Modulation of Alzheimer’s amyloid $\beta$ peptide self-assembly – Insights into molecular mechanisms of peptide aggregation associated with Alzheimer’s disease

Axel Abelein
Modulation of Alzheimer’s amyloid $\beta$ peptide self-assembly

Insights into molecular mechanisms of peptide aggregation associated with Alzheimer’s disease

Axel Abelein
To my family
List of Papers

The following papers, referred to in the text by their Roman numerals, are included in this thesis.

PAPER I: **Hydrophobicity and conformational change as mechanistic determinants for nonspecific modulators of amyloid \(\beta\) self-assembly**

PAPER II: **Transient small molecule interactions kinetically modulate amyloid \(\beta\) peptide self-assembly**

PAPER III: **Formation of dynamic soluble surfactant-induced amyloid \(\beta\) peptide aggregation intermediates**

PAPER IV: **The zinc ion – a minimal chaperone mimicking agent for retardation of amyloid \(\beta\) peptide fibril formation**

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List of Additional Papers

PAPER i: Biophysical studies of the amyloid $\beta$-peptide: interactions with metal ions and small molecules

PAPER ii: The hairpin conformation of the amyloid $\beta$ peptide is an important structural motif along the aggregation pathway
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**Translated Sections**

**Populärvetenskaplig sammanfattning**

**Populärwissenschaftliche Zusammenfassung**

**Acknowledgements**

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## Abbreviations

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<tr>
<td>aSN</td>
<td>α-synuclein</td>
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<tr>
<td>Aβ</td>
<td>Amyloid β</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>AICD</td>
<td>APP intracellular C-terminal domain</td>
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<td>APOE</td>
<td>Apolipoprotein</td>
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<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>CD</td>
<td>Circular Dichroism</td>
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<tr>
<td>cmc</td>
<td>Critical Micelle Concentration</td>
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<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
</tr>
<tr>
<td>H(S/M)QC</td>
<td>Heteronuclear Single/Multiple Quantum Coherence</td>
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<tr>
<td>IDP</td>
<td>Intrinsically Disordered Protein</td>
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<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
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<tr>
<td>PrPc</td>
<td>Cellular Prion Protein</td>
</tr>
<tr>
<td>rf</td>
<td>Radio frequency</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SAXS</td>
<td>Small-Angle X-ray Scattering</td>
</tr>
<tr>
<td>SDS/LiDS</td>
<td>Sodium/Lithium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
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<tr>
<td>TROSY</td>
<td>Transverse Relaxation-Optimized Spectroscopy</td>
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1. Introduction

Life is a fascinating matter - its sensitivity and complexity are unique and despite thousands of years of human curiosity many aspects still remain a black box to the human mind. Life is based on a fine-tuned molecular system of a multiplicity of biological and biochemical processes. Understanding of these processes has been closely linked to advances in the general health status of humanity promoted by medical progress and developments. Different levels of complexity and detailing have historically determined the domains of biology, chemistry and physics. Approaches to obtain a better comprehension about how processes on a molecular level influence the nature of life have triggered the appearance of new scientific disciplines, which can be summarized by the term life sciences. Biophysics, biochemistry, biotechnology and biomedicine belong to this field of research although there is nothing like a strict differentiation and the sub-disciplines greatly overlap. This thesis belongs to this broad research area.

Biological function in living organisms is ultimately associated with a functioning workflow of molecular processes where the cell is the basic unit of this machinery. A cell contains various biomolecules, among them proteins and nucleic acids, that execute different functions [1; 2]. Nuclei acids, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), serve as an information storage via the nucleic acid sequence. Proteins, in contrast, fulfill a number of different functions, e.g. ion transport, enzyme catalysis, etc. [1; 2] Proteins consist of polypeptide chains that are usually folded into a three-dimensional structure (see following section). Short polypeptide chains, which may contain up to around 50 amino acids, are referred to peptides. Due to the short length, peptides often lack a well-defined 3D-structure. But also a number of proteins possesses less defined regions with high conformational freedom and classified as the group of intrinsically disordered proteins (IDPs) [3]. A structural misarrangement of the polypeptide chain, which frequently leads to protein aggregation, is often linked to biological dysfunction, including several human diseases (see following sections).

An better understanding of the protein/peptide aggregation mechanism is a major aim of this thesis, which is centered around the amyloid β peptide and its aggregation pathways implicated in Alzheimer’s disease. Various biophysical methods, including optical and nuclear magnetic resonance (NMR)
spectroscopy, have been applied to study the molecular mechanism of Aβ self-assembly and modulation of this process.

