Amyloid Aggregates:
Detection and Interaction

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Cover: Illustration of interactions between amyloid-β protofibril (spheres molecule in the middle, white-gray) and proteins (surrounding molecules, presented with different color) in human biofluid.
(credit: C. Lendel)

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Amyloid Aggregates: *Detection and Interaction*

Abstract

The research on protein aggregation and amyloid formation is motivated by the fact that amyloid formation in tissue is harmful and associated with several debilitating diseases including Alzheimer's disease (AD) and systemic amyloidosis such as transthyretin (ATTR) amyloidosis. Nevertheless, their beneficial roles in Nature have recently been identified, and artificial self-assembling of amyloid structure for various applications are emerging. In this thesis, disease-related amyloid aggregates were studied with a focus on their detection and interactions with other proteins in biofluid. Also, the usefulness of functionalized self-assembled amyloid structure as a detection system for pathological amyloid is investigated.

New affinity proteins based on Affibody molecules were developed targeting stable protofibrils formed by an engineered version of amyloid-β (Aβ) peptide, called $A\beta_{42}CC$. The developed affinity proteins also recognize protofibrils of wild-type $A\beta_{42}$, and showed selective binding to protofibrils over other aggregated forms of Aβ. Binding kinetics of these new binders to $A\beta_{42}CC$ protofibrils were determined. These proteins have potential to be used in diagnostic or even therapeutic applications.

An enhanced method was developed for the detection of small ATTR aggregates. A nanofibril, which was functionalized with the antibody-binding Z domain was the new molecule in the improved method. The efficiency of the new method for sensitive detection of ATTR aggregates was studied. The result of this study was very encouraging and could potentially be used in the future for high sensitivity detection of ATTR aggregates.

The potential interactions of $A\beta_{42}CC$ protofibrils and $A\beta_{42}wt$ fibrils with other proteins in serum and cerebrospinal fluid from patients with AD and non-AD were studied. More than hundred proteins with diverse functionality were identified to bind to $A\beta_{42}CC$ protofibrils and $A\beta_{42}wt$ fibrils. It was shown that different Aβ conformations have a distinct set of binding partners, and the binding is enhanced upon aggregation of Aβ. Many of the identified proteins may have potential as AD biomarkers.

In conclusion, this thesis has developed new research tools and a methodology to detect amyloid aggregates as well as studied potential interactions of these aggregates with other proteins, which could advance our understanding about protein aggregation and disease.

Keywords: Alzheimer's disease, amyloid, detection, functional amyloid, interaction, protein aggregation, transthyretin.

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To my parents

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Elisabet Wahlberg*, M. Mahafuzur Rahman*, Hanna Lindberg, Elin Gunneriusson, Benjamin Schmuck, Christofer Lendel, Mats Sandgren, John Löfblom, Stefan Ståhl, Torleif Härd (2017). Identification of proteins that specifically recognize and bind protofibrillar aggregates of amyloid-β. *Sci Rep,* vol. 7 (1), pp. 5949.

II M. Mahafuzur Rahman, Benjamin Schmuck, Henrik Hansson, Gunilla T. Westermark, Torleif Härd, Mats Sandgren (2018). Enhanced detection of pathological ATTR aggregates using a nanofibril-based assay. *Manuscript*.

III M. Mahafuzur Rahman, Henrik Zetterberg, Christofer Lendel, Torleif Härd (2015). Binding of human proteins to amyloid-β protofibrils. *ACS Chem Biol,* vol.10 (3), pp. 766–774.

IV M. Mahafuzur Rahman, Gunilla T. Westermark, Henrik Zetterberg, Torleif Härd, Mats Sandgren (2018). Protofibrillar and fibrillar amyloid β-binding proteins in cerebrospinal fluid. *JAD*, DOI 10.3233/JAD-180596, E-pub ahead of print.

* These authors contributed equally to this work.

Other scientific contribution, not included in the thesis

V Anatoly Dubnovitsky, Anders Sandberg, M. Mahafuzur Rahman, Iryna Benilova, Christofer Lendel, Torleif Härd (2013). Amyloid-β protofibrils: Size, morphology and synaptotoxicity of an engineered mimic. *PLoS One,* vol. 8 (7), pp. e66101.

The contribution of M. Mahafuzur Rahman to the papers included in this thesis was as follows:

I Experimental work and design including protein production, aggregate formation, affinity capture assay, SPR kinetics assay and data analyses, and taking part in writing.

II Planning of the project together with co-authors, experimental work and wrote the first version of the manuscript.

III Designing the project together with C. Lendel. All experimental work and data analysis except mass spectrometry, and participated in the writing of the manuscript.

IV Planning and performing the experimental work, data analysis and writing the first version of the manuscript.

Abbreviations

α-Syn	α Synuclein
3D	Three-dimensional
Aβ	Amyloid β
AβPP	Amyloid β protein precursor
Ab-bNF	Antibody-binding nanofibril
ABD	Albumin binding domain
AD	Alzheimer's disease
AFM	Atomic force microscopy
AICD	AβPP intracellular domain
ApoE	Apolipoprotein E
ATTR	Transthyretin amyloidosis
ATTRwt	Wild-type ATTR amyloidosis
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GO	Gene ontology
hATTR	Hereditary ATTR amyloidosis
HPA	Human protein atlas
IAPP	Islet amyloid polypeptide
NFTs	Neurofibrillary tangles
NMR	Nuclear magnetic resonance spectroscopy
p-tau	Hyperphosphorylated tau
PAI	Peptide abundance indices
PD	Parkinson's disease
PET	Positron emission tomography
RNA	Ribonucleic acid
SpA	Staphylococcal protein A
SPR	Surface plasmon resonance

TEM	Transmission electron microscopy
ThT	Thioflavin T
TTR	Transthyretin
TTR-bNF	TTR antibody bound to Ab-bNF

1 Introduction

Life is a fine-tuned complex biological system and more than 200 different cell types interact and make up the human body. A cell comprises several different biomolecules, and *nucleic acids* and *proteins* are two of the four major macromolecules. The nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) store and transfer all information required for the synthesis of a protein. Proteins are the most versatile biomolecules in living organisms and they perform a large variety of functions including metabolism, processing, storage and transport of other molecules as well as providing structure and mechanical support (Lehninger et al., 1992, Berg et al., 2002). A protein consists of amino acids linked together via peptide bonds into a polypeptide chain, and the unique order of the amino acids defines the primary structure of the molecules. The polypeptide chain folds upon itself and forms secondary structural elements, α-helix and β-sheet. These elements are further organized to produce the three-dimensional (3D) protein structure, the tertiary and quaternary structure.

Proteins are synthesized on ribosomes using information encoded by mRNA, and these proteins must subsequently fold into their unique 3D structure in order to function (Fig. 1.1, top panel) (Tyedmers et al., 2010). It is generally accepted that the necessary information needed for the proper folding of a protein lies in its primary structure. This is known as the thermodynamic hypothesis of protein folding, and was established through a series of experiments performed by Anfinsen and colleagues in the 1950s (Anfinsen et al., 1954, Anfinsen et al., 1955). As tribute to his work Anfinsen was awarded the 1972 Nobel Prize in Chemistry (Anfinsen, 1973). However, in the crowded cellular environment, a newly synthesized polypeptide faces several challenges including environmental stress, mutation or translational errors that increase the risk of misfolding and aggregation. To avoid this situation, the cell has evolved an advanced protein quality control machinery, which is comprised of a specialized group of proteins referred to as molecular chaperones. Chaperones, also known as heat shock

proteins (HSPs), have the capacity to assist folding of the newly synthesized protein as well as enable partially misfolded protein to refold into its correct 3D conformation and thereby gain functional activity (Fig. 1.1). About 20-30% of proteins synthesized in a mammalian cell need chaperone assistance to adopt the correct conformation (Hartl et al., 2011).

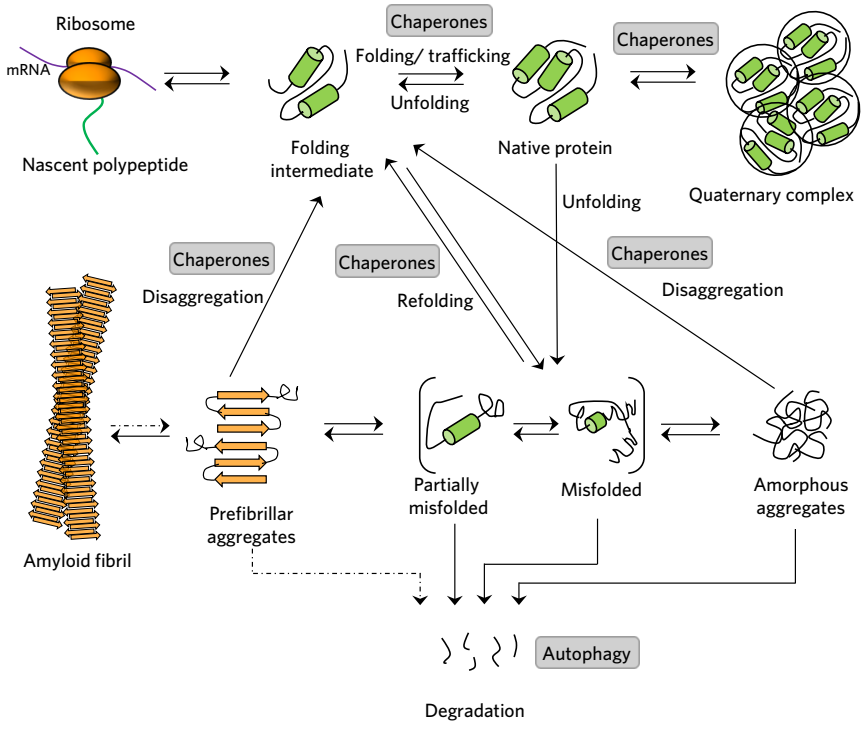

Figure 1.1 Upon release from the translation machinery, the nascent polypeptide folds into native conformation, when necessary with the assistance from chaperones, and subsequently form the higher order structures (top panel). However, due to environmental stress or other reasons, the newly synthesized protein can be misfolded. The misfolded protein can either be degraded by the cellular degradation system or refold into its native conformation through chaperone machinery. If the cellular quality control is insufficient and refolding or degradation fails, the partially misfolded and misfolded proteins can form either amorphous aggregates or a highly ordered structural conformation called amyloid. The amorphous aggregates and pre-amyloid aggregates may be disaggregated or degraded. Figure modified from refs. Tyedmers et al., 2010, Hartl et al., 2011.

In addition, the cell uses an extensive proteostasis network, composed of several hundred proteins that are responsible for trafficking, disaggregation, and proteolytic cleavage. Prior to clearance, misfolded proteins undergo ubiquitination that directs them to the proteasome and subsequent degradation (Ciechanover and Schwartz, 2002, Glickman and Ciechanover, 2002), while aggregated proteins are ubiquitinated and degraded through aggrephagy, a

selective form of macroautophagy (Yamamoto and Simonsen, 2011). However, in certain scenario the proteostasis network may be insufficient or malfunctioning, and misfolded proteins deposit in the intracellular or extracellular compartment. Misfolded proteins that escape degradation can adopt a highly ordered fibrillar conformation rich in β-sheet known as *amyloid* (Fig. 1.1, bottom panel).

The deposition of amyloid in tissue have severe consequences and is involved in several debilitating diseases including Alzheimer's disease and Parkinson's disease. However, not all amyloid structures are harmful, some have a useful biological function and are termed *functional amyloid*. Also, amyloid structures can be functionalized with desired function.

Detection of disease-related amyloid aggregates and examination of the interaction of such aggregates with other proteins is the focus of this thesis. The usefulness of functionalized amyloid in the pathological amyloid detection systems is also investigated.

2 The amyloid fold of proteins

The term amyloid was first introduced in 1854 by the German physician Rudolf Virchow, and is derived from the Latin *amylum* and the Greek *amylon* that means cellulose or starch-like. Nevertheless, in 1859 Friedreich and Kekule demonstrated that the composition of amyloid was not carbohydrate, but proteinaceous (reviewed in ref. Sipe and Cohen, 2000). In the following sections, the general characteristics of amyloid, the beneficial and the harmful roles of amyloid, how amyloid is formed, and polymorphisms of amyloid will be discussed.

2.1 Characteristics of amyloid

Amyloid is characterized by a *fibrillar* morphology, typical structural *cross-β sheet* conformation, and affinity for specific *dyes* (Divry and Florkin, 1927, Cohen and Calkins, 1959, Eanes and Glenner, 1968). Amyloid fibrils found in tissue deposits (Fig. 2.1a) appear as unbranched twisted fibers of ca. 7–10 nm in diameter and up to several microns long when analyzed by transmission electron microscopy (TEM) (Fig. 2.1b) (Cohen and Calkins, 1964) or atomic force microscopy (AFM) (Chamberlain et al., 2000). All amyloid, regardless of which peptide or protein that builds the fibril, share a structural conformation known as cross-β sheet structure, which was first analyzed by *X-ray fiber diffraction* in 1968 (Eanes and Glenner, 1968). The X-ray fiber diffraction pattern of amyloid fibril shows two reflections, one at 4.7 Å, which corresponds to distance between two neighboring β stand in β-sheet packing and one at ca. 10 Å, which corresponds to β stands within β-sheet (Fig. 2.1c). More recent high-resolution structural data obtained from X-ray crystallography, solid-state NMR, and small angle X-ray scattering complemented well with the X-ray fiber diffraction pattern in which the β-sheet secondary structure is arranged perpendicular to the fibril axis (Fig. 2.1di) through the intramolecular hydrogen bonds between

strands in the β-sheet (Knowles et al., 2007). Amyloid exhibit apple green birefringence in polarized light upon binding to *Congo red* (Holder Puchtler, 1962) and an intense fluorescent signal with 440 nm excitation and 490 nm emission maxima upon binding to thioflavin T (ThT) (Vassar and Culling, 1959).

