 1, some examples of amyloidosis are listed together with the proteins associated with the different diseases. Depending on where in the body amyloid is present, the disease is classified as either systemic amyloidosis, which affects more than one organ or system, or as localized amyloidosis, which affects only one organ or tissue type (Westermark et al., 2002).

<table>
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<td>Gesolin amyloidosis</td>
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<td>Fibrinogen amyloidoses</td>
<td>Fibrinogen</td>
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<td>AA amyloidosis</td>
<td>Fragments of serum amyloid A protein</td>
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<tr>
<td>Medullary carcinoma of the thyroid</td>
<td>Calcitonin</td>
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Tabell 1: Examples of amyloidosis and the protein associated with the disease.

**Alzheimer’s disease**

In 1906, the German psychiatrist and neuropathologist Alois Alzheimer gave a lecture in which he described a form of dementia that later, after a suggestion from Emil Kraepelin, became known as Alzheimer’s disease (AD). Alois Alzheimer described the case of Auguste Deter, who at the age of 51 had developed progressive...
cognitive impairment, focal symptoms, hallucinations, delusions and psychosocial incompetence. After her death at the age of 55, Alzheimer examined her brain and discovered plaques, neurofibrillary tangles and arteriosclerotic changes (Maurer et al., 1997).

When this first case of AD was reported, the average life expectancy was much shorter than today and thus incidences of the disease were relatively uncommon. Today, AD is the most common cause of dementia and in individuals aged over 60 years, is more prevalent than stroke, musculoskeletal disorders, cardiovascular diseases and cancer. Worldwide, 30 million people suffer from AD, and as the average life expectancy increases, it is predicted that the number of cases will quadruple in the next 40 years (Minati et al., 2009).

AD exists in both familial and sporadic forms. The familial form, which is responsible for about 5% of cases, is caused by mutations in single genes that are inherited in an autosomal-dominant way. The inherited form often has an earlier age of onset than the sporadic form, which seldom occurs before the age of 65. Age is in fact the primary risk factor for the sporadic form; over the age of 65, the risk of AD doubles every 5 years (Ferri et al., 2005).

The disease is psychologically characterized by a decline in certain cognitive functions as well as changes in personality and behavior. One of the earliest symptoms is impairment of recent memory (Selkoe, 1991; D. M. Walsh et al., 2005). Physiologically, AD is characterized by the presence of extracellular neurotic plaques composed of the amyloid β (Aβ) peptide and intracellular neurofibrillary tangles composed of the hyperphosphorylated tau protein (Walsh & Selkoe, 2004b). A reduction in brain volume is typically observed in AD patients post-mortem.

Since 1992, the amyloid cascade mechanism has been the most important hypothesis defining the neuropathological hallmarks of AD (Hardy & Higgins, 1992). This hypothesis considers that Aβ deposits, which are caused by an imbalance in Aβ production and clearance, are the causative agent for AD and that neurofibrillary tangles, cell loss, vascular damage and dementia are consequences of these deposits. However, neuropathological studies in humans and evidence from transgenic mouse models of AD (Lambert et al., 1998;
Lue et al., 1999; McLean et al., 1999; Mucke et al., 2000) have prompted a paradigm shift. Instead of assuming that fibrils are the cause of AD, it is now proposed that fibril precursors and soluble oligomeric structures are the key species involved in the pathogenicity of AD (Cleary et al., 2005; Haass & Selkoe, 2007; Haass & Steiner, 2001; Kirkitadze et al., 2002; Klein et al., 2001; Klein et al., 2004; Walsh & Selkoe, 2004a). This paradigm shift has led to a revised amyloid cascade hypothesis, in which intermediates formed at an early stage of the aggregation process of amyloid β are implicated as the main causative agents of AD (Hardy & Selkoe, 2002). The main challenges facing researchers are the identification of the specific Aβ species responsible for cell toxicity and elucidation of the mechanism by which the Aβ aggregates lead to neurodegeneration.

**Amyloid β precursor protein and Aβ**

The precursor protein of the Aβ peptide, which is the major component of plaques associated with Alzheimer’s disease (AD), is known as the amyloid β precursor protein (APP). APP is a transmembrane glycoprotein whose biological function is not clear, but it seems to be involved in neurite outgrowth, synaptic function, induction of apoptosis and cell adhesion (Koo, 2002).

The APP gene is located in chromosome 21, the same chromosome as that triplicated in Down’s syndrome (Robakis et al., 1987). Individuals with Down’s syndrome exhibit Alzheimer-type pathology if they reach the age of 50, and this observation pointed towards a possible link between the APP gene and AD (Oliver & Holland, 1986; Tanzi et al., 1987).