1.1 Protein folding and misfolding

The proper fold of a protein is closely linked to its biological function in the cell machinery. However, biological function may also be conveyed without a well-defined structure as exemplified by the class of IDPs [3]. After translation from messenger RNA the unstructured polypeptide chain folds and adopts a three-dimensional structure [1;2;4]. The questions of how proteins fold and how they know to fold that way remain still unanswered [5]. The native state should be energetically favored, i.e. be at a minimum in the protein folding energy landscape [4;6;7]. But as Levinthal pointed out in the year 1968, the native fold cannot be obtained by a simple random search through all possible conformations [8]. Such a search would require \( \sim 10^{27} \) years, while observed folding processes occur on a time-scale of seconds or less. However, it was shown that if energy minimization is biased by a small energy term against unfavorable configurations the time frame will fall in a biologically relevant range [9]. The folding process may thus be described by a funneled energy landscape where the protein folds energetically downhill [5;10]. This folding pathway includes transition states with energy barriers that have to be overcome and different folding intermediates. The native folded state should yet not be considered as an end-state but rather as a dynamic equilibrium with (partly) unfolded states and these dynamics are believed to be important for biological function [4]. Interestingly, although folding intermediates may only be weakly populated, NMR relaxation dispersion methods (see Chapter 3.3.2) provide a unique tool to investigate these states. And recently, a structure of such a low-populated folding intermediate was determined [11].

Besides the regular native fold proteins can also adopt low energy structures that are not on-pathway toward the native structure [7;12;13]. This process is generally referred to as protein misfolding [12], which is often closely linked to human diseases (see Chapter 1.2). Fortunately, the cell provides control and degradatory systems, such as molecular chaperones that assist protein folding and take care of misfolded species [4;12]. However, if the cell internal degeneration machinery is unbalanced, misfolded proteins can assemble to large, well-defined structures, called amyloid fibrils (see Chapter 2.3.3). Also protein misfolding can be understood in terms of an energy landscape. Compared to native folding the energy landscape is much "routher" for protein aggregation meaning that energy minima are less well-defined, which may be the origin of a large variety of different fibril morphologies [7] (see also Chapter 2.3). It was proposed that formation of protein assemblies is a generic property
of polypeptide chains, i.e. most proteins share this feature, not only a disease-associated subgroup, and self-assemble under certain conditions [12–14].

As many proteins have, at least transiently, intrinsically disordered regions [3] these parts are able to interact intermolecularly and form high-order protein assemblies. Furthermore, the native state can be destabilized by nonnative conditions, like temperature, pressure and pH, which cause the protein to (locally) unfold [12]. Mutations, which may be associated with certain familial forms of diseases, can substantially destabilize the native state. This can be exemplified by a mutated variant of human lysozyme, which was shown to feature much higher propensity to aggregate than the native form [15;16].

It would be desirable to predict why some proteins and peptides aggregate very easily, while others are rather aggregation-inert. And, indeed, some properties have been identified that significantly influence the aggregation propensity of proteins/peptides, among them hydrophobicity, charge and secondary structure propensity [12]. With the help of these parameters aggregation propensities can be predicted and algorithms have been developed to compute aggregation-prone sequences [17–19]. In general, high hydrophobicity and $\beta$-sheet propensity may promote aggregation, whereas a high, global or local, net charge decreases the aggregation propensity [12;17].

For some biological processes protein aggregation is even desired. These self-assemblies are summarized under the term functional amyloids that appear in different organisms and execute various specific biological functions (reviewed in [12;20]). The supposedly most prominent example is spidroin, which is needed for the production of spider silk. Also, some prion proteins, which are usually associated with infectious diseases (see Chapter 1.2), belong to this class and have diverse biological functions [12;20].

1.1.1 Folding free energy

The folding process can be understood as an equilibrium of folded ($F$) and unfolded ($U$) state characterized by the folding and unfolding constants, $k_F$ and $k_U$:

$$ U \overset{k_F}{\underset{k_U}{\rightleftharpoons}} F $$

The energy difference between these states can physically be described by the difference in Gibbs free energy, which is determined by the enthalpy and conformational entropy of the state:

$$ \Delta G = \Delta H - T \Delta S $$

Enthalpy can directly be related to "heat" or energy in the system, whereas entropy describes the "disorder" of all possible configurations the system can
adopt [21, 22]. There is a direct relationship between the observable measure, the equilibrium constant $K_{eq} = k_U/k_F$, and the Gibbs free energy by $\Delta G = -RT \ln K_{eq}$. In the simplest model the enthalpy and entropy are assumed not to be explicitly temperature-dependent, marked as $H^0$ and $S^0$, and the Gibbs free energy is, thus, linearly dependent on temperature: $\Delta G(T) = \Delta H^0 - T\Delta S^0$.