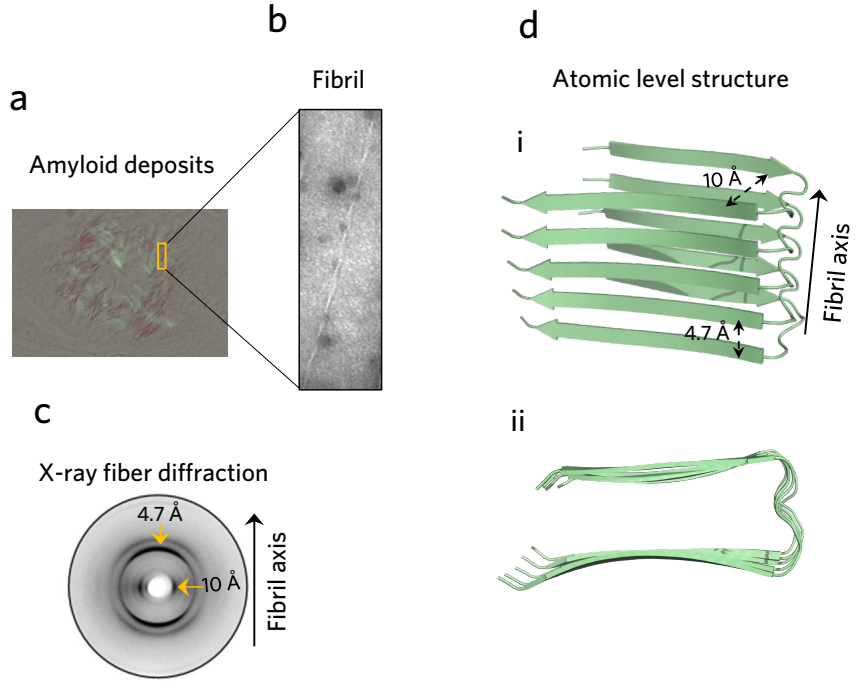

Figure 2.1 Structural features of amyloid. **a**) A micrograph showing amyloid-β deposits stained with Congo red in a section from the brain of an AD patient. **b**) TEM micrograph of an amyloid fibril, produced from synthetic wild-type $A\beta_{42}$ peptide, showing unbranched twisted character. **c**) X-ray fiber diffraction from amyloid fibrils showing cross-β diffraction pattern. **d**) 3D structure obtained from an $A\beta_{42}$ fibril using NMR (Luhrs et al., 2005), showing β-sheet packing at atomic level in which β-sheet running perpendicular to the fibril axis (i) and top view of a β-sheet in fibril arrangement (ii). Congo red stained micrograph was kindly provided by Westermark GT, diffraction photograph was adopted from ref. Serpell, 2014 with permission and Aβ fibril structure was produced using PDB accession no. 2BEG (NMR structure of $A\beta_{42}$ fibril).

2.2 Amyloid—what they do

A broad range of fatal degenerative diseases arises from the deposition of amyloid in different tissues. At present, a total of 36 peptides or proteins have been identified to form amyloid in human, and each protein is associated with a specific disease (Sipe et al., 2016). A selection of amyloid-forming proteins and associated diseases are listed in Table 2.1.

Table 2.1 Examples of peptides or proteins that form extracellular amyloid or intracellular amyloid inclusions in human and their associated diseases.

Peptide/protein[a]	Target organs	Associated disease
α-Synuclein (α-Syn)	CNS	Parkinson's disease, Dementia with Lewy bodies, and Multiple system atrophy
Amyloid-β peptide (Aβ)	CNS	Alzheimer's disease, Hereditary cerebral hemorrhage with amyloidosis
Apolipoprotein A-I (ApoAI)	Heart, liver, kidney, PNS, testis, and skin	ApoAI amyloidosis
Apolipoprotein A-II (ApoAII)	Kidney	ApoAII amyloidosis
Huntingtin exon 1 (HttEx1)	CNS	Huntington's disease
Immunoglobulin heavy chain (AH)	All organs except CNS	AH amyloidosis
Immunoglobulin light chain (AL)	All organs except CNS	AL amyloidosis
Islet amyloid polypeptide (IAPP)	Islets of Langerhans, insulinomas	Type II diabetes and insulinoma
Lysozyme (Lys)	Kidney	Lysozyme amyloidosis
Serum amyloid A (AA)	All organs except CNS	AA amyloidosis
Transthyretin (TTR)	Heart, PNS, eye	SSA, FAP, and FAC

Table adapted from Sipe et al., 2016.
CNS, Central nervous system; PNS, Peripheral nervous system; SSA, Senile systemic amyloidosis; FAP, Familial amyloid polyneuropathy; FAC, Familial amyloid cardiomyopathy.

Amyloid fibrils are mainly deposited in the extracellular space. However, intracellular inclusion is not rare *e.g.*, Aα-synuclein (AαSyn) deposition in Parkinson's disease (PD) and Islet amyloid polypeptide (AIAPP) deposits in islet of Langerhans. Amyloid deposits can occur in a single organ or in multiple organs. When amyloid is deposited in a single organ, it is termed *localized amyloidosis*. In localized amyloidosis, the amyloid protein is synthesized in the organ affected by the deposition. When amyloid is deposited in multiple organs, the condition is termed *systemic amyloidosis* (Sipe et al., 2016). In systemic amyloidosis, the amyloid proteins are mainly produced by the liver or by plasma cells.

Besides humans, animals can also develop amyloidosis. Of the 36 proteins or peptides currently known to form amyloid in human diseases, nine proteins or peptide are also known to form amyloid in animals such as immunoglobulin light chain (AL) and serum amyloid A (AA), which form amyloid in cow, cats, horse and dog (Sipe et al., 2016).

The amyloid structure was first discovered in the context of disease, but not all amyloid structures are harmful and a group of amyloid structures have biological functions. Amyloid with beneficial functions in living systems is known as *functional amyloid* (Fowler et al., 2006). Many organisms ranging from bacteria to human use amyloid structure to gain a variety of functions (Fowler et al., 2007, Knowles and Mezzenga, 2016). For example, curli fibrils produced from the CsgA protein are used by *Escherichia coli* (*E. coli*) for biofilm formation (Chapman et al., 2002), and fibrils of the Pmel17 protein is involved in mammalian skin pigmentation (Fowler et al., 2006). Notably, in recent years, amyloids have become the center of interest in nanomaterial sciences for functionalization in various technological applications. Amyloid in functional aspect will be discussed in more depth in chapter 4.

2.3 Mechanism of amyloid fibril formation

The amyloid fibril formation process is modeled as a nucleation-dependent polymerization, with three phases: a lag phase, an elongation phase, and a steady state (Jarrett and Lansbury, 1992) (Fig. 2.2, red curve). The native folded state of the protein/peptide needs to be unfolded to form fibril. The fibril formation is initiated by the formation of a *nucleus* during the *lag phase*, a thermodynamically unfavored state in the aggregation process. The nucleus has a different conformation than the soluble protein, which is rich in β-sheet structure (Serio et al., 2000), and presumably composed of oligomers although monomer has also been implicated (Eisele et al., 2015). Once the nucleus is formed, monomeric species are rapidly added to the growing polymer and formed aggregates that are more thermodynamically stable (Jarrett and Lansbury, 1993). Fibril growth occurs during the *elongation phase*. The fibrillation can be catalyzed by secondary nucleation where the surface of preformed fibril or fibril fragmentation act as nucleation sites (Fig. 2.2) (Cohen et al., 2011, Cohen et al., 2013), and dominate the reaction. The lag phase can be shortened or abolished by addition of pre-formed fibrils, so-called *seeds* (dotted curve in Fig. 2.2) (Jarrett and Lansbury, 1992). The end reaction state is a *steady state* where the maximum fibril growth has been reached and no monomer species are available to be incorporated into the growing fibril.

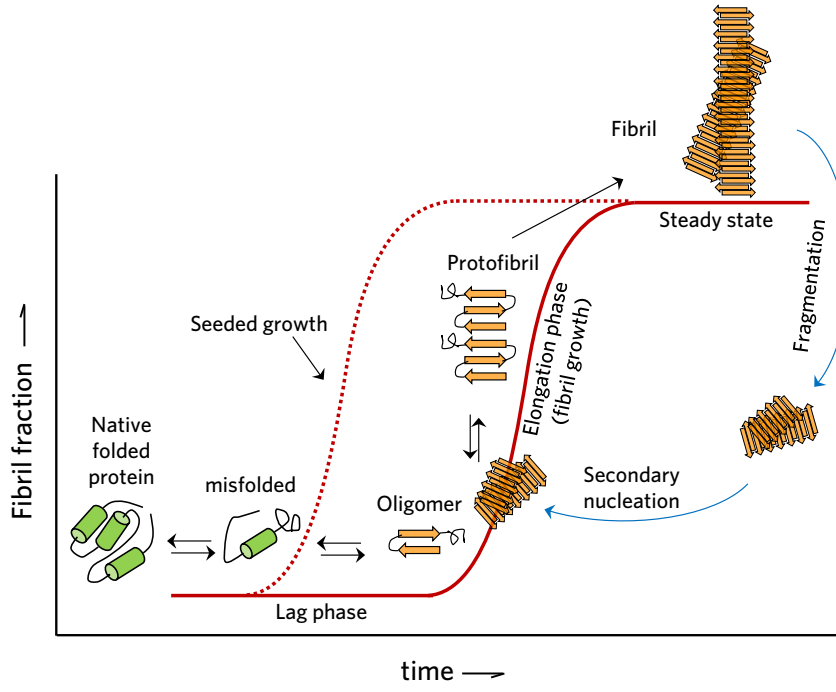

Figure 2.2 Illustration of amyloid protein fibrillation pathway. A nucleus is formed during the lag phase, and addition of monomer results in the formation of aggregates, which subsequently grow into mature fibrils. Preformed fibrils or fibril fragmentation may catalyze nucleation and this is called secondary nucleation.

2.4 Polymorphism of amyloid fibril

Amyloid-forming peptides self-assemble into fibrils with multiple morphologies. Unlike the functional structures, the amyloid fibrils have not been under evolutionary pressure to maintain a single thermodynamically stable conformation, which is probably the cause of different fibril morphologies (Pedersen et al., 2006). Polymorphism may arise already during the lag phase of the amyloid fibrillation pathway during which metastable intermediates are formed, and/or within the fibrillar form (Kodali and Wetzel, 2007). In addition, polymorphism can occur at the atomic level, *e.g*, fibrils formed by the D23N-Aβ_{40} peptide appeared with different atomic structures. The D23N-Aβ_{40} is known as the Iowa mutation, which is associated with early-onset of AD. Some of the fibrils formed by D23N-Aβ_{40} exhibit the in-register parallel β-sheet structure, but a majority of fibrils exhibit antiparallel β-sheet packing (Tycko et al., 2009, Härd, 2014). Likewise, structural polymorphism may play important roles in clinical phenotypes and pathology. Indeed, a recent study has

demonstrated differences in disease progressions between two AD patients who displayed distinct fibril patterns in their plaques (Lu et al., 2013).

3 Amyloid in disease pathology

Deposition of amyloid in tissue can be harmful and is associated with numerous diseases. Two such diseases, Alzheimer's disease (AD) and transthyretin (ATTR) amyloidosis will be discussed in the following sections, since they are central to this thesis.

3.1 Alzheimer's disease

AD is the most common cause of dementia and accounts for 60–70% of all diagnosed cases (Alzheimer's Association, 2018). AD was first described by the German psychiatrist and neuropathologist, Alois Alzheimer, in 1906 and the disease was named after him. During a brain autopsy of his 55-year-old patient, Auguste Deter, who had been suffering from progressive memory loss and confusions, he noted the presence of abnormal neuritic plaques around neurons and twisted neurofibrils inside the neuron. These two distinctive pathologies are today known as *amyloid plaque* and *neurofibrillary tangles* (NFTs), the two pathological hallmarks of AD (Alzheimer et al., 1995, O'Brien and Wong, 2011).

The amyloid plaque consists of several proteins of which amyloid-β (Aβ) is the major one. Aβ is an enzymatic cleavage product of the *amyloid-β protein precursor* (AβPP). Aβ is produced both in non-AD and AD brains. In non-AD brain, Aβ peptides are usually degraded, whereas in AD brain, Aβ accumulates as plaque outside of the neuron. The NFTs are found inside the cells and mainly composed of tau protein. Tau is a microtubule-associated protein in the neuron that interacts with tubulin and promotes its assembly into microtubules and stabilizes the microtubule network (Iqbal et al., 2010). In the AD brain, tau becomes hyperphosphorylated (p-tau), sequesters normal tau and disassembles the microtubules network, and consequently the p-tau forms tangles (Alonso et al., 2001).

AD may develop because of genetic abnormalities; however, the percentage of genetic disorder-related AD is very low (<1%) (Bekris et al., 2010). Mutations in the AβPP may lead to an increased Aβ production and subsequent AD development. The Swedish mutation, which is a double mutation (KM670/671NL) of AβPP that is known as APPSW, lies adjacent to the β-secretase cleavage site and is one example of a mutation that results in an increased generation of Aβ (Goate et al., 1991, Bekris et al., 2010). Also, mutations in the genes PSEN1 and PSEN2 are linked with early-onset AD. PSEN1 and PSEN2 encodes for presenilin 1 and presenilin 2, respectively; and are subunits of the enzyme γ-secretase, which cleaves AβPP and releases Aβ (see in the following section). The gene responsible for producing AβPP is located on chromosome 21. Individuals with Down syndrome, also known as trisomy 21, have an extra copy of chromosome 21, and is more susceptible to AD since the extra copy of chromosome 21 results in an increased production of Aβ fragments (St George-Hyslop et al., 1987).