APP exists in three splicing forms: APP695, APP751 and APP770. APP695 is expressed in neuronal cell membranes (Haass et al., 1991). As shown in Figure 4, the Aβ region of APP spans the membrane, and APP is processed through two competing pathways: one is non-amyloidogenic and the other is amyloidogenic. The initial cleavage takes place near to the membrane and is catalyzed by the enzyme α-secretase or β-secretase, releasing soluble extracellular N-terminal α-sAPP or β-sAPP, respectively. α-secretase cleaves APP within the Aβ region, and thereby prevents the release of the full-length Aβ peptide.
Figure 4: Processing of APP through two different pathways: one non-amyloidogenic and one amyloidogenic.

The C-terminus fragment (α-CTF) is cleaved by γ-secretase, releasing a harmless p3 fragment. β-secretase cleavage, on the other hand, leaves the Aβ region attached to the C-terminus fragment (β-CTF), and after further processing by γ-secretase, the Aβ peptide is released. In both cases, an APP intracellular domain (AICD) is generated by γ-secretase cleavage. γ-secretase is able to cleave β-CTF at different sites, resulting in the formation of different isoforms of Aβ with lengths ranging from 39 to 43 amino acids, of which 40 or 42 amino acids are the most common (Teplow, 1998). The Aβ42 peptide is more prone to aggregation and is also more toxic than Aβ40. The accumulation of Aβ in the brain is a consequence of an imbalance in production and clearance of Aβ and/or an increase in the Aβ42/Aβ40 ratio (Kuperstein et al., 2010; Mawuenyega et al. 2010).
The amino acid sequence of Aβ42 is:

Asp - Ala - Glu - Phe - Arg - His - Asp - Ser - Gly - Tyr - Glu - Val - His -
His - Gln - Lys - Leu - Val - Phe - Phe - Ala - Glu - Asp - Val - Gly - Ser -
Asn - Lys - Gly - Ala - Ile - Ile - Gly - Leu - Met - Val - Gly - Gly - Val -
Val - Ile – Ala

The Aβ peptide possesses an amphiphilic structure with a hydrophilic N- and hydrophobic C-terminus. The peptide overall is highly hydrophobic, containing about 45% hydrophobic amino acids. Due to its hydrophobic nature, it is known to interact with lipid or model membranes and forms an α-helical structure (Kirkitadze et al., 2001; Terzi et al., 1997). However, when integrated in an amyloid fiber, the Aβ peptide forms β strands, which can be visualized as a hairpin with two anti-parallel β strands in the C-terminus of Aβ. The Aβ40 hairpin is stabilized by electrostatic interactions between the negatively charged aspartic acid and the positively charged lysine. These “hairpins” stack on top of each other in amyloid Aβ fibrils forming cross-β structure (Figure 5). (Luhrs et al., 2005; Petkova et al., 2002; Serpell et al., 2000).

![Figure 5: In the amyloid fiber, the C terminal of the Aβ peptide forms two β strands which can be visualized as a hairpin. The circles represent amino acids where the blue circles are positively charged and the red circles are negatively charged. Stabilizing interactions are indicated in the bending region of the Aβ40 hairpin Right: A schematic representation of the parallel cross-β structure unit in Aβ40 fibrils.](image)

The formation of intermediate species precedes amyloid formation. These β-sheet-rich oligomeric intermediates are referred to as prefibrillar aggregates and display a wide distribution of sizes and morphologies (Figure 6). Morphologies included in this category are low molecule weight oligomers, which are less than 8-mers and are formed in equilibrium with monomeric Aβ (Bitan et al., 2003; Walsh et al., 2002); protofibrils, which are curvilinear structures shorter
than 200 nm (Harper et al., 1997; Hartley et al., 1999; Walsh et al., 1997); Aβ-derived diffusible ligands (ADDLs) or globulomers, which are spherical aggregates of 3-5 nm diameter (Lambert et al., 1998); Aβ-annular assemblies, which are shaped like donuts with an outer diameter of 8-12 nm (Bitan et al., 2003; Kayed et al., 2009; Lashuel et al., 2003); and Aβ*56 (56kD) assemblies (Lesné et al., 2006)

Non-amyloidogenic monomer Soluble oligomers Amyloid fibrils

Beta sheet structured species Protofibrils

Figure 6: Schematic of a possible pathway for Aβ fibril formation.

**Aβ variants**

There are several well-known pathogenic mutations in the APP gene, both inside and outside the region encoding Aβ. Mutations outside the Aβ gene are located near the β- or γ-secretase cleavage sites and interfere with the cleaving process. Most of the mutations inside the region encoding Aβ result in an amino-acid shift at or nearby position 22. This position resides in the hairpin-forming region of the peptide, and mutations here have been shown to reduce the stability of the bend (Grant et al., 2007; Krone et al., 2008).