However, folding is a complex process and to account for hydration and protein-protein interactions a heat capacity has to be introduced by $\Delta C_P = \left( \frac{\partial H}{\partial T} \right)_P$. The Gibbs free energy can then be described, with respect to a reference temperature $T_{ref}$, by:

$$\Delta G(T) = \Delta H_{T_{ref}} - T\Delta S_{T_{ref}} + \Delta C_P(T - T_{ref}) - T\Delta C_P \ln(T/T_{ref}) \quad (1.3)$$

The sign of $\Delta C_P$ distinguishes between polar (−) and apolar (+) solvation and, hence, $\Delta C_P$ describes the hydrophobic effect of protein folding [22]. The folded state possesses, in general, plenty of intramolecular chemical bonds, including hydrogen bonds, electrostatic and Van-der-Waals interactions, that contribute to the enthalpy of the state. In contrast, the unfolded state is favored by entropy since the conformational freedom is larger when the protein is disordered. The specific values of $\Delta H$, $\Delta S$ and $\Delta C_P$, which are dependent on the environmental conditions, determine hence the stability of a state. [10, 22]

1.2 Misfolding Diseases

Protein misfolding is associated with a variety of human diseases that all share the accumulation of misfolded proteins or peptides as one hallmark [12, 23]. In these diseases soluble proteins/peptides cannot any longer provide their native functions but self-assemble into large aggregates. The end-state aggregates feature a high degree of conformational order and are referred to as amyloid fibrils (see Chapter 2.3.3), which themselves accumulate to microscopic depositions called amyloid plaques. These characteristics are summarized by the term amyloidosis, i.e. the property to form amyloid material [12]. A selection of the most prominent examples of human diseases that have their origin in protein misfolding and their associated amyloid-forming protein/peptide is compiled in Table 1.1. Many of these diseases are neurodegenerative, meaning that the amyloidosis occurs in the brain. Others exhibit amyloid formation in other tissues than the brain and are referred to non-neuropathic amyloidoses. Despite the fact that amyloid material is closely associated with these diseases the link between (neuro-)toxicity and amyloids still remains unclear [24]. For a few diseases simply the large load of amyloid material disturbs organ tissues and their functions, e.g. amyloidosis associated with the cerebral vessels (see Table 1.1), but for many others there is no such strong correlation [24].
### Table 1.1: Selection of human protein misfolding diseases and their associated protein or peptide that forms amyloid deposits. Table adopted from [12; 24]

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aggregating protein/peptide</th>
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<td><strong>Neurodegenerative diseases</strong></td>
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<tr>
<td>Alzheimer’s disease</td>
<td>Amyloid $\beta$ peptide (A$\beta$)</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>$\alpha$-synuclein ($\alpha$SN)</td>
</tr>
<tr>
<td>Dementia with Lewy bodies</td>
<td>$\alpha$-synuclein ($\alpha$SN)</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin with polyglutamine expansion</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>Gerstmann-Sträussler-Scheinker</td>
<td>Prion protein</td>
</tr>
<tr>
<td>Creutzfeldt-Jacob Disease</td>
<td>Prion protein</td>
</tr>
<tr>
<td>Frontotemporal dementia with Parkinsonism</td>
<td>Tau</td>
</tr>
<tr>
<td><strong>Non-neuropathic amyloidoses</strong></td>
<td></td>
</tr>
<tr>
<td>ApoA(I/II/IV) amyloidosis</td>
<td>N-terminal fragments of apolipoprotein A(I/II/IV)</td>
</tr>
<tr>
<td>AL amyloidosis</td>
<td>Immunoglobulin light chains or fragments</td>
</tr>
<tr>
<td>AA amyloidosis</td>
<td>Fragments of serum amyloid A protein</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>Amylin (islet amyloid polypeptide)</td>
</tr>
<tr>
<td>Hereditary cerebral hemorrhage with amyloidosis, Icelandic type</td>
<td>Variant of cystatin C</td>
</tr>
<tr>
<td>Hereditary cerebral hemorrhage with amyloidosis, Dutch type</td>
<td>Amyloid $\beta$ peptide</td>
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Instead, the soluble form or smaller aggregated species, often referred to *neurotoxic oligomers*, correlate with the degree of dysfunction [12; 23; 24]. A detailed discussion about neurotoxicity in Alzheimer’s disease is provided in Chapter 2.2.3.
2. Alzheimer’s disease and the amyloid \( \beta \) peptide