Age is the main risk factor for late-onset AD (Fratiglioni et al., 1991), and the disease affect 3% of the people in the age group 65–74, 17% of the people in the age group 75–84, and nearly 50% of the people of age 85 years or older (Qiu et al., 2009). Nevertheless, AD is not a part of normal aging, and age is not itself sufficient to cause AD. A second risk factor of developing AD is epsilon 4 allele of apolipoprotein E (apoEe4) (Saunders et al., 1993). The apoE gene exists in three isoforms, e2, e3, and e4. The function of apoE is to clear or degrade Aβ, and the e4 isoform is thought to be the least efficient among the three isoforms of apoE. Inheriting the e4 allele confers reduced clearance of Aβ and an increased risk of developing Aβ plaque in the brain. The risk may be higher if two copies of e4 are inherited (Strittmatter et al., 1993).

3.1.1 AβPP metabolism and the Aβ peptide

AβPP is a 695–770 residue transmembrane protein with a large extracellular domain, a transmembrane region in which Aβ is partly embedded, and a short cytoplasmic tail (Fig. 3.1) (Selkoe, 2001). The protein is produced in large amounts in the brain (Lee et al., 2008). The exact functions of AβPP remain unclear. However, it has been suggested that AβPP play vital roles in brain development, neural migration, synaptogenesis and neurogenesis (reviewed in ref. Coronel et al., 2018).

AβPP is proteolytically cleaved by two distinct pathways: *non-amyloidogenic pathway* which does not produce Aβ, and *amyloidogenic pathway* which produces Aβ. In the non-amyloidogenic pathway, AβPP is first cleaved by α-secretase within the Aβ region between residues 16 and 17. This

cleavage releases a soluble extracellular fragment called sAβPPα, while the C-terminus CFTα fragment remain anchored to the membrane. The CFTα fragment is then cleaved by γ-secretase, which generates a non-amyloidogenic short peptide called p3 and an AβPP intracellular domain (AICD). Because the α-secretase cleaves AβPP in the middle of the Aβ which precludes an intact Aβ generation (Fig. 3.1) thus the fragment (p3) is non-amyloidogenic (O'Brien and Wong, 2011). α-secretase belongs to the ADAM enzyme family, which have been shown to take part in cell interactive events such as cell-cell fusion (Lammich et al., 1999). γ-secretase is a multiprotein complex made of presenilin 1 or 2, nicastrin, and Aph-1 and Pen-1 (Bergmans and De Strooper, 2010).

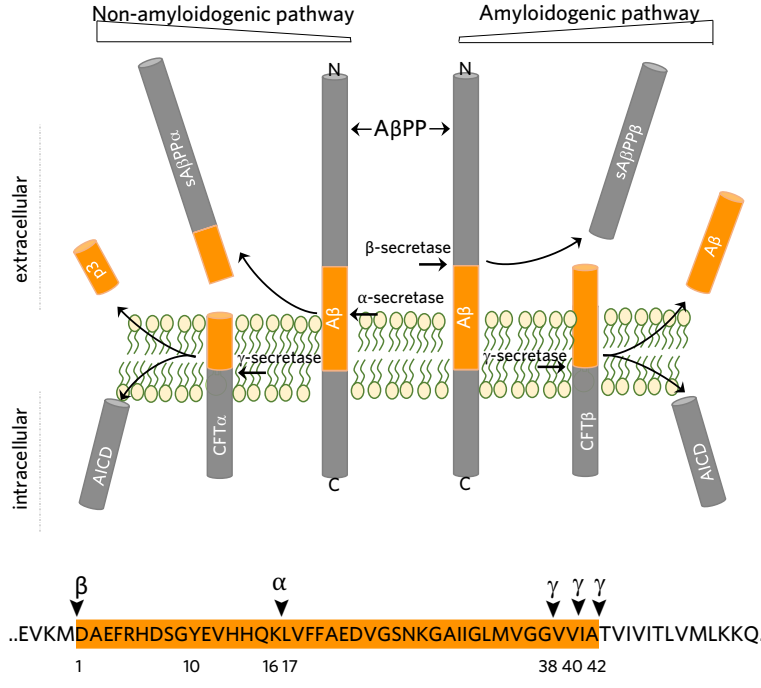

Figure 3.1 Processing of AβPP through the non-amyloidogenic pathway involve α- and γ-secretase cleavages with the release p3; and the amyloidogenic pathway involve β- and γ-secretase cleavages, and the release of the Aβ peptide. The bottom panel presents the Aβ region in the AβPP. Possible cleavage into Aβ sequence by different proteases are indicated.

Production of Aβ via amyloidogenic pathway is initiated by cleaving AβPP with β-secretase (also called BACE1; an aspartic protease) at the N-terminus of Aβ sequence, which produces in the sAβPPβ and CFTβ fragments. The subsequent cleavage of CFTβ fragments by γ-secretase produces different isoforms of Aβ peptides while leaving the AICD fragment in the cytoplasm. The most common isoforms of Aβ are the 40-residue fragment ($Aβ_{40}$) and the 42-residue fragment

($A\beta_{42}$). $A\beta_{42}$ is more aggregation-prone than $A\beta_{40}$ (Burdick et al., 1992, Jarrett et al., 1993). Aβ is constantly produced from AβPP and immediately degraded by enzymes such as neprilysin, insulin degrading enzyme, and endothelin converting enzyme (Wang et al., 2010).

3.1.2 Aβ aggregation and neurotoxicity

Aβ aggregation occurs through two pathways. One pathway involves the formation of low molecular weight oligomers without secondary structure (Hoyer et al., 2008) and subsequent formation of amorphous aggregates that do not convert into fibril (off-pathway, Fig. 3.2). On the pathway to fibrillation, the disordered monomer adopts a β-hairpin conformation as a constituent of soluble oligomers (Hoyer et al., 2008), which further form larger *protofibrils* (Harper et al., 1997, Walsh et al., 1997), and eventually undergo a structural interconversion into fibrils (Fig. 3.2).

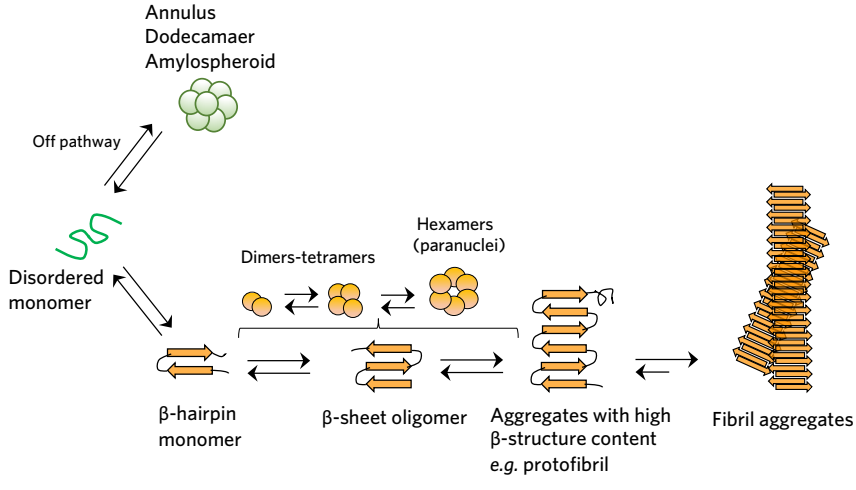

Figure 3.2 Illustration of Aβ aggregation via the off- and on-pathway. Disordered monomer forms low molecular weight oligomers that lack a regular secondary structure, which enters the off-pathway and remain as amorphous aggregate. On the on-pathway, disordered monomer first form β-hairpin structure which end up in fibrils through a dynamic intermediates state.

Previously, much attention was given to fibrils found in the brain of AD patients. However, the role of fibrils in AD pathogenesis is debated. The plaque burden in AD brain does not correlate well with the degree of impaired memory and cellular dysfunction (Kayed and Lasagna-Reeves, 2013), and removing the plaque from the brain in an AD mouse model does not revert the observed dysfunctionality (Hardy, 2009). The focus of research has shifted from fibrils towards the soluble *intermediate aggregates* such as oligomers or protofibrils

(Walsh and Selkoe, 2007, Sengupta et al., 2016). It has become evident that these soluble oligomers do correlate well with the severity of disease (Lue et al., 1999, McLean et al., 1999), and are more toxic to neurons than the fibrils (Kuperstein et al., 2010, Benilova and De Strooper, 2013). A variety of soluble aggregates have been identified *in vivo* and *in vitro* ranging from dimers-tetramer (Shankar et al., 2008, Klyubin et al., 2008), pentamer and hexamers (paranuclei) (Roychaudhuri et al., 2009), and Aβ*56 (Lesné et al., 2006). It is thought that the toxicity of soluble oligomers depends on the size of the Aβ assemblies. An inverse correlation has been proposed between the size of soluble aggregates and the potency of their toxicity (Sengupta et al., 2016). Even the smallest oligomers *i.e.* dimers isolated from the cortex of AD brain were found to be toxic compared to dimers isolated from non-AD brain (Jin et al., 2011, Härd, 2011). However, much of the details about oligomer formation and toxicity remain elusive. New proteins that specifically recognize intermediate aggregates would be valuable tools in basic research as well as in diagnosis and therapeutics. In *paper I* of this thesis, new affinity proteins with selective binding capacity towards Aβ protofibrils are developed.

3.1.3 Studies of soluble Aβ aggregates

The biochemical and biophysical studies of soluble Aβ aggregates are very challenging due to the heterogeneity among aggregates and the high propensity of these aggregates to form fibrils. To control the various aggregates state (*i.e.* stabilize it), many methods have been developed including protein engineering, chemical cross-linking, and the use of mixed solvent or detergent (reviewed in Härd, 2011).

In this thesis (*paper I*, *III*, and *IV*), protofibrils formed by an engineered version of Aβ peptide, called *AβCC*, are used. The AβCC model will be discussed in the section below.

The AβCC protofibril—a mimic of wild type Aβ

The Aβ peptide is predominantly disordered, and does not adopt a unique conformation in solution. However, it has been demonstrated that the Aβ peptide adopts a β-hairpin conformation (Hoyer et al., 2008). The structure of the $A\beta_{40}$ peptide in complex with Affibody molecules ($Z_{A\beta3}$; see the following section) was studied using NMR in which Aβ was observed to adopt a β-hairpin conformation (Fig. 3.3a). The β-hairpin conformation was characterized by antiparallel β-stands formed by residues 17-23 and 30-36 and stabilized by intramolecular hydrogen bonds. The N-terminus of the Aβ peptide was not well defined in the NMR spectra, and it has therefore been suggested to be disordered.

However, the secondary structure of the Aβ peptide in Aβ:$Z_{Aβ3}$ complex resembled well with the Aβ conformation in fibrils (Hoyer et al., 2008).

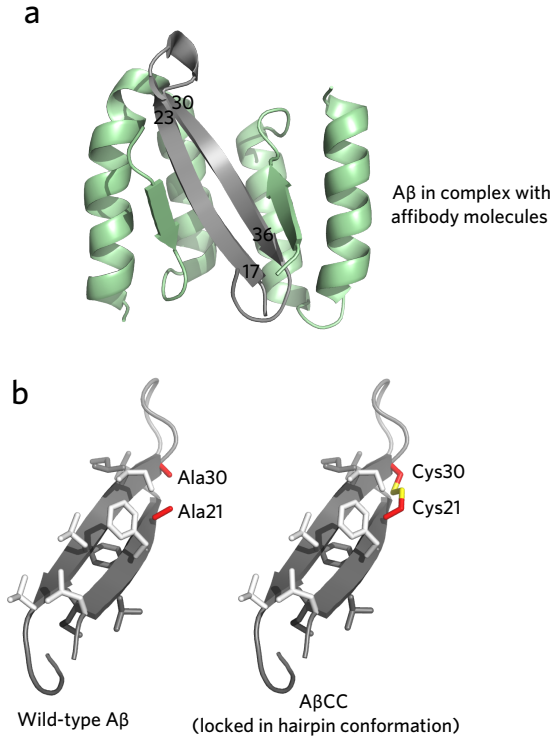

Figure 3.3 a) The β-hairpin conformation of the Aβ peptide (in gray) observed in complex with $Z_{Aβ3}$ dimer (in green). **b**) (To the left) A β-hairpin conformation showing that the two alanine residues (Ala21 and Ala30) on opposite β-stands are in close proximate and were subjected to mutations. (To the right) Model of the AβCC variant in which Ala21 and Ala30 have been mutated to Cys21 and Cys30, which creates an intramolecular S-S bond (colored yellow), thereby locking the β-hairpin conformation. The 3D structure of Aβ:$Z_{Aβ3}$ complex was produced using PBD accession no. 2OTK and the structures in panel b was adapted from T. Härd.

In a later study, two cysteines were introduced in the β-hairpin conformation, one at position 21 (A21C) and one at position 30 (A30C). These two cysteine residues form an intermolecular disulfide bond, which locks the β-hairpin conformation (Fig. 3.3b) (Sandberg et al., 2010). The double cysteine variant of the peptide is called *AβCC*. The AβCC form oligomers but not fibrils unless a reducing agent is added (Sandberg et al., 2010). It has been demonstrated that the protofibril formed by AβCC is a good mimic of wt-protofibrils as determined by a number of methods including, ThT fluorescence, TEM, AFM, circular dichroism, analytical ultracentrifugation, immunohistochemistry, cell toxicity,

and a structural model based on solid-state NMR and Rosetta modeling (Sandberg et al., 2010, Dubnovitsky et al., 2013, Lendel et al., 2014).