One thoroughly studied mutation at this location is the Arctic mutation (E22G), which was first identified in a family in the north of Sweden (Nilsberth et al., 2001). This mutation causes an early onset form of AD, and the resulting variant has been shown to be more neurotoxic than Aβ42 when expressed in the central nervous system (CNS) of *Drosophila melanogaster*, causing a 74% reduction in fly longevity (Brorsson et al., 2010a; Luheshi et al., 2007). *In vitro*, the Arctic peptide forms more prefibrillar species and fewer well-defined fibrils than the wild type (Figure 7). The introduction of a single point mutation (I31E) into the Arctic peptide (here denoted as
the Rescue variant) resulted in no significant neurodegeneration when this peptide was expressed in Drosophila, and in vitro, this variant does not cause accumulation of prefibrillar aggregates but instead produces a large amount of well-defined fibers (Figure 7). These findings support the current hypothesis that it is the early formed oligomeric species that are responsible for the Aβ cell toxicity.

A key question is why does the Arctic peptide form fewer well-defined fibrils than the wt and why does the I31E mutation enhance the propensity for fibril formation? If we compare a schematic model of the β-hairpin motif of Aβ42 (Luhrs et al., 2005) with models of the Arctic and Rescue peptides, we notice that for the Arctic peptide, the amino acid at position 22 is located in the middle of one of the two β strands, and therefore introduction of a negatively charged amino acid could destabilize interactions between the β strands (Figure 8). The Rescue peptide has the I31E mutation located in the turn between the β strands. Thus, in a similar way as for the Aβ40 wt hairpin, the negatively charged glutamic acid may interact with the positively charged lysine, stabilizing the β hairpin (Figure 8). This possible stabilization of the Aβ hairpin could explain why the Rescue peptide is more prone to form fibrils.
Another Aβ variant we have studied is Aβ40 E3R. When this peptide was expressed in the CNS of *Drosophila*, it reduced the longevity by 30% compared to flies expressing the nontoxic Aβ40 wt (Brorsson et al., 2010a). When comparing aggregates formed by Aβ40 wt and Aβ40 E3R, the latter more toxic variant gave rise to a larger number of prefibrillar species and was less prone to form fibrils than the nontoxic Aβ40 wt (Figure 9).

Although the E3R mutation is located outside the β-hairpin forming region of the peptide, introduction of a negatively charged amino acid, Arginine, at this location does somehow disturb fibril formation compared to the Aβ40 wt. Interestingly, for the Aβ42 peptide the N-terminals is not included in the fiber-forming sequence whereas for the in Aβ40 fibrils the N-terminus is buried inside the fiber (Olofsson et al., 2007). Therefore, the E3R mutation in the N-terminal of the Aβ40 might disrupt the fiber formation process.
Aims

The current consensus is that early formed oligomeric intermediates of the Aβ peptide are responsible for the pathogenicity of AD (Cleary et al., 2005; Haass & Selkoe, 2007; Klein et al., 2001; Lambert et al., 1998; Lord et al., 2009). Therefore, it is important to study the prefibrillar species involved in cellular damage and elucidate their biophysical properties. The aim of the work described in this thesis was to determine what properties of these oligomeric species can be linked to toxicity. This was accomplished by comparing the aggregation behavior and examining the resulting aggregated species for different variants of the Aβ peptide. These Aβ variants induce different degrees of neurotoxicity when expressed in the CNS of Drosophila melanogaster. Figure 10 summarizes properties of the Aβ peptides studied. The work has resulted in the completion of two papers, which are briefly summarized in the final section of the thesis.
Figure 10: Overview of five variants of the Aβ peptide A) Hairpin model of the peptide. B) Described morphology of aggregated species. C) Relative toxicity when expressed in the CNS of Drosophila visualized as red bars.
Methodology

Protein preparation
When investigating protein aggregation, it is important to start with monomeric solutions without any preformed aggregates, which can seed the aggregation process. Aβ peptides are difficult to handle because of their high propensity to aggregate. Synthetic Aβ peptides can be dissolved in low pH solutions of trifluoroacetic acid (TFA) and 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP). This method has been shown to be successful for solubilizing synthetic Aβ peptides (Brorsson et al., 2010b).

In vitro fibrillation
The development of the fibrillation process is highly dependent on the conditions used during aggregation. These conditions (peptide concentration, temperature, buffer properties, quiescent or shaken incubation) also affect the morphology of the species formed (Johansson et al., 2006). This has to be taken into account when designing the experiment.

Fluorescence spectroscopy
Fluorescence spectroscopy can be used as a highly sensitive method for the characterization of protein conformational transformation. Extrinsic dyes can bind covalently to proteins or attach noncovalently through hydrophobic or electrostatic interactions (Hawe et al., 2008). Fluorescence refers to the phenomenon where a
molecule absorbs light of a given wavelength, displacing an electron from its ground state to a higher energy level. When the excited electron returns to the ground state, energy is released as a photon, which is observed as fluorescence. As a result of internal conversion, some of the energy in the excited state is lost due to rotational and vibrational relaxation. Thus, the emitted light is usually of lower energy, and thereby longer wavelength, than the absorbed light. The difference between the excitation and emission energy is known as the Stokes shift. The process is illustrated by a Jablonski diagram (Figure 11). Environmental factors affect the fluorescence intensity maximum of a fluorophore (Hawe et al., 2008).

![Jablonski diagram illustrating the fluorescence of a fluorophore.](image)

**Thioflavin T (ThT)** is a benzothiazole dye (Figure 12) which, since its introduction in 1959, has been used to determine the presence of amyloid fibrils in tissue samples and for real-time monitoring of the self-assembly kinetics of fibril formation in vitro (Naiki et al., 1989; Vassar & Culling, 1959). When ThT binds to amyloid fibrils, it displays dramatic shifts in the excitation maximum from 385 to 450 nm and emission maximum from 445 to 482 nm. ThT fluorescence depends on the presence of cross-β structure in fibrils and it does not recognize the proteins in its native form, amorphous aggregates or non-amyloidogenic oligomers (LeVine, 1999). A typical ThT curve acquired during fibril formation shows an initial nucleation polymerization process, which begins with a lag phase dominated by
monomeric species, followed by a nucleating phase where β-sheet-rich oligomers act as nuclei for the aggregation process. During the growth phase, monomers add to the growing fibrils. Eventually the process reaches a steady state (Figure 12).

Pentamer formyl thiophene acetic acid (p-FTAA) is a luminescent conjugated oligothiophene (LCO) that was introduced in 2009 (Åslund et al., 2009). LCOs have a flexible backbone and can adopt different geometrical forms. However, interactions with a protein can cause the backbone to become conformationally restricted, altering the fluorescence. A planar conformation results in a red shift in the fluorescence emission spectra, whereas a twisted backbone gives rise to a blue shift. Intermolecular stacking of LCOs also produces a red shift. LCOs emit a remarkably intense fluorescence with a distinct emission profile when bound to amyloid aggregates (Klingstedt & Nilsson, 2010; Nilsson et al., 2007). The emission curve of p-FTAA exhibits a double peak at 505 and 540 nm when bound to Aβ aggregates (Figure 13). P-FTAA can be used to detect non-thioflavinophilic pre-fibrillar aggregates of Aβ that appear prior to the aggregates recognized by ThT (Klingstedt et al., 2011; Åslund et al., 2009).

Figure 12: Left) the structure of ThT. Right) ThT fluorescence curve probing an Aβ aggregation process.

Figure 13: Left: The structure of p-FTAA. Right: Emission spectra of p-FTAA binding to aggregated species of Aβ.
8-Anilino-1-napthalenesulfonate (ANS) is widely used in protein-folding studies (Figure 14). When ANS binds to solvent-exposed hydrophobic regions on protein surfaces, its emission intensity increases and its emission maximum shifts from about 505 nm (in water) towards shorter wavelengths (Hawe et al., 2008; Semisotnov et al., 1991; Stryer, 1965).

![Figure 14: The structure of ANS.](image)

**Transmission electron microscopy (TEM)**

In TEM, a beam of high energy electrons is passed through a specimen to generate an image of the analyzed sample. The beam of electrons is generated by an electron gun and is focused and oriented by electromagnets. When the electrons hit the sample, some are lost as a result of absorption and scattering, while others are transmitted directly through the specimen and eventually impact on a phosphorescent microscope screen, producing an image. The contrast in the image depends on the amount of lost electrons; a shadow with high contrast is achieved when only a small number of electrons pass through the specimen. Regions in the sample containing nuclei of high atomic number are associated with increased scattering of electrons and appear dark against a brighter background. Proteins, which are mainly composed of carbon (with a rather low atomic number), do not give images with high contrast, and therefore staining is used to obtain better images of protein samples. Uranyl acetate, which binds to biological materials with a high affinity and gives high contrast images in TEM, is often used for the negative staining of protein samples.
Cell toxicity
To assess the toxicity of a molecule, a response can be measured after applying it to human SH-SY5Y neuroblastoma cells. One way to analyze the response is to study the appearance of cells with a microscope since toxic molecules can alter the shape of cells (Figure 15). Another common assay used to detect cellular effects examines metabolism of the dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT). In this assay, the yellow tetrazole dye is reduced to a purple formazan by mitochondrial activity, which serves as an indicator of cell viability (Green et al., 1984; Weidner et al., 2011).