Affibody molecules—a class of engineered affinity proteins

Until 30 years ago, it was believed that the immune system is the only source of affinity proteins *i.e.* antibodies, and remain a primary choice for the detection of specific antigen to this day. However, antibodies have some limitations such as their large size, production complexity, and cost, which has motivated researchers to develop of non-antibody proteins as an alternative. Affibody molecules are a kind of affinity proteins, which originated from an engineered domain called Z domain (see below) of staphylococcal protein A (SpA). SpA is located on the surface of *Staphylococcus aureus* cells and has five homologous IgG-binding domains, denoted as E, D, A, B, and A, that are capable of binding to the Fc region of antibodies from different species and subclasses (Moks et al., 1986). The B domain was initially engineered with a few modifications to increase chemical stability towards hydroxylamine and to facilitate cloning, and the mutated domain was denoted *Z domain* (Nilsson et al., 1987). The Z domain is 58-residue (6.5 kDa) scaffold protein with three α-helices that form a bundle-like structure. Its small size, high solubility and easy production, absence of internal cysteine residues, rapid folding kinetics, and high-affinity binding to targets convinced researchers to explore its potentials as future non-antibody affinity protein (Nygren, 2008). So-called *Affibodies* are the next generation of the Z domain. In 1995, the first Affibody combinatorial library was created by randomizing 13 solvent-exposed residues on helix 1 and 2 of the Z domain scaffold (Nord et al., 1995). After two years, in 1997, high-affinity proteins were isolated from the library using phage display technology (Nord et al., 1997).

During the last two decades, Affibody-based proteins have been designed for numerous targets for use in a variety of applications including in bio-separation (Rönnmark et al., 2002, Hedhammar and Hober, 2007), as detection agents (Karlstrom and Nygren, 2001), as blocking agent or inhibitor (Jonsson et al., 2009, Hoyer et al., 2008), and *in vivo* tissue imaging (Sorensen et al., 2014).

The Affibody $Z_{A\beta3}$ was selected targeting $A\beta_{40}$ peptide and demonstrated to bind $A\beta_{40}$ with high affinity with a K_D of 17 nM (Hoyer et al., 2008, Hoyer and Härd, 2008). Moreover, $Z_{A\beta3}$ acts as an *amyloid inhibitor*, and has been shown to inhibit Aβ fibrillation by sequestering monomeric Aβ from Aβ aggregation solution (Hoyer et al., 2008, Luheshi et al., 2010). Furthermore, it has also been demonstrated that the $Z_{A\beta3}$ dissociates pre-formed aggregates, albeit very slowly, by shifting the dynamic monomer-oligomer equilibrium (Luheshi et al., 2010). In addition, the potency of $Z_{A\beta3}$ to inhibit Aβ aggregation and to reduce neurotoxicity has also been tested *in vivo* using a *Drosophila* fruit fly model of

AD (Luheshi et al., 2010). The proven potency of $Z_{A\beta3}$ has made it an important research tool and has been used in many studies. For instance, the stable Aβ protofibril (described in previous section) was developed by exploiting $Z_{A\beta3}$:Aβ complex. Besides Aβ, $Z_{A\beta3}$-based inhibitors have been developed for other amyloidogenic proteins *e.g.*, the HI18 inhibitor against IAPP aggregation, which is involved in type II diabetes (Mirecka et al., 2016) and AS69 against AαSyn aggregation involved in PD (Mirecka et al., 2014). All studies mentioned above were performed by targeting non-aggregated species (soluble peptide). In *paper I* of this thesis, we have identified Affibody-based affinity proteins targeting the protofibrillar aggregates of Aβ.

3.1.4 Aβ-associated proteins and AD

Studies of postmortem amyloid plaques have shown that several proteins *e.g.*, α-1 antichymotrypsin (Abraham et al., 1988), agrin (Cotman et al., 2000), apolipoprotein E (apoE), clusterin and collagen, (Liao et al., 2004), heparin sulfate proteoglycan (Sandwall et al., 2010) and serum amyloid P (Kalaria et al., 1991) are *colocalized* with Aβ. It is thought that Aβ interacts with other proteins in human biofluid, which may lead to Aβ toxicity and the subsequent development of AD. An important part of the work presented in this thesis is focused on identifying the binding partners of soluble pre-fibrillar and fibrillar Aβ aggregates in human biofluid (*papers III* and *IV*).

3.1.5 Diagnosis and treatment of AD

Currently, no single test can confirm AD. Several clinical tests and assessments are usually conducted for diagnosis of AD. These tests include medical and family history, cognitive tests like mini-mental state examination (MMSE; the most commonly used test), physical and neurological examination to check overall neurological health, and blood test to rule out other potential causes for memory impairments and confusions (Alzheimer's Association, 2018). At present, cerebrospinal fluid (CSF) biomarkers, reduced levels of $A\beta_{42}$, and elevated levels of total tau and p-tau, have increasingly been used at clinical laboratory for diagnosis AD (Zetterberg and Blennow, 2013, Niemantsverdriet et al., 2017). Also, imaging techniques such as positron emission tomography (PET) is used in research settings and clinical trials. Advanced PET techniques can be used to detect the amount of Aβ plaque in the brain (Sehlin et al., 2016). However, AD can only be diagnosed with complete accuracy only after death of the patient through a brain autopsy to confirm the presence of amyloid plaques and neurofibrillary tangles.

Today no cure exists for AD. However, some symptomatic treatments are available that delay the disease symptoms. Currently, two drugs—cholinesterase inhibitors and memantine, are approved by the US food and drug administration (FDA). These drugs act by counterbalancing the neurotransmitter disturbances (Yiannopoulou and Papageorgiou, 2013). Cholinesterase inhibitors protects or delay the degradation of acetylcholine, an important chemical messenger, and are prescribed for mild to moderate AD case. For moderate to severe AD, the NMDA receptor antagonist memantine is used (McKeage, 2009). Treatments targeting the pathological entity/reason of disease, referred to as disease-modifying treatments, are underway for AD where Aβ is the prime target. Many of these treatments are currently at different phases of clinical trials. Approaches used in such treatments include anti-amyloid strategies, decreasing the production of Aβ or rapid clearance by inhibiting β- or/and γ-secretase or activating α- secretase, halt or prevent Aβ aggregation by developing anti-aggregating compounds, and removal of toxic Aβ aggregates, by for instance using active and passive anti-amyloid immunotherapy (reviewed in refs. Yiannopoulou and Papageorgiou, 2013, Folch et al., 2018, Cummings et al., 2018).

3.2 Transthyretin amyloidosis

The transthyretin (TTR) protein was discovered in the 1940s and was initially called prealbumin since it migrates ahead of albumin during protein gel electrophoresis (Kabat et al., 1942). Later the name was changed to transthyretin, a name that reflects its dual functionality—transportation of thyroxine and retinol in the circulation (Robbins, 2002).

TTR is a homotetrameric protein with a molecular weight of 55 kDa, which is predominantly produced in the liver and released into the plasma (Felding and Fex, 1982). Additionally, a small amount of TTR is produced in the choroid plexus of the brain and released into CSF (Weisner and Roethig, 1983, Herbert et al., 1986). Also, the retina of the eye and the endocrine cells of the pancreatic islets are reported to be TTR production sites (Hamilton and Benson, 2001). In healthy individuals, the TTR tetramer functions as a transporter for thyroxin and retinol in plasma and CSF. However, mutations in the TTR can lead to a destabilization of the tetramer, which results in an increased propensity to aggregate and accumulate as amyloid in various organs. The aggregation-prone TTR protein is called ATTR, and the disease is called ATTR amyloidosis.

3.2.1 Structure of TTR

The X-ray crystal structure of human TTR was solved in 1971 (Blake et al., 1971), which revealed a structure with high β-sheet content. A native TTR monomer is composed of 127 amino acids and forms eight β-stands denoted A-H and a small α-helical structure between E and F stands (Fig. 3.4a). The eight β-stands form two β-sheets, the β-stands D, A, G, and H form one β-sheet and the second β-sheet is formed by β-stands C, B, E, and F. The two β-sheets are organized face-to-face forming a β-barrel. Two monomers dimerize through intramolecular extensive hydrogen bonding between H-stands of each monomer. Hydrogen bonds between the F stands are also present, but to a lower extent with only one pair in the dimeric structure. The structure is further organized into a tetramer where two dimers interact through hydrophobic contact involving the loop region between β-stands A and B and β-stands G and H (Fig. 3.4b) (Hörnberg et al., 2000). Assembling two dimers into a tetramer in this manner provides a hydrophobic channel for thyroxin binding. Two thyroxin binding sites are available per tetramer (Fig. 3.4b).

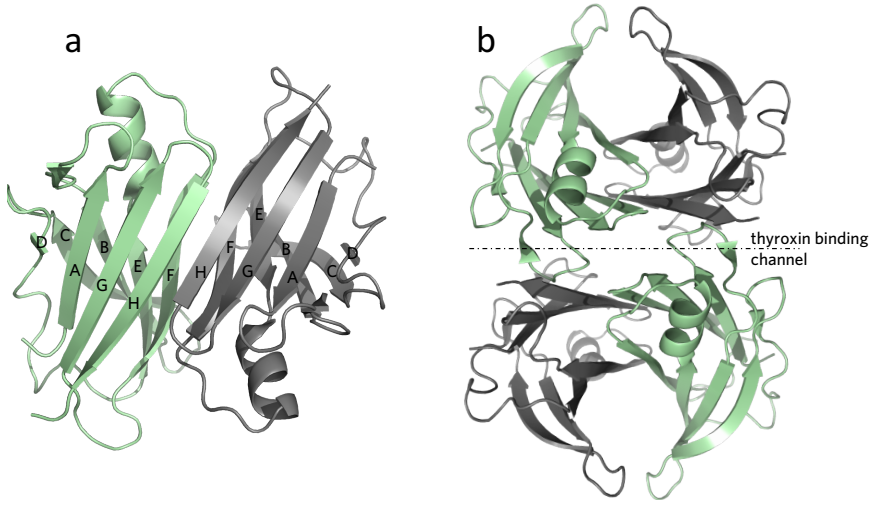

Figure 3.4 Cartoon presentation of TTR structure. (**a**) Self-assembly of two monomeric units (one colored green and the other gray) into dimer and (**b**) two dimers into a tetrameric functional unit. Thyroxin binding channel is indicated with dotted arrow. The structures were produced using PDB accession no. 1F41, a model structure of human wt-TTR solved using X-ray crystallography at 1.5 Å resolution.

3.2.2 ATTR fibrillation

It has been well established that the TTR protein becomes aggregation-prone upon dissociation into partially unfolded monomer, which then rapidly self-assemble into oligomers before forming insoluble fibrillar aggregates (Fig. 3.5)

(Foss et al., 2005). However, unlike the classical amyloid aggregation pathway (discussed in chapter 2.3), the ATTR aggregation occurs via a non-nucleated mechanism known as downhill polymerization (Hurshman et al., 2004). The dissociation of tetramer into monomers is the rate-limiting step in the aggregation process. Thus, seeding does not accelerate ATTR aggregation as demonstrated by *in vitro* kinetic data (Hurshman et al., 2004, Eisele et al., 2015). Nevertheless, a more recent study has demonstrated that patient-derived amyloid seeds accelerate monomeric and tetrameric TTR fibrillation *in vitro* (Saelices et al., 2018). Conditions that favor TTR dissociation and subsequent aggregation are debated. Some studies have shown that acidic conditions are required for tetramer dissociation and fibril formation (Lai et al., 1996, Lashuel et al., 1999), while others have demonstrated that the fibrillation can occur at physiological pH (Quintas et al., 1997, Quintas et al., 1999).

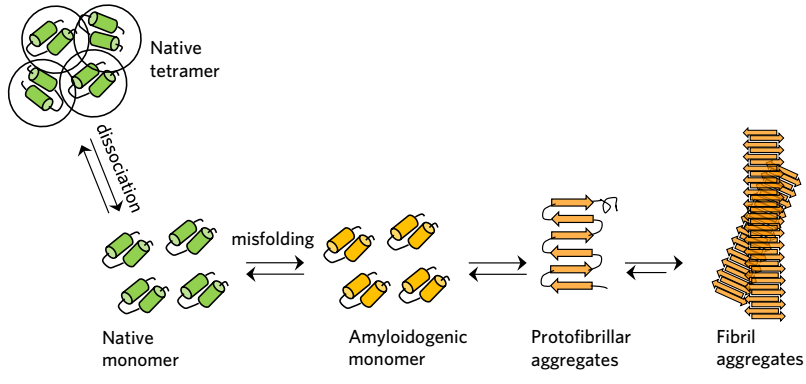

Figure 3.5 Illustration of ATTR fibrillation, which is initiated by dissociation of native folded tetramer into monomer which are further converted into amyloidogenic monomer. Subsequently, the amyloidogenic monomer assembled into higher ordered aggregates en route to fibril.

3.2.3 ATTR-derived amyloidoses

ATTR amyloidosis exits in two main forms: *hereditary ATTR amyloidosis* (hATTR), which affects multiple organs *e.g.*, peripheral nerves, heart, eyes and kidney and *wild-type ATTR amyloidosis* (ATTRwt), which primarily affects the heart. The hATTR is associated with mutations in the TTR genes and is a lethal autosomal dominant disorder. As of October 2018, 137 different mutations have been identified in the TTR gene (http://amyloidosismutations.com/attr.html). The most common TTR mutation globally is ATTRV30M (valine to methionine substitution at position 30), which is associated with hATTR with polyneuropathy. Another example on a polyneuropathy mutation is ATTRL55P, which is considered to be the most pathogenic variant (Lashuel et al., 1999) and

is associated with an early age onset (at about 15-20 years) polyneuropathy (Jacobson et al., 1992).