Figure 15: Human SH-SY5Y neuroblastoma cells. Left: Control cells with normal shapes. Right: Cells with altered shapes after treatment with toxic $\alpha\beta$ species.
Results and Discussion

Identification of distinct physiochemical properties of toxic prefibrillar species formed by Aβ peptide variants (Paper I)

In this study, we examined the aggregation behavior of three Aβ variants by the combined use of different fluorescent probes monitored by fluorescence spectroscopy during in vitro fibril formation. ThT fluorescence was used to probe the formation of amyloid fibrils, p-FTAA probed prefibrillar non-thioflavinophilic species and ANS fluorescence gave information on the hydrophobicity of the aggregates formed. To determine the morphologies of aggregates during the aggregation process, we employed TEM. To study the toxicity of aggregated species, human SH-SY5Y neuroblastoma cells were exposed to Aβ aggregates formed at different stages of the aggregation process.

The three peptides used in this study were Aβ42 wt, the Arctic variant (Aβ42 E22G) and the Rescue variant (Aβ42 E22G/I31E). It is previously shown that the Arctic peptide is more prone to forming prefibrillar species and also more neurotoxic than Aβ42 wt when expressed in the CNS of Drosophila melanogaster, whereas the Rescue variant abolished pathogenicity (Brorsson et al., 2010a; Luheshi et al., 2007).
The results from fluorescence spectroscopy during the aggregation process of Aβ42 showed that the p-FTAA and ANS signals increased rapidly at the start of the experiment, whereas the ThT signal increased more slowly, indicating that species recognized by p-FTAA (p-FTAA⁺) were formed more rapidly than species recognized by ThT (ThT⁺) (Figure 16A) and these early formed species had exposed hydrophobic patches. We propose that the early formed p-FTAA⁺ species were later converted to ThT⁺ aggregates since the p-FTAA signal decreased while the ThT signal continued to increase during the later part of the experiment. TEM images captured when the p-FTAA signal was still increasing showed a mixture of aggregates with different morphologies, whereas long fibrils were the dominant aggregates in TEM images taken at the end of the aggregation experiment (Figure 16B & C).

Figure 16: Aggregation of the Aβ42 peptide. A) Progression of aggregation probed by ThT (black line), p-FTAA (grey line) and ANS (dotted line). B) TEM images of peptide samples captured after 2.5 h, and C) at the end of the aggregation experiment. Scale bar = 500 nm. D) Results of MTT viability assay after exposing human SH-SYSY neuroblastoma cells to Aβ 42 aggregates formed after 0h, 1h, 5h and 25 h incubation. Viability is expressed as proportion of the MTT fluorescence for untreated cultures (Control).
Cell toxicity experiments revealed that the aggregates formed early in the aggregation process (after one hour) exhibited the highest toxicity to neuroblastoma cells (Figure 16D). At the end of the aggregation experiment, when well-defined fibrils were the dominant species present in the TEM images, no cell toxicity was observed. The main conclusions which can be drawn from the Aβ42 experiments are that early formed and small p-FTAA⁺ hydrophobic species are toxic to cells, whereas the larger aggregates which form later in the aggregation process do not induce cell death.

**Arctic variant**

When probing the aggregation process of the Arctic peptide with p-FTAA and ANS, a significant fluorescence signal was observed for both probes at the start of the experiment, indicating that p-FTAA⁺ hydrophobic prefibrillar species appeared immediately after dissolving the peptide in the buffer (Figure 17A).

The ThT fluorescence signal increased progressively from the beginning of the aggregation until the end of the experiment. TEM

![Figure 17: Aggregation of the Arctic peptide. A) Progression of aggregation probed by ThT (black line), p-FTAA (grey line) and ANS (dotted line). TEM images recorded at the end of the aggregation experiments of Arctic aggregates in the presence of B) ThT, C) p-FTAA, and D) ANS. Scale bar = 500 nm.](image-url)
images captured at the end of the aggregation process showed that the Arctic peptide resulted in the accumulation of a large quantity of prefibrillar species, which were most likely responsible for the high p-FTAA signal observed at the end of the experiment (Figure 17 B-D).

By studying the cell toxicity data for the Arctic peptide, it was apparent that this peptide induced the most cell death when applied to cells immediately after dissolving it in the buffer (Figure 18A,C & F). Aggregates formed at later time points did not induce cell death (Figure 18 A, D, E, G & H).

Figure 18: Cell toxicity and TEM images of Arctic aggregates. A) Viability of human SH-SY5Y neuroblastoma cells after exposure to Arctic aggregates using the MTT viability assay. Viability is expressed as proportion of the MTT fluorescence for untreated cultures. Microscope images of cells after exposure to aggregates of the Arctic peptide formed at C) 0 hours and after D) 12 h and E) 30 h. TEM images of the Arctic peptide at F) 0 h and after G) 12 h, and after H) 30 h of incubation. Scale bar = 500 nm.
The high cell toxicity was most probably induced by the p-FTAA+ hydrophobic species detected in the fluorescence profiles at the very beginning of the aggregation experiment. These instantly formed toxic species could be any of the aggregates visible in the TEM image captured at 0 hours (Figure 18 F).

**Rescue variant**

The fluorescence profiles observed for the Rescue peptide differed from the Aβ42 and Arctic profiles. In the case of the Rescue peptide, a long lag phase was present with all three probes, and the fluorescence intensity for p-FTAA was very low compared to ANS and ThT (Figure 19A).

![Fluorescence profiles](image)

**Figure 19**: Aggregation of the Rescue peptide. A) Progression of aggregation probed by ThT (black line), p-FTAA (grey line) and ANS (dotted line). TEM images captured at the end of the aggregation experiments in the presence of B) ThT, C) p-FTAA and D) ANS. Scale bar = 500 nm.

Thus, no prefibrillar species were formed which could be detected with p-FTAA. In line with the low p-FTAA signal, no prefibrillar species were observed in the corresponding TEM images of the samples captured at the end of the aggregation (Figure 19B-D).
The TEM images suggested that the aggregation process of the Rescue peptide only resulted in an accumulation of mature fibrils, sometimes with a twisted appearance, and these fibrils were well recognized by ThT and ANS. When aggregated under these conditions, the Rescue peptide did not induce cell death (Figure 20A-D). This result supports the Drosophila study, which showed that the Rescue peptide did not exert any significant neurotoxicity (Broersen et al., 2010a).

**Figure 20: Cell toxicity and TEM images of Rescue aggregates.** A) Results of MTT viability assay after exposing human SH-SY5Y neuroblastoma cells to Rescue aggregates formed at 0h, and after 12h and 25 h incubation. Viability is expressed as proportion of the MTT fluorescence for untreated cultures (Control). TEM images of the Rescue peptide at B) 0 h and after C) 12 h, and D) 30 h of incubation. Scale bar = 500 nm.

**Arctic and Rescue under Shaken conditions**

Since stirring and shaking protein samples can affect amyloid fibril formation (Lee et al. 2007; Xue et al., 2009), we repeated the aggregation experiments with the Arctic and Rescue peptides under conditions where the peptide samples were shaken. The resulting TEM images of the Arctic peptide revealed a small difference in the morphologies of the aggregates formed under quiescent versus shaken conditions; fibrils formed under quiescent conditions were longer compared to those formed under shaken conditions.
In both cases, prefibrillar species were evident (Figure 21A & B). More strikingly, aggregates formed by the Rescue peptide under shaken conditions had a completely different morphology, exhibiting short fibrillar species compared to the long and well-defined fibrils formed under quiescent conditions (Figure 21C & D).

Figure 21: Differences between aggregates formed during quiescent and shaken conditions. TEM images recorded at the end of the aggregation experiments in the presence of ThT: A) Arctic variant under quiescent conditions. B) Arctic variant under shaken conditions, C) Rescue variant under quiescent conditions, and D) Rescue variant under shaken conditions. E) Aggregation of Rescue variant under shaken conditions probed by ThT (black line), p-FTAA (grey line) and ANS (dotted line). F) MTT viability assay after exposing human SH-SY5Y neuroblastoma cells to Rescue aggregates formed after 1h and 30 hours incubation under shaking conditions. Viability is shown as a proportion of the MTT fluorescence for untreated cultures (Control). Light microscope images of cells, showing differences in their appearance. G) Control and H) after exposure to Rescue aggregates formed after 1 h. I) TEM images of Rescue aggregates formed after 1 h under shaken conditions. Scale bar = 500 nm.
Interestingly, the fluorescence profiles for the aggregation process of the Rescue peptide under shaken conditions were entirely different from the profiles detected under quiescent conditions (Figure 21E). We found that under shaken conditions, the Rescue peptide formed species that were well recognized by p-FTAA and ThT. These species, which according to the ANS profile contained exposed hydrophobic patches, appeared almost immediately but were rapidly converted to aggregates that could only be recognized by ThT. The cell toxicity data revealed that the only Rescue samples that caused any significant cell death were species formed after 1 hour under shaken conditions (Figure 21F-H). TEM images captured at this stage of the aggregation process showed the presence of a substantial amount of prefibrillar species (Figure 21I). From these results, we can conclude that the p-FTAA+ hydrophobic prefibrillar species that were formed after 1 hour were responsible for the cell death that was mediated by the Rescue peptide when incubated under shaken conditions.