The ATTRV122I mutation, which is frequently found among individuals of African descent (Jacobson et al., 1996), and the ATTRL111M mutation, which is common in the Danish population (Nordlie et al., 1988), are two examples of hATTR with cardiomyopathy.

In old age, the wt-TTR can also forms amyloid (Cornwell et al., 1988, Westermark et al., 1990). In approximately 25% of all individuals above the age of 80 years, some cardiac amyloid deposit composed of wt-TTR can be detected (Westermark et al., 1990, Tanskanen et al., 2008).

3.2.4 Diagnosis and therapies for ATTR amyloidosis

Commonly, tissue biopsy followed by Congo red staining is used for the identification of amyloid in tissue and typing of amyloid deposit is most commonly performed by western blot or mass spectrometry (Suhr et al., 2000). During this procedure, a small piece of subcutaneous fat tissue (sometimes from other sites) is used as smear and subjected to Congo red staining. When amyloid is present, this is detected as an apple-green birefringence under a polarized microscope. However, Congo red staining is reliant on the size of the aggregates that can be detected, and therefore small aggregates could be overlooked. In *paper II* of this thesis, we attempted to develop an assay for detection of early ATTR deposits.

Previously, liver transplantation (LT) was thought of as the first-line treatment for hATTR amyloidosis. In LT treatment, the mutant ATTR producing liver is replaced by a wt-TTR producing liver (Suhr et al., 2000). However, LT is limited by the availability of organs.

The fact that tetrameric TTR needs to dissociate into monomer prior to the formation of amyloid, many drugs have been developed to stabilize the tetramer or halt dissociation and thereby prevent TTR amyloid formation. Notably, two drugs—diflunisal and tafamidis, have shown promising results in clinical trials. Tafamidis has been approved for treatment of hATTR amyloidosis in European countries and Japan (Sekijima, 2015) and has recently completed a phase II/III trials (Maurer et al., 2018). Structural studies demonstrated that tafamidis binds to the thyroxin binding sites (TTR dimer-dimer interface, see in Fig. 3.4) of the tetramer and in this way stabilizes the two dimeric species into tetramer (Bulawa et al., 2012). Other promising therapeutics on the way are antisense oligonucleotides (Benson et al., 2017) and siRNAs (Rizk and Tuzmen, 2017) for suppression of mutant and wt-TTR synthesis. These drugs are currently in phase III trials.

4 Beneficial roles of amyloid

Nonpathogenic amyloid structures are ubiquitous in Nature. Numerous organisms including bacteria, fungi, insects, and mammals—including humans, utilize amyloid structures for a diverse range of biological functions. The current chapter will discuss the examples of natural amyloid as well as the potentials of amyloid for artificial functional materials.

4.1 Beneficial formation of amyloid in Nature

The discoveries of *functional amyloid* in yeast have changed the way we perceived amyloid. Of the many cases known, the Sup35p and Ure2p proteins, both from *Saccharomyces cerevisae*, are well-studied amyloid structures in yeast (reviewed in ref. Chien et al., 2004). The Sup35p in its non-amyloid form functions as a translation termination factor that mediates the termination of protein synthesis at stop codons. However, the aggregation of Sup35p into amyloid disrupts its function and the protein no longer acts as a translational terminator. Thus, the translation continues through the stop codon, and this is thought to be associated with introduction of new phenotypes (Serio and Lindquist, 1999). In addition, it has been demonstrated that the formation of Sup35 fibrils allow yeast cells to withstand both heat and chemical stress better than yeast cells that do not contain Sup35 amyloid (True and Lindquist, 2000). This suggests that the conversion of Sup35p into fibrils in yeast cells may be the results of evolutionary selection (Lancaster et al., 2010). Like Sup35p, the Ure2p protein can also form amyloid. In its soluble form, Ure2p regulates nitrogen catabolism by repressing the activity of transcription factor Gln3p, which controls the expression of nitrogen catabolic gene DAL5 (Coffman et al., 1994). In the presence of a good nitrogen source, yeast blocks the expression of DAL5 (Chien et al., 2004). The conversion of Ure2p into amyloid form prevent this action, which causes constitutive activation of Gln3p, and thus upregulation of

DAL5, which enables yeast to utilize poor nitrogen sources through pathways controlled by DAL5.

Functional amyloids have also been discovered in gram-negative bacteria such as *E. coli* and *Pseudomonas*. Curli fibril, assembled from the protein CsgA, is a type of functional fiber believed to constitute the major proteinaceous component of the extracellular matrix produced by *E. coli*. (Arnqvist et al., 1992). The assembly of curli fibril is tightly controlled and assisted by several co-factors such as CsgB, which favors the nucleation process, and CsgG, which transports CsgA to the extracellular space. Curli fibrils are thought to be involved in a range of activities including colonization of inert surfaces and biofilm formation (Vidal et al., 1998), and mediate binding to the host proteins (Olsén et al., 1993).

Before the discoveries of these functional amyloid structures, amyloid found in human were thought to be associated with diseases only. Therefore, the identification of functional amyloid in humans, such as Pmel17 and various peptide hormones in secretory granules of endocrine system, has forced us to re-evaluate how we look at amyloid. The Pmel17 has been demonstrated to catalyze melanin biosynthesis in melanosomes (Fowler et al., 2006), and storage of peptide hormones in pituitary secretory granules as aggregates are believed to play critical role in the regulation of their release (Maji et al., 2009). Table 4.1 presents a selection of functional amyloid structures that occur in Nature for various physiological functions.

Table 4.1 Examples of amyloid-like structure in natural use.

Species	Protein	Physiological function of resulting fibril
Bacteria		
Escherichia coli	CsgA	Curli fibrils involved in biofilm formation
Pseudomonas	FapC	Fibrils in biofilm
Streptomyces coelicolor	Chaplins	Modulation of water surface tension
Fungi		
Saccharomyces cerevisae	Sup35p	New phenotypes by enabling stop codon read-through
	Ure2p	Enables yeast to uptake poor nitrogen source
Animalia		
Insect and fish	Chorion	Structure and protection of the eggshell
Nephila clavipes	Spidroins	Formation of silk fiber of the web
Homo sapiens	Pmel17	Catalyzes synthesis of melanin

Table adapted from refs. Fowler et al., 2007, Knowles and Mezzenga, 2016.

4.2 Functionalization of amyloid

The mechanical robustness, as well as the fact that amyloids are used in Nature, makes these structures attractive as the building block for artificial functional materials known as *nanofibril-based material*. The amyloid fibril-forming protein can be engineered as a fusion comprising functional biomolecules such as enzymes, antibody-binding domain, and metal-binding peptide. The fibril-forming protein will spontaneously self-assemble to form nanofibrils with desired functionalities. Numerous of nanofibril-based materials have been developed for various applications in the field of biotechnology, medicine, and biomaterial sciences. For examples, nanofibril-based materials have been designed for use as cell culture scaffolds (Kasai et al., 2004, Gras et al., 2008), as vehicles for drug delivery (Silva et al., 2013), for capturing carbon dioxide from the environment (Li et al., 2014), and as efficient biosensors (Sajanlal et al., 2011, Hauser et al., 2014). A selection of fibril-forming proteins and their emerging applications are listed in Table 4.2.

Table 4.2 Examples of emerging application of non-pathological and pathological amyloid fibril.

Fibril protein	**Emerging applications**
β-lactoglobulin	Biosensor, catalysis, hybrid, transfection, nanocomposites
Lysozyme[a]	Artificial bones, hybrids, cell scaffolds
Sup35p	Biosensors, hybrids
α-synuclein[a]	Hybrids, sensors
CsgA/*E. coli* Curli	Under water adhesives
TTR1[a]	Cell scaffolds
$A\beta_{42}$[a]	Light harvesting, cell scaffolds, cell differentiation

[a]also form pathological deposit in human disease
Table adapted from Knowles and Mezzenga, 2016.

A functionalized nanofibril that has a high binding capacity to antibodies (hereafter called Ab-bNF for antibody-binding nanofibril), was recently assembled in our research group (Schmuck et al., 2017). The Ab-bNF is used in *paper II* of this thesis. The composition and efficiency of the Ab-bNF will briefly be discussed below.

4.2.1 Antibody-binding nanofibrils

In the Ab-bNF, fibrils formed from the N-terminal fragment (1-61) of Sup35 are exploited as frame for displaying the antibody-binding functional Z-domain (introduced in chapter 3.1.3). The Ab-bNF was assembled by co-fibrillation of the functional antibody-binding domain fused to the fibril-forming protein

(Sup35(1-61)-ZZ, chimeric protein) with fibril-forming protein alone (Sup35(1-61), carrier protein). An optimized ratio between the chimeric and carrier proteins provided adequate space on the surface of the fibrils, which reduce steric hindrance, and thus maximizes the functionality of the Ab-bNF. The Ab-bNF assembled using a molar ratio of carrier protein to chimeric protein of 1:0.33 has a binding capacity of 1.8 mg antibody per mg Ab-bNF, which is almost 20-fold higher binding capacity compared to the gold standard Protein A Sepharose used for antibody purification (Schmuck et al., 2017).

5 Present investigations

5.1 Scope of this thesis

This thesis focuses on two aspects: detection of Aβ and ATTR aggregates *in vitro* and *ex vivo*, and investigation of potential interactions of protofibrillar and fibrillar aggregates of Aβ with proteins in biofluid.

Specific aims

Paper I: to identify new affinity proteins that specifically bind to Aβ protofibrils.

Paper II: to devise a method with high sensitivity for detection of TTR *in vitro* and ATTR deposits in tissue samples.

Paper III: to investigate potential interactions between Aβ protofibrils and proteins in human serum and cerebrospinal fluid.

Paper IV: to explore differences of binding profiles of cerebrospinal fluid proteins to protofibrillar and fibrillar aggregates of Aβ.

5.2 Methodological considerations

This section aims to highlight main methods as well as methods developed in the course of the thesis work. Details of experimental procedures used in each paper are described in the methods section of the corresponding paper.

5.2.1 Surface plasmon resonance (*paper I*, *III* and *IV*)

Surface plasmon resonance (SPR)-based biosensors provide a real-time and label-free technique to study biomolecular interactions (Jonsson et al., 1991). In this method, one binding partner is immobilized on a gold coated surface of a sensor chip while the other interacting-partner is injected in a continuous flow of solution. Polarized light is directed to the under surface of the gold film where surface plasmon is generated at a critical angle (resonance angle) of the incident light. Binding or dissociation of biomolecules at the sensor surface changes the local refractive index and produces a shift in the resonance angle (Schuck, 1997). The SPR provides more comprehensive information of the interactions between two molecules in terms of affinity, kinetics, concentration, and thermodynamics compared to other techniques such as isothermal calorimetry (ITC) and bio-layer interferometry (BLI). ITC cannot provide kinetic (association and dissociation constant) while BLI has very low sensitivity.

The SPR experiments presented in this thesis were executed on a Biacore X100 system (GE Healthcare). The ligands, $A\beta_{42}$CC protofibrils or $A\beta_{42}$ fibril, were immobilized separately onto CM5 sensor chips (GE Healthcare) using standard amine coupling chemistry. Selected proteins were allowed to pass over the immobilized chip surface, and the association and dissociation of the binding kinetics were recorded. Collected data was fitted to 1:1 binding kinetic model or heterogeneous binding model with two binding sites.

5.2.2 Immunoassay (*paper II*)

We have developed an enhanced detection method to be used for the detection of small amounts of ATTR aggregates. The method is based on the Ab-bNF (introduced in chapter 4.2.1). An enhanced signal in an amyloid detection system can be achieved if an increased number of antibodies can be linked to the antigen avoiding steric hindrance. Using the Ab-bNF, whose surface contains the antibody-binding Z domain (Fig. 5.1a), we could increase the concentration of primary antibody in close association to the antigen, which then provides an increased number of binding sites for the secondary antibody that results in signal amplification (Fig. 5.1b).

Anti-human TTR polyclonal (1899) antibody produced in rabbit was loaded onto the Ab-bNF by incubation at room temperature for 45 min. 1.8 µg antibody was added to per µg Ab-bNF. Hereafter the TTR antibody (1899) bound Ab-bNF is referred to as TTR-bNF. After incubation, TTR-bNF was pelleted and washed to remove unbound antibodies followed by dilution in 1× phosphate buffered saline prior to addition to microplate well containing TTR or on tissue section containing ATTR aggregates. The detection procedure was similar to

that of conventional microplate immunoassay or the immunolabeling of tissue section. Goat anti-rabbit antibody conjugated with Alexa Fluor 488 (GAR-Alexa 488) (Thermo Fisher) was used for the detection of TTR-bNF bound to TTR or ATTR aggregates in tissue section.

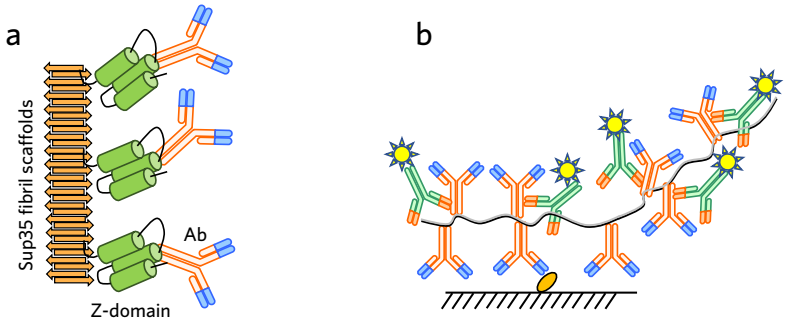

Figure 5.1 Concept of signal enhancement using Ab-bNF. (**a**) Anti-human TTR polyclonal (1899) antibody was bound to Ab-bNF via Z domain displayed on the surface, and (**b**) incubated with TTR antigen, the additional antibodies on the fibril surface amplify the number of secondary antibody binding sites thereby enhancing the signal.