Taken together, our experimental data indicate a correlation between the propensity of the Aβ peptide to form small prefibrillar assemblies early in the aggregation process in vitro and its toxicity towards human neuroblastoma cells. This correlation is also likely to apply to the neurodegenerative properties of the peptide when expressed in the CNS of Drosophila. Moreover, these prefibrillar species are characterized by their ability to give rise to p-FTAA fluorescence and the presence of exposed hydrophobic patches. We also found that larger aggregates were not able to induce cell death. We observed that the Rescue peptide, which was previously shown to have no neurodegenerative properties when expressed in the CNS of Drosophila, produces no toxic aggregates in vitro under quiescent conditions but forms toxic aggregates under shaken conditions. The biophysical properties of the species formed under quiescent and shaken conditions were markedly different; under quiescent conditions, the peptide only formed long mature fibrils and no p-FTAA+ hydrophobic aggregates, whereas under shaken conditions, the peptide produced p-FTAA+ prefibrillar species with exposed hydrophobic patches.
Dissecting the aggregation events of Alzheimer’s disease associated Aβ peptide variants by the combined use of different fluorescent probes. (Paper II)

In a previous study in which two variants of Aβ40 peptides were expressed in the CNS of Drosophila melanogaster, it was found that an Aβ40 peptide with a single point mutation, E3R, caused a reduction in longevity by 30% compared to Aβ40 wt (Brorsson et al., 2010a). To elucidate the correlation between the biophysical properties of Aβ species and their neurodegenerative effects, we here studied the in vitro aggregation process of these two Aβ40 peptides by the combined use of four different fluorescent probes and transmission electron microscopy. ThT was used to detect the formation of amyloid fibrils, while ANS was used to probe the hydrophobic properties of the aggregates formed. We used two different luminescent conjugated oligothiophenones (LCOs), p-FTAA, which has been shown to detect non-thioflavinophilic Aβ species preceding the formation of amyloid fibrils and the novel nonamer formyl thiophene acetic acid (n-FTAA) (Hammarström et al., 2010; Klingstedt & Nilsson, 2010; Klingstedt et al., 2011; Lindgren & Hammarström, 2010; Nilsson et al., 2007; Åslund et al., 2009). The morphologies of species formed were analyzed by TEM and fluorescence microscopy.

From the fluorescence profiles obtained during the aggregation process of Aβ40 wt, we observed that after a short lag phase, species which gave rise in p-FTAA fluorescence were formed earlier than species that gave rise in ThT, ANS and n-FTAA fluorescence (Figure 22A & B). By the time p-FTAA reached its maximum fluorescence, the signals due to ThT and n-FTAA fluorescence had started to increase, whereas the ANS signal was still negligible. When the ThT fluorescence reached its maximum value, the p-FTAA fluorescence was decreasing. Thus, aggregates that gave emission from p-FTAA (p-FTAA+) were formed prior to species that yielded in ThT fluorescence (ThT+). This is in line with previous studies, which showed that p-FTAA recognized non-thioflavinophilic prefibrillar species (Hammarström et al., 2010; Klingstedt et al., 2011; Åslund et al., 2009). In addition, our ANS data indicate that these prefibrillar species do not contain exposed hydrophobic patches. The steady decrease in the p-FTAA fluorescence signal that occurred alongside the increasing ThT fluorescence signal implies increased stacking of
p-FTAA due to the conversion of p-FTAA\(^+\) prefibrillar species to more densely organized ThT\(^+\) amyloid fibrils.
Figure 22: The aggregation behavior and morphology of Aβ40 wt and Aβ40 E3R. Progression of the aggregation of Aβ40 wt (A and B) and Aβ40 E3R (E) probed by ThT (black line), p-FTAA (grey line) n-FTAA (green line), and ANS (red line). TEM images of Aβ40 wt and Aβ40 E3R taken at the start (C) Aβ40 wt, (F) Aβ40 E3R) and end (D) Aβ40 wt, (G) Aβ40 E3R) of the aggregation experiment. Scale bar = 500 nm. Fluorescence profiles of Aβ40 wt (black line) and Aβ40 E3R (grey line) probed by H) pFTAA (relative fluorescence), I)ThT, J)ANS and K) n-FTAA.

Figure 22E illustrates the fluorescence profiles for Aβ40 E3R. The p-FTAA fluorescence clearly increases rapidly at the start of the experiment, indicating that p-FTAA+ species appeared immediately after dissolving the peptide in the buffer. Interestingly, at this time point several species were detected concurrently in the TEM images (Figure 22F) which may be responsible for this p-FTAA fluorescence. These p-FTAA+ species were later on also recognized by ANS and n-FTAA, suggesting that the Aβ40 E3R peptide, in contrast to Aβ40 wt, produces p-FTAA+ species which contain hydrophobic patches (Figure 22E). The ThT fluorescence profile exhibit a longer lag phase followed by an increase in the signal after which the fluorescence intensity started to level off and then remained fairly constant during the remainder of the experiment. The steady decrease in the p-FTAA fluorescence signal indicates the accumulation of more densely organized aggregates resulting in increased stacking of the probe as was also detected for Aβ40 wt. Notably, the p-FTAA+ species formed by Aβ40 E3R exhibited a longer lifetime than those formed by Aβ40 wt as deduced from the ThT and p-FTAA fluorescence curves, which showed that the conversion of p-FTAA+ species to ThT+ aggregates occurred earlier and at a higher rate for Aβ40 wt compared to Aβ40 E3R (Figure 22H & I).