An advantage with this enhancement method is that it links many antibodies to the same binding site on the antigen, which increases the signal from the same antigen compared to the signal from a conventional immunoassay and thereby offer a more sensitive system for the detection of small pathogenic aggregates. A disadvantage of this method is that it requires a large amount of antibody compared to conventional method. However, method development is still at an early stage, and this obstacle should be included in the further optimization strategies.

5.2.3 Protein pull-down assay (*paper III* and *IV*)

We established a protein pull-down assay to investigate the potential binding of Aβ protofibril and fibril to proteins in serum and CSF. Previously, affinity chromatography-based method was developed for this purpose (Calero et al., 2012). This method involved in preparation of affinity matrix by preincubation of Aβ with activated cyanogen bromide (CNBr) sepharose and subsequent packing a chromatography column with the matrix. The sample of interest was run over the column, and bound proteins were eluted. A similar approach was demonstrated by Medvedev *et al*. using a different sepharose matrix, *i.e.* Aff-Gel10 (Medvedev et al., 2015). However, these protocols are time-consuming and require expensive instruments such as a liquid chromatography system. Moreover, the volume of the eluate from this setup is large and often needs to be

concentrated for downstream analysis, which increases the risk of losing material in the process. We developed a simple capturing assay which does not require expensive instruments and can be performed in an Eppendorf tube. The other strength of this assay is that the elution volume can be limited to a few µL.

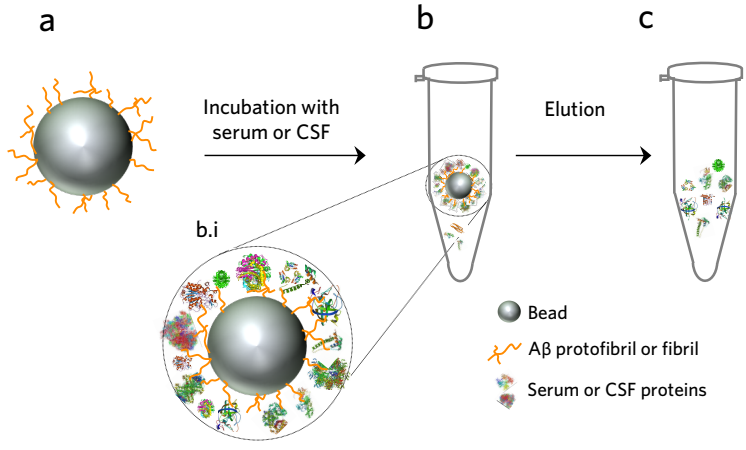

Figure 5.2 Illustration of the pull-down assay used to investigate the binding of serum and CSF proteins to protofibrillar and fibrillar aggregates of Aβ. (**a**) Aβ aggregates were coupled onto the Dynabeads M-280. (**b**) Bead-Aβ complex was incubated with serum or CSF samples for protein binding (**b.i**). (**c**) Proteins bound to Aβ were eluted by heating in SDS-PAGE sample buffer.

We used M-280 magnetic Dynabeads (Invitrogen, catalog #14204) as matrix for coupling the ligands *i.e.* Aβ aggregates. The bead surface contains activated tosyl groups, which bind to amino groups of the ligands by formation of amine bonds. The bead is ideal for coupling ligands containing primary amino group and has high coupling efficiency. The ligands ($A\beta_{42}$CC protofibril or $A\beta_{42}$ fibrils, 100 µg) were incubated with 5 mg beads in 0.1 M Na-phosphate at pH 7.4 overnight at 37 °C for covalent binding, followed by an additional 1 h incubation in PBS pH 7.4 with 0.5% Tween-20 to block remaining binding sites on the bead surfaces. After this process, the bead-Aβ complex is ready to study its interaction with proteins in serum or CSF (Fig. 5.2a). For capturing Aβ-binding partners in serum or CSF, bead-Aβ complex (0.5 mg) was incubated with serum (150 µL) or CSF (200 µL) at 37 °C for 1h (Fig. 5.2b,bi). Beads were then washed rigorously three times, and bound proteins were eluted in 12 µL SDS-PAGE sample buffer (Bio-Rad) by heating at 70 °C for 10 min (Fig. 5.2c).

The pull-down assay was further accompanied by LC-MS/MS identification of bound proteins and SPR biosensor-based validation of binding.

In the following sections of this thesis, the work in the appended published papers and manuscript are reviewed. *Paper I* and *II* are presented separately, and then the *paper III* and *IV* are presented together. Conclusions follow each of these reviews. The chapter ends with general discussion and possible future directions of this thesis work.

5.3 New affinity proteins to detect Aβ protofibrils (*paper I*)

The field of AD research is in great needs of potential methods to detect and distinguish soluble Aβ protofibril from other aggregated forms and monomer. However, antibodies targeting pre-fibrillar Aβ have previously been developed (Kayed et al., 2003, Englund et al., 2007, Kayed et al., 2007). This study focuses on *in vitro* development of new affinity protein, alternative to antibodies, to detect Aβ protofibrils.

5.3.1 Selection and validation

Affibody-based affinity proteins were selected by phage display selection (Gronwall et al., 2007). Protofibrils formed from $A\beta_{42}$CC variant were used as target molecules in the selection method. Six rounds of selection and phage amplification were performed. For the post-selection screening, 744 randomly chosen colonies were expressed directly from phagemid vector in fusion to an albumin binding domain (ABD) and screened for binding to $A\beta_{42}$CC using an enzyme linked immunosorbent assay (ELISA). Twenty-five potential candidates, EC_{50} values in the range of 15 to 90 nM, were identified and among these the five most promising binders were denoted as $Z_{A\beta42cc\_1}$, $Z_{A\beta42cc\_2}$, $Z_{A\beta42cc\_3}$, $Z_{A\beta42cc\_4}$, and $Z_{A\beta42cc\_5}$ (hereafter collectively called Aβpf-binders) were considered for further characterization.

These top five binders were then tested for their selective binding to protofibrils over other forms of Aβ. $A\beta_{42}$wt protofibrils were also included in this experiment. ABD-fused Aβpf-binder was immobilization on ELISA plate via an anti-ABD antibody followed by incubation with various form of Aβ. To detect bound Aβ to Aβpf-binder, conformation independent monoclonal antibody 6E10, or conformation-specific antibody mAb1C3 (has some specificity for $A\beta_{42}$ protofibrils (Englund et al., 2007)) were used.

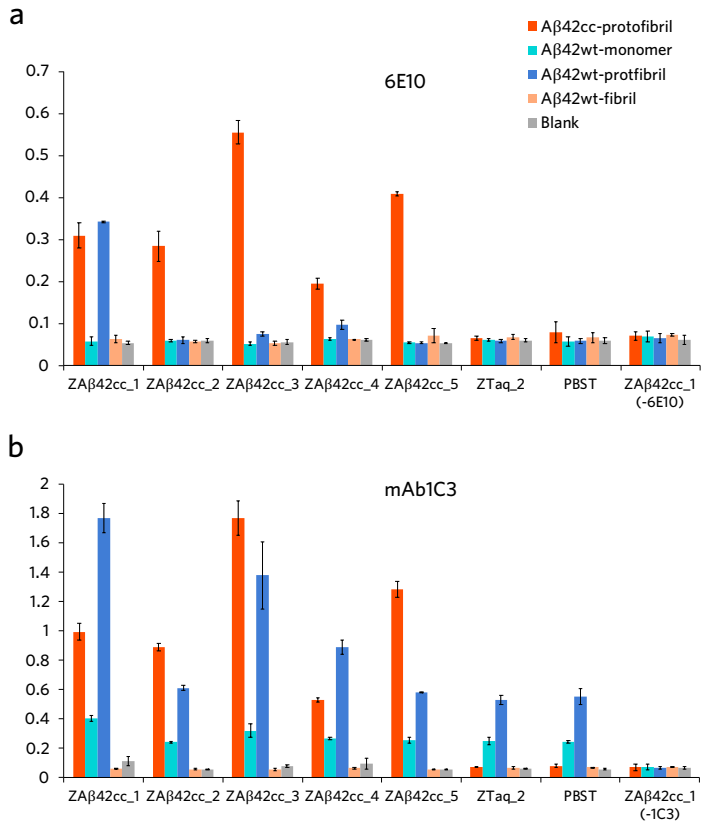

Figure 5.3 Selective binding of the top five Aβpf-binders to various forms Aβ tested by ELISA using monoclonal antibody 6E10 (**a**) and mAb1C3 (**b**). A non Aβ-binder Affibody molecule, ZTaq_2, PBST, and PBST replacing antibodies were used as controls. Values are means of duplicate experiments.

As shown in Figure 5.3, all tested Affibody molecules bound well to $A\beta_{42}CC$ protofibrils, and none of them bound to either monomer or fibrils. Moreover, incubation with 6E10 showed that $Z_{A\beta42cc\_1}$ also bind to $A\beta_{42}wt$ protofibrils (Fig. 5.3a). Notably, the conformation-specific mAb1C3 experiment revealed that not only $Z_{A\beta42cc\_1}$, but also $Z_{A\beta42cc\_3}$ and possibly $Z_{A\beta42cc\_4}$ bound to wt-protofibrils (Fig. 5.3b). Why the two other Aβpf-binders *i.e.*, $Z_{A\beta42cc\_2}$ and $Z_{A\beta42cc\_5}$ did not recognize the wt-protofibrils can be explained by the fact that the wt-protofibrils might form mature fibrils during 1 h incubation time and washed off before detection. The interconversion of wild-type protofibrils to mature fibrils happens rapidly (Luheshi et al., 2010). The situation can also be true for the experiment where 6E10 antibody is used (Fig. 5.3a).

An affinity capture assay with shorter incubation time was then carried out. This assay involved immobilization of Aβpf-binder on nickel-charged resin employing a His6 tag followed by 5 min incubation with various Aβ species. SDS-PAGE analysis confirmed the selective binding of Aβpf-binder to protofibrils over other species (Fig. 5.4a). The results suggest that the tested binders ($Z_{A\beta42cc\_1}$ and $Z_{A\beta42cc\_4}$), recognized $A\beta_{42}$wt protofibrils and $A\beta_{42}$CC protofibrils with similar selectivities/intensities (Fig. 5.4b), which was the expected outcome of this experiment.

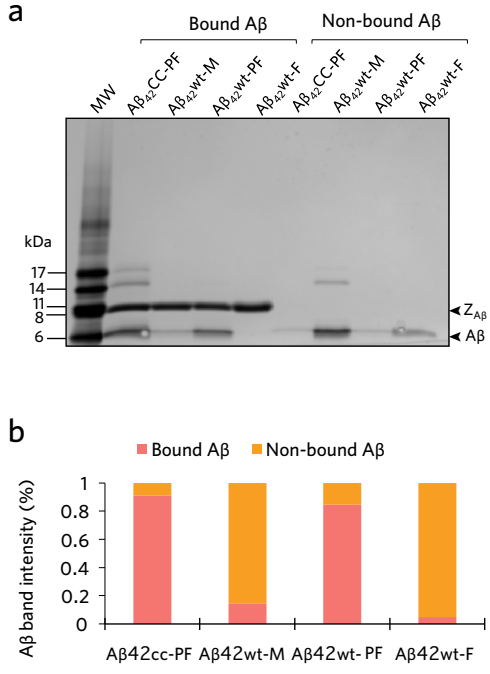

Figure 5.4 (**a**) Representative SDS-PAGE analysis of the samples obtained from affinity capture assay using $Z_{A\beta42cc\_4}$ as ligand. Aβ species that bind to $Z_{A\beta42cc\_4}$ are eluted together with $Z_{A\beta42cc\_4}$ (indicated as bound to Aβ) and Aβ species that did not bind to $Z_{A\beta42cc\_4}$ are recovered in supernatant (indicated as non-bound to Aβ). The band corresponding to Affibody and Aβ are indicated by arrow head. (**b**) SDS-PAGE band intensities corresponding to bound and non-bound Aβ. The data was normalized with respect to each Aβ species, *i.e.* the sum of the intensities for bands originating from one Aβ species is equal to 100%.

5.3.2 Binding kinetics of Aβpf-binders to protofibril

All tested Aβpf-binders showed low nanomolar (nM) binding affinity[1] to $A\beta_{42}$CC protofibrils, determined by SPR. Average dissociation constants, K_D,

1. Kinetic data can be found in Table S1 in the supplementary information of paper I

were determined to be ≈1.7 (±0.6) nM. However, in order to detect Aβ protofibrils in biological samples, for instance in CSF, even stronger binders are required. The concentration of protofibrils in the AD CSF is in the picomolar range (Georganopoulou et al., 2005), while in the brain of AD patient the concentration has yet to be determined but is presumed to be very low. Using conformation-specific antibodies, efficient capture of Aβ protofibrils with concentration in the picomolar range was demonstrated previously (Englund et al., 2007, Yang et al., 2015).

A recent study had shown that the affinity of the Affibody molecules to a protein containing multiple similar binding surfaces (Aβ protofibril might have such surfaces) can be improved 1000-fold by making dimeric construct (Lindborg et al., 2013). We therefore constructed 14 dimers and studied their binding to $A\beta_{42}$CC protofibrils by SPR. However, the affinity improvement with dimeric construct was not as great as expected. All dimeric Aβpf-binders showed either equal or up to one order of magnitude stronger affinity compared to the monomers. Further studies, in particularly, structural comparisons of monomeric and dimeric constructs as well as the surface on protofibrils, would be required to understand why higher affinities are not achieved with dimeric construct.

5.3.3 Binding topology

Mapping of the chemical properties of selected side chains onto the Aβpf-binders revealed three chemically distinct regions of the Z-domain surface as *positive-nonpolar-polar*. The surface pattern of Aβpf-binders presumably matches a corresponding surface epitope on the protofibrils. In fact, a recently developed structural model of protofibril contains surfaces that appear to match the selected Affibody molecules (Lendel et al., 2014). However, further structural studies are required to confirm the binding site on the protofibrils.

5.3.4 Conclusions

In this study, we have identified Affibody-based potential proteins that specifically recognize and bind $A\beta_{42}$CC protofibrils. Importantly the binders also recognize protofibrils formed by $A\beta_{42}$wt peptide. The binders have potential to be used for detection of $A\beta_{42}$ protofibril over other aggregated forms and monomer both in research as well as diagnostics and therapeutic applications.

Future work should be devoted to extensive characterization of these binders—in particular structural studies to get insight into the structural basis for the interactions with protofibrils. It would also be relevant to investigate the potential of these affinity proteins in brain imaging. Another exciting future

study would be designing a new functional Aβ protofibril-binding protein nanofibril, where the Aβ-pf binder will be displayed on the fibril surface, a similar strategy used in *paper II*.

5.4 Development of method for sensitive detection of ATTR aggregates (*paper II*)

The tissue damage caused by amyloid deposition is not repairable, and early detection of amyloid deposit can provide early treatments thus prevent progressive tissue damage. In this study, we improved an ATTR detection system by exploiting nanofibril functionalized with TTR antibody (TTR-bNF) to detect small amount TTR amyloid.

5.4.1 Signal enhancement in microplate immunoassay

To test the efficacy of the TTR-bNF, we performed a microplate immunoassay using a stable monomeric TTR (mono-TTR) (Jiang et al., 2001), as antigen. The mono-TTR was immobilized onto the microtiter plate well. TTR-bNF charged with ≈13.5 μg anti-human TTR polyclonal (1899) antibody was added to the well. After incubation, TTR-bNF bound to mono-TTR was detected using GAR-Alexa 488. In parallel, a reference assay was performed using the same procedure except that 1 μg antibody was added to wells with no fibrils included.

Using the improved assay, sensitivity was enhanced 128-fold compared to the reference assay (Fig. 5.5a). A control experiment that involved immobilization of mono-TTR onto a microtiter plate wells followed by incubation with increasing concentration of primary antibody showed that fluorescence could not be increased by adding more antibody without loading onto the fibrils (Fig. 5.5b). This demonstrated that the enhanced signal is due to TTR-bNF in the system.

The results showed that the concept for signal enhancement worked as expected. However, the current protocol has limitations. For example, the limit of detection cannot be precisely calculated from the collected data using the current setup due to the large standard deviation that was observed. Possible reasons for the large standard deviation could be the diversity of the fibril lengths as well as longer fibrils employed in the assay. The use of a uniform fibril length could be critical, since it determines the number of primary antibodies introduced into the system. In addition, longer fibrils are heavier and could be more susceptible to uncontrolled release from the surface due to shearing forces during the washing steps. The irregular data points in our assay support this hypothesis. Our Ab-bNFs are quite long and exhibit fibrils of diverse lengths

ranging from 1-2.4 µm analyzed by TEM. Another factor that possibly contribute to the large variation between data points is uneven distribution of fibril in solution.

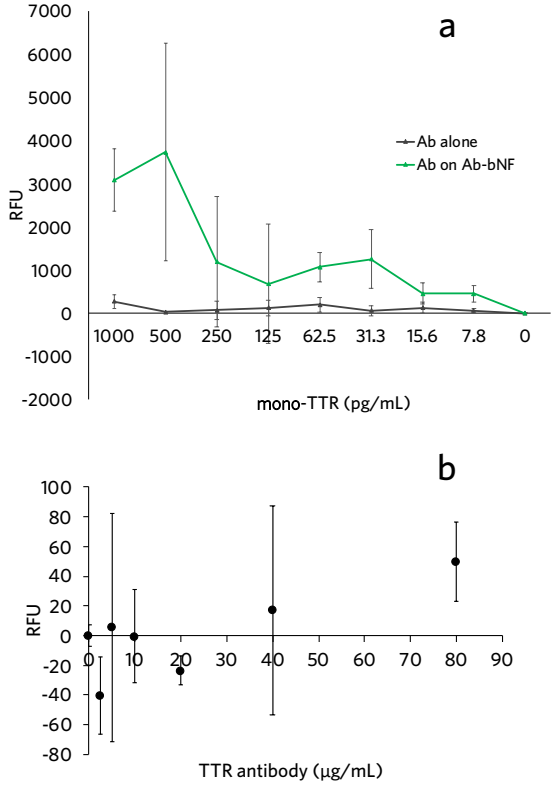

Figure 5.5 Signal enhancement in microplate immunoassay. **(a)** Comparison of TTR-bNF assay (green) with reference assay (black) (**b**) A plot showing that the florescence cannot be increased by adding extra amount of antibody if they are not loaded onto fibrils. Each data point represents mean ± SD (n=3).

Introduction of shorter fibrils of ca. 500 nm into the system could prevent uncontrolled release from surface and also minimize the uneven distribution in the solution, as demonstrated in a previous study (Men et al., 2009). Fibrils with shorter length can be produced by adding more seeds during fibril assembly. Also, a relatively large volume of fibrillation solution (than the amount used) can be beneficial to produce small fibrils as well as their even distribution in the solution. In addition, the strategic testing of the fibril compositions *i.e.* different ratios of fibril-forming proteins (Sup35) and fusion protein (Sup35-ZZ) should be performed. Importantly, the reproducibility of the Ab-bNF should be a key to obtain the defined fibrils lengths and fibrils distribution.

5.4.2 Enhanced signal in immunolabeling of *ex-vivo* tissue

Next, we tested the usefulness of the method in immunolabeling of patient-derived tissue containing ATTR aggregates. This assay involved incubation of tissue section with TTR-bNF containing either 9 µg or 13.5 µg primary antibody followed by detection of bound TTR-bNF using GAR-Alexa 488. For comparison, decreasing concentrations of primary antibody, 1 µg, 0.5 µg or 0.25 µg was directly added to the section.

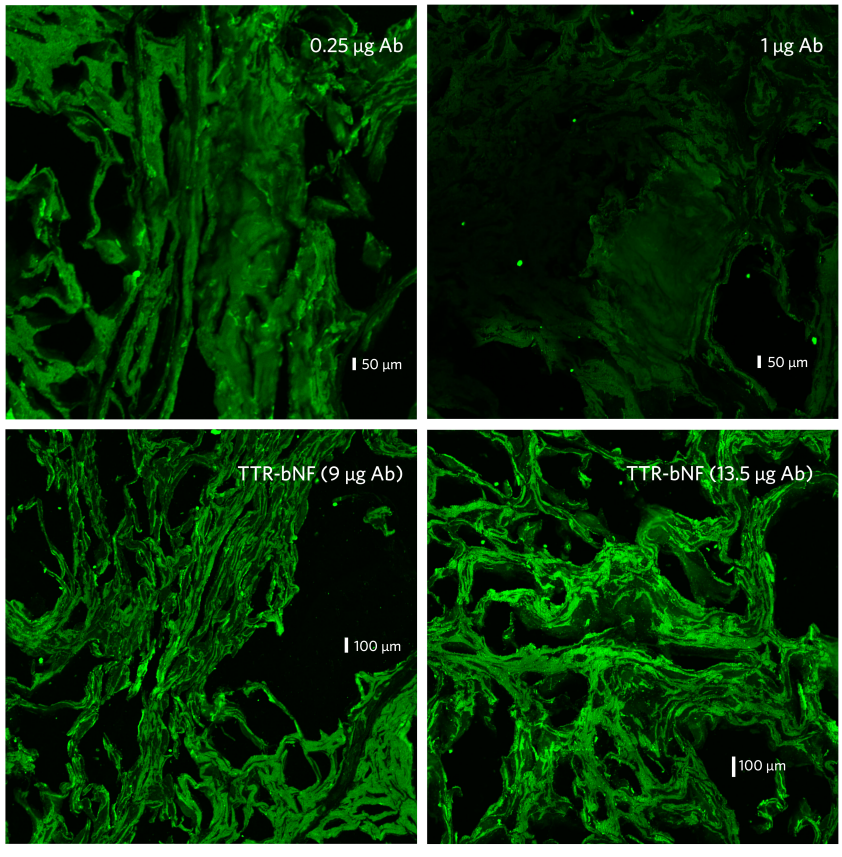

Figure 5.6 Enhanced fluorescence in immunolabeling of patient-derived tissue containing ATTR aggregates using the TTR-bNF. *Top two images*, the regular assay where primary antibody was directly added to the tissue sections. *Bottom two images*, the TTR-bNF assay where primary antibody was bound to the fibrils before addition to the tissue sections.

As shown in Figure 5.6, amyloid specific signal was observed in section incubated with 0.25 µg of antibody. In comparison the section incubated with 1 µg (4-fold more) antibody exhibited a weaker signal, which is not uncommon when tissue section is incubated with higher antibody concentration. As

expected, section incubated with TTR-bNF carried 9 μg antibody, which is almost 10-fold more antibodies compared to above reference, results in strong fluorescent signals. Notably, much stronger signal is observed in section incubated with TTR-bNF that carried 13.5 μg antibody compared to signal observed in section incubated with TTR-bNF that carried 9 μg antibody (Fig. 5.6, bottom images). This result shows that the enhanced signal is indeed due to TTR-bNF. Moreover, the enhanced signal is well associated with amyloid deposit in tissue, and the unspecific binding of amyloid fibril (TTR-bNF) to the tissue could be excluded. The result indicates that the method has potential to detect small aggregates deposited in tissue, and further evaluation should be performed on tissue with very small amyloid deposits.

5.4.3 Conclusions

The results produced in the pilot study are promising, but further optimizations are warranted. With further development the detection system could provide a new method for the detection of small amyloid aggregates. Currently, we are focusing on producing short fibrils with uniform length.

Interesting future studies would be to explore the method in the detection of other amyloid aggregates such as Aβ and IAPP aggregates.

5.5 Identification of Aβ interactome in biofluid (*paper III* and *IV*)

Numerous proteins are colocalized with Aβ in the AD plaque, and may associate with soluble Aβ in CSF and serum. Identification of Aβ-interacting partners in serum and CSF is of great interest for studying Aβ-related neurotoxicity in AD as well as biomarker discovery. Potential binding partners of pre-fibrillar and fibrillar form of Aβ in serum, and AD and non-AD CSF is investigated in *paper III* and *IV*.

5.5.1 Aβ sequesters numerous proteins in biofluid

We identified a couple of hundred proteins that bound to various forms of Aβ. In *paper III*, we studied the binding of serum proteins to $A\beta_{42}$CC protofibrils. The binding partners of $A\beta_{42}$CC protofibrils in serum were captured using the pull-down assay outlined the chapter 5.2.3. The MS analysis of the captured proteins revealed that 51 different proteins bound to $A\beta_{42}$CC protofibrils from serum. Beads coated with glycine were used as control. A few proteins were found to bind the control beads, but the binding level was low (Fig. 5.7), which

was further confirmed by the peptide abundance indices (PAI) values (Sanders et al., 2002).

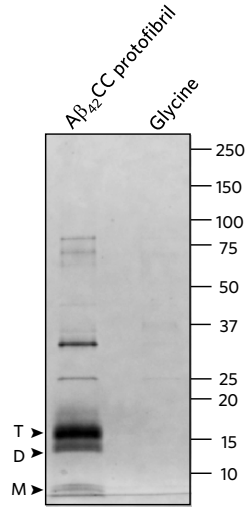

Figure 5.7 An SDS-PAGE analysis of protein samples extracted from serum using $A\beta_{42}$CC protofibril- and glycine-coated breads showing that more proteins are extracted by beads coated with $A\beta_{42}$CC protofibril than beads coated with glycine. The arrows indicate bands corresponding to $A\beta_{42}$CC monomer (M), dimer (D), and trimer (T).

CSF is perhaps more biologically relevant to AD pathology (Blennow et al., 2010) compared to serum. We studied the binding of CSF proteins to $A\beta_{42}$CC protofibrils (*paper III*) and $A\beta_{42}$wt fibrils (*paper IV*). Of twelve CSF samples used, six were from AD and six from non-AD patients (see Table 5.1 for the clinical features of CSF sample).

Table 5.1 Patient characteristics and CSF parameters. Patients were designated as AD or non-AD as previously described (Hansson et al., 2006).

	n	M/F (n)	Age (y)	$A\beta_{42}$ (ng/L)	t-tau (ng/L)	p-tau (ng/L)
non-AD	6	1[a]/3	71.2 ± 7.1[b,c]	808.5 ± 253.7	262.7 ± 145.9	40 ± 17.8
AD	6	3/3	65.8 ± 8.7	454 ± 140.2	782 ± 94.4	93.1 ± 11.3

[a]two samples without recorded gender.
[b]two samples without recorded age.
[c]Calculated for four samples.
non-AD, non-Alzheimer's disease; AD, Alzheimer's disease; t-tau, total tau; p-tau, phosphorylated tau
Values are expressed as mean ± SD.