Since ThT probes highly ordered structures, we can conclude, based on the differences in ThT fluorescence amplitudes, that Aβ40 wt aggregates contain more ordered structures than Aβ40 E3R (Fig 22I). TEM images captured at the end of aggregation confirmed that mature fibrils were formed in the Aβ40 wt sample, but no mature fibrils could be detected in the Aβ40 E3R sample (Figure 22D & G). According to the ANS profiles, species formed by Aβ40 E3R were considerably more hydrophobic than those formed by Aβ40 wt (Figure 22J). Interestingly, ANS was the only probe that showed higher fluorescence intensity for Aβ40 E3R than for Aβ40 wt,
indicating that the former amorphous aggregates contain a large number of hydrophobic patches.

A further observation made in the present study was that the novel probe n-FTAA not only detects prefibrillar species but also has a high affinity for fibrillar ThT⁺ structures, which were formed to a higher extent by Aβ40 wt compared to Aβ40 E3R (Figure 22I & K).

**Correlation between neurotoxicity and biophysical properties of aggregates formed by Aβ40 wt and Aβ40 E3R**

A key question is if differences in biophysical properties between Aβ40 wt and Aβ40 E3R can be linked to the different levels of neurotoxicity that these two Aβ 40 variants exert in the CNS of *Drosophila* flies? Both the Aβ40 wt peptide and the Aβ40 E3R peptide formed species early in the aggregation process which were recognized by p-FTAA and n-FTAA. Notably, there was a significant difference in hydrophobicity between these prefibrillar species; aggregates formed by the toxic Aβ40 E3R peptide had exposed hydrophobic patches, whereas the Aβ40 wt aggregates did not. In fact, ANS was the only probe in our study that gave higher fluorescence intensity with Aβ40 E3R than with Aβ40 wt. Another observation was that the nontoxic Aβ40 wt peptide was considerably more prone to form mature amyloid fibrils, as detected by ThT fluorescence and TEM, compared to the more toxic Aβ40 E3R, which only formed a small amount of ThT⁺ aggregates and no mature fibrils were evident by TEM. These observations are in line with our previous study where we compared the aggregation behavior of the highly toxic Arctic peptide and the nontoxic Rescue peptide. In that study, we found a clear link between the toxicity of the peptide and the propensity to form prefibrillar species with exposed hydrophobic patches at an early stage of the aggregation process; the Arctic peptide had a high propensity to form p-FTAA⁺ hydrophobic prefibrillar species, whereas the Rescue peptide only formed long ThT⁺ fibrils. A correlation between toxicity and exposure of hydrophobic surfaces has previously been reported (Bolognesi et al., 2010).
The aim of the work described in this thesis was to determine what properties of Aβ species can be linked to toxicity. When I compared the aggregation behavior and the resulting aggregated species for different Aβ peptide variants possessing different levels of neurotoxic activity when expressed in the CNS of *Drosophila*, I found a clear link between the toxicity of the Aβ peptide and the propensity to form prefibrillar species with hydrophobic patches early in the aggregation process. Figure 23 summarizes the properties of the Aβ variants determined in this work.

The physiochemical properties of toxic Aβ species are distinct characterized by: 1) being of a specific size; neither the monomeric form nor the accumulated mature fibrils of the Aβ peptide show toxicity, only prefibrillar assemblies formed early in the aggregation process exhibit cell toxicity and 2) having exposed hydrophobic surfaces; only those Aβ peptide variants that showed neurodegeneration in *Drosophila* were able to form hydrophobic prefibrillar species.

Dissecting the physiochemical properties of toxic Aβ species is essential in order to find an effective treatment for AD. Recent data have shown that shifting the equilibrium from toxic prefibrillar species towards nontoxic amyloid fibrils can reduce Aβ toxicity and may be a potential therapeutic approach to combat AD (Bieschke et al., 2011; Caesar et al., 2012). Furthermore, in line with my findings other studies have implied that the exposure of hydrophobic surfaces is crucial for the toxicity of Aβ aggregates promoting interference of the aggregates with membrane structures (Bolognesi et al., 2010; Campioni et al., 2010; Kremer et al., 2000). Therefore, preventing theses hydrophobic interactions could also be a strategy for AD therapy.
Figure 23: Summary of the main characteristics of five variants of the Aβ peptide. A) Hairpin model of the peptide. B) Relative toxicity when expressed in the CNS of Drosophila visualized as red bars. C) TEM images of aggregated species, scale bar = 500 nm. D) Aggregation probed by ThT (black), p-FTAA (grey) and ANS (red) E) Described morphology of aggregated species. F) Fluorescent images of aggregates stained with p-FTAA.
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