Like the serum experiment, several proteins were extracted by both $A\beta_{42}$CC protofibril- and $A\beta_{42}$wt fibril-coated beads from CSF samples. Also, the same low-level binding was observed to controls coated with glycine or tryptophan

(Fig. 5.8). Through the MS analysis we identified 74 and 201 proteins[2], extracted by $A\beta_{42}$CC protofibril- and $A\beta_{42}$ fibril-coated beads from CSF, respectively. However, while comparing $A\beta_{42}$CC protofibrillar binding proteins with $A\beta_{42}$wt fibrillar binding proteins, the $A\beta_{42}$wt fibril-binding proteins list were corrected by subtracting[3] proteins below 20 kDa and above 250 kDa (56 proteins subtracted[4]) which results the number of $A\beta_{42}$wt fibril-binding proteins to 145. These numbers show that more proteins are extracted by $A\beta_{42}$ fibril-coated beads than beads coated with $A\beta_{42}$CC protofibril. Notably, many proteins were identified to bind $A\beta_{42}$wt fibril but not $A\beta_{42}$CC protofibrils. Hence, we called these $A\beta_{42}$wt fibril-specific proteins (*paper IV*).

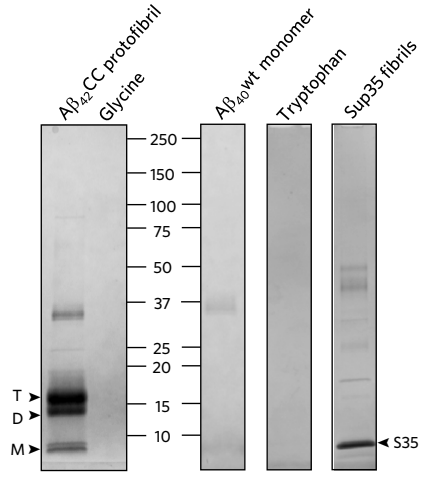

Figure 5.8 Representative SDS-PAGE analyses of protein samples extracted from CSF by $A\beta_{42}$CC protofibrils, glycine, $A\beta_{40}$wt monomer, tryptophan, and Sup35 fibrils coated beads. More proteins bind to $A\beta_{42}$CC protofibril-coated beads than beads coated with glycine, $A\beta_{40}$wt monomer, and tryptophan. Protein binding pattern to Sup35 fibrils is different compared to binding to other tested samples. Arrow heads indicate bands corresponding to $A\beta_{42}$CC monomer (M), dimer (D) and trimer (T), and Sup35 (S35).

Substantial variation of protein binding among the CSF samples were noted. Such differences are expected due to heterogeneity among the CSF samples. The number of identified proteins did not show obvious correlation with total protein content or sex. However, a positive correlation between age and the number of

2. The complete list of protofibril-binding proteins can be found in Supporting Information S1 of paper III, and fibril-binding proteins are found in Supporting Table S1of paper IV.

3. The rationale for this subtraction is, in the protofibril-binding experiment, the pull-down eluate was run on SDS-PAGE and proteins migrating between the MW of 20-250 kDa were cut out and analyzed by LC-MS/MS, whereas, in the fibril-binding experiment, the whole eluate was subjected to LC-MS/MS analysis.

4. The subtracted proteins list is found in Table S3 in the Supporting information of paper IV.

identified proteins, bound to $A\beta_{42}$wt fibril, was noted in samples from patients diagnosed with AD[5] (*paper IV*). Proteins identified to bind to $A\beta_{42}$CC protofibrils did not reveal any major differences between AD *vs.* non-AD group (*paper III*), nonetheless, proteins found to bind $A\beta_{42}$wt fibrils showed some differences between AD *vs.* non-AD group (*paper IV*).

To further access the specificity of the interaction, we studied the binding of CSF proteins to non-disease related nanofiber formed by yeast protein Sup35. Fibrils formed by Sup35 are similar in structure to Aβ fibrils (Nelson et al., 2005). One AD and one non-AD CSF sample was tested. Protein binding pattern to Sup35 fibrils was different compared to other tested ligands (Fig. 5.8). The MS analysis reveals twenty different proteins were bound to Sup35 fibrils (*paper III*). Notably, all these twenty proteins were also found to bind $A\beta_{42}$wt fibrils (*paper IV*). This result indicates that the surface of the fibrils might have specific binding epitopes for these proteins or the proteins have binding sites for fibrils.

However, several proteins identified in the present study as binding to different Aβ conformations have previously been reported to be associated with Aβ in AD plaque (Kalaria et al., 1993, McGeer et al., 1994, Liao et al., 2004), which indicates that our studies are of relevance to the situation *in vivo*. Moreover, protein identified in our studies may have potential to modulate Aβ fibrillation. Indeed, some of the proteins in our list have been shown to modulate amyloid formation and toxicity, for examples apolipoprotein A-I (Paula-Lima et al., 2009) and apolipoprotein E (Drouet et al., 2001), complement component C3q (Pisalyaput and Tenner, 2008) and clusterin (Yerbury et al., 2007). Hence, further investigation of protein identified in our studies might lead to new insight into the pathways of AD neurodegeneration.

5.5.2 Aβ species higher in order of aggregation binds more proteins

As stated in previous section, $A\beta_{42}$wt fibrils attract more proteins than $A\beta_{42}$CC protofibrils (74 for protofibrils *vs.* 145 for fibrils). In *paper III*, we studied the binding of CSF proteins to monomeric Aβ to compare the outcome with proteins identified to bind to two other Aβ conformations. One CSF sample was tested. For this test, we chose to use the $A\beta_{40}$ monomer since the $A\beta_{40}$ isoform is less prone to aggregation than $A\beta_{42}$ (Burdick et al., 1992, Jarrett et al., 1993). A few weak gel bands were visible on an SDS-PAGE in samples from $A\beta_{40}$ experiment (Fig. 5.8, lane: $A\beta_{40}$wt monomer). The MS result revealed only nine proteins in $A\beta_{40}$wt sample, compared to an average 30 and 80 proteins for $A\beta_{42}$CC protofibrils and $A\beta_{42}$wt fibrils, respectively. The Aβ species higher in order of

5. See Table S2 in supplementary information of paper IV.

the aggregation pathway (monomer → oligomer/protofibril → fibril) binds more proteins, which suggests that the binding of proteins to Aβ is enhanced upon aggregation of Aβ. A more recent study performed by Salza and co-workers (Salza et al., 2017) supports our finding. The protein binding differences among Aβs and other ligands can be explained by several factors including the differences in surface area accessibility, charge and tertiary structure of the binding surface, as well as the structural differences of pre-fibrillar and fibrillar aggregates.

5.5.3 Validation of protein binding to different Aβ conformations

To confirm the binding event of proteins with $Aβ_{42}$CC protofibril and/or $Aβ_{42}$wt fibrils, SPR-based biosensor studies was performed on selected proteins. The SPR results validated the pull-down assay results as well as Aβ conformation-dependent protein binding. The results are summarized in Table 5.2. The Aβ conformation-dependent protein binding in our study agrees well with an earlier study (Salza et al., 2017), and indicates that different Aβ nanostructure has a distinct set of binding partners.

Table 5.2 Binding kinetics of the selected proteins to $Aβ_{42}$CC protofibrils and $Aβ_{42}$wt fibrils, determined by SPR assay. Also includes a comparison of pull-down assay results in terms of yes/no binding with SPR results.

Protein	**SPR assay**		**Pull-down assay**	
	Bound to PF (K_D)	**Bound to F (K_D)**	**Bound to PF**	**Bound to F**
Agrin	n.d	3.5 nM	×	√
Antithrombin III	0.3 μM	n.t	√	√
Apolipoprotein A-I	3 μM	n.t	√	√
Apolipoprotein E	3 nM	0.3 nM	√	√
Complement C3	0.6 μM	n.t	√	√
Dickkopf-protein 3	n.d	26.2 nM	×	√
Neurocan	n.d	11.7 nM	×	√
Osteopontin	n.d	n.d	×	√
SPARC-like protein 1	n.d	6.2 nM	×	√

PF, protofibrils; F, fibrils; n.d, not determined; n.t, not tested; √, yes; ×, no

As seen in Table 5.2, all tested proteins showed high affinity, in the range of low micromolar to subnanomolar, to the corresponding Aβ partner.

5.5.4 Comparison of protofibrillar and fibrillar Aβ binding-proteins

Gene ontology (GO) annotation (Mi et al., 2013) into molecular function and cellular component were performed. The analysis showed that $A\beta_{42}$wt fibril-binding proteins possess binding activity, in contrary, $A\beta_{42}$CC protofibril-binding proteins possess catalytic activity. Both aggregate binders are predominantly extracellular proteins. The extracellular matrix is an important component of the brain, and is associated with various functions such as networking (Dauth et al., 2016). It is thought that the Aβ fibril formation is nucleated on extracellular matrix (Murphy and LeVine, 2010).

By utilizing the Human Protein Atlas (HPA) tissue-enriched database (Uhlén et al., 2015), we identified ten proteins with brain-specific expression pattern that associated with $A\beta_{42}$wt fibril only, but not to the $A\beta_{42}$CC protofibrils. Fibrils are the principal component in the amyloid plaque (Chauhan et al., 2004, Chander et al., 2007), and they might have more frequent interaction with brain proteins.

We identified some proteins, bound to $A\beta_{42}$wt fibril, which may enable the discrimination between non-AD and AD CSF and could be of relevance of AD biomarkers. Agrin, an extracellular matrix heparan sulphate proteoglycan expressed in neurons (Donahue et al., 1999), is an example of such proteins. In our study, agrin was found to be abundant in AD CSF. Our result agrees well with the existing literature. For examples, Berzin *et al*. (2000) observed increased levels of agrin in CSF of patients with AD compared non-AD CSF while Cotman *et al*. (2000) reported that agrin is colocalizes with Aβ in amyloid plaques and they also showed that agrin bind to Aβ and accelerates Aβ fibrillation. Alpha-1-microglobulin/bikunin (AMAB) was found to be abundant in non-AD CSF, which corroborates earlier data (Ramström et al., 2003).

5.5.5 Conclusions

Aβ was found to interact with a broad range of proteins in serum and CSF. The binding of proteins to Aβ is likely governed by the Aβ conformation, and protein binding is enhanced upon aggregation of Aβ. Protofibrillar and fibrillar Aβ-binding protein represent distinct functional categories, and fibril-binding proteins are enriched in the brain. Additionally, several of the identified proteins might have potential to discriminate between AD *vs.* non-AD CSF. Taken together, our results demonstrate that Aβ aggregates might appear as multiprotein aggregates *in vivo* and that the presence of protein binding partners might be important when investigating the cytotoxic mechanisms of protein aggregates.

An interesting future project would be to identify Aβ interacting partners in the brain tissue extracts, which would provide greater insight into the Aβ interactome in the actual brain environment. A second future project would be to test the potential of new identified Aβ-binders for modulating *in vitro* Aβ fibrillation and toxicity in cell culture experiment. Another interesting topic of the future study would be to investigate the potential of the binders identified here as candidate biomarkers for AD.

5.6 Concluding remarks

The molecular biology underlying Aβ aggregation and synapse dysfunction in AD is poorly understood. However, from a situation where most of the research was focused on the mature Aβ fibrils, the focus has been shifted to soluble pre-fibrillar Aβ aggregates *e.g.*, oligomers and protofibrils. Considerable evidence suggests that oligomers or protofibrils are the most toxic species among other Aβ species and are correlate well with neuronal death in the AD brain (Walsh et al., 2002, Walsh and Selkoe, 2007). However, many questions remain to be answered, for instance how do protofibrils exert their toxicity and initiate neurodegeneration? Among possible mechanisms, their interactions with other proteins have been implicated in these effects (Lee et al., 2017). No significant amount of research has been carried out to investigate this aspect. Our studies on Aβ-binding protein will add to the knowledge about the complex Aβ-interactome and its toxicity.

Although recent studies have helped us to advance our understanding about protofibril assembly and their structural properties, much of the details surrounding oligomers or protofibrils formations remain elusive. The fact that these soluble pre-fibrillar aggregates are heterogeneous and metastable and therefore poses a challenge for their detection. Because of its strong association with AD, the protofibrils are also the prime target for the development of AD treatments, in particular, disease-modifying therapies. Several good antibodies have been developed for the detection of oligomer or protofibril (Kayed et al., 2003, Englund et al., 2007, Kayed et al., 2007). The apparent advantage of our new protofibril binders compared to antibodies is their small size. This would enable future applications in both therapy and brain imaging since small molecules are the primary choice for efficient tissue penetration or passage across the blood-brain barrier.

As a complementary to the traditional amyloid detection techniques *e.g.* Congo red stating, the development of new methods based on biotechnology will become valuable tools for clinical identification of amyloid in tissue. Our approach of using nanofibrils in amyloid detection system will be an addition in

this regard. However, further optimizations and validation are required before it can be used in clinical practice.

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Popular science summary

Proteins are the most fundamental molecules in living organisms and are involved in almost all biological processes. They are synthesized on ribosomes as linear amino acids chains. These chains must then be folded into the correct conformation, called native fold of a protein, in order to gain functional activity. However, due to mutations, increased production or changes in the cellular microenvironment, proteins can be misfolded. If these misfolded proteins escape the cellular control system that normally remove the misfolded proteins, proteins can aggregate into a highly ordered structure called amyloid fibrils. Deposition of amyloid fibrils in tissue is linked to several diseases, for examples, Alzheimer's disease (AD) and transthyretin (ATTR) amyloidosis. To provide the best possible treatments, a more complete understanding of the disease development and the design of improved diagnostic methods are essential.

In this thesis, we developed new research tools and method for the detection of protein aggregates that caused AD and ATTR amyloidosis. These developments have possible applications in the diagnosis of these diseases as well as therapeutics. Additionally, we identified many proteins in biofluid that interact with AD-causing protein aggregates. This knowledge will help us to gain a better understanding of AD.

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