2.1 Alzheimer’s disease

Alzheimer’s disease (AD) is the most prevalent dementia type and belongs to the family of neurodegenerative disorder diseases, which includes other prominent examples such as Parkinson’s disease and Huntington’s disease (see Table 1.1) [23; 25]. The psychiatrist and neuropathologist Alois Alzheimer was the first who assigned specific symptoms of his patient Auguste Deter to AD in 1907 [26]. Now, more than 100 years later, AD is diagnosed in about one of nine persons over the age of 65 years [25]. The percentage of people carrying AD drastically increases with age and about one third of people 85 years or older have the disease, while only ca. 4 % of AD patients are younger than 65 [25]. Most of the AD cases are sporadic, meaning that they do not feature any family history and only a small number, i.e. less than 1 % of all cases, is associated with the familial form of AD [23 25]. Familial AD is implicated with mutations in the \( \beta \)-amyloid precursor protein (APP), presenilin-1 or -2 genes [23 27 28]. But also other gene defects may cause familial AD since several cases have been reported that do not involve any of the genes mentioned above [27].

AD causes neuronal damage that manifests itself in characteristic symptoms of dementia patients such as memory loss, general confusion and problems with speaking and writing. Eventually, the disease leads to death of the patient. [25 29]

Age is the major risk factor for developing the disease [25 30]. A slightly increased risk exists to develop both the sporadic and familial forms when inheriting the Apolipoprotein \( \varepsilon 4 \) (APOE \( \varepsilon 4 \)) allele, in particular when two copies are inherited [25 27]. The APOE has three different alleles named APOE \( \varepsilon 2 \), APOE \( \varepsilon 3 \) and APOE \( \varepsilon 4 \) and it was found that more than 50 % of AD patients carry the APOE \( \varepsilon 4 \) allele [30]. An increased risk for developing AD by a factor of 2.8 was reported for carriers of one copy of APOE \( \varepsilon 4 \), while two copies gave an about 8 times increased risk compared to cases without the APOE \( \varepsilon 4 \) allele [31]. The presence of this genotype, however, does not necessarily provoke the disease and the percentage of people carrying two copies of APOE \( \varepsilon 4 \) is
only small so that it cannot account for the majority of AD cases \[25; 30; 31\]. In contrast to APOE ε4, the APOE ε2 allele is putatively protective against developing AD \[27\]. Additionally, other risk factors have been suggested, such as gender (slightly higher risk for women), smoking, cardiovascular disease, diabetes, cholesterol levels in midlife, etc. \[25; 30\]

Until today no successful cure or therapeutic strategy has been found \[25\] despite the immense efforts that have been made in scientific and industrial research to investigate AD. Besides the desolate situation for the patients and their families, this causes also significant demands and costs on the society to provide health (long-term) care and hospice to the patients \[25\].

### 2.2 The amyloid β peptide

One hallmark of AD is the deposit of amyloid plaques and neurofibrillar tangles in the brain \[23; 28; 29; 32\]. The major compound of the amyloid plaques is a small peptide called amyloid β peptide (Aβ), which was first isolated by Glenner and Wong in 1984 \[33; 34\], while neurofibrillar tangles mainly consist of aggregated tau protein. Aβ features a molecular mass of about 4.3 kDa and consists of 38-43 residues with the two most common forms Aβ_{40} and Aβ_{42}.

#### 2.2.1 Aβ as a cleavage product

Aβ is a cleavage product obtained from processing of the amyloid precursor protein (APP), which is a 695-770 residue transmembrane protein (Figure 2.1) \[28; 32; 35\]. The biological function of APP is still unknown and experiments with APP gene knocked-out mice showed unchanged viability and fertility but reduced weight and locomotor activity compared to controls \[27; 36\]. Interestingly, several studies reported metal binding to APP and an active role of APP in metal homeostasis has been suggested \[37; 38\].

APP is a membrane protein with one transmembrane helix where the C-terminus is intracellular and the Aβ domain is localized both in the membrane and extracellularly (Figure 2.1). Processing of APP can be subdivided in non-amyloidogenic and amyloidogenic pathways and occurs through the enzymes α- , β- and γ-secretase. \[23; 28; 32; 35\]

In the non-amyloidogenic pathway APP is cleaved by the α-secretase, which acts on the extracellular membrane side between residue 16 & 17 in the Aβ sequence, and releases the extracellular fragments, called α-APPs. The remaining part C83 is cleaved subsequently by the γ-secretase in the membrane, releasing a non-amyloidogenic fragment p3 and the APP intracellular C-terminal
domain (AICD). The α-secretase is suggested to belong to the so-called ADAM family of metalloproteases [39].

In contrast, the amyloidogenic pathway is mediated by the β- and γ-secretases and generates extracellular release of amyloidogenic Aβ. The β-secretase liberates extracellular β-APPs and subsequent cleavage of the remaining C99 fragment by the γ-secretase produces Aβ and intracellular AICD [23, 28, 32, 35]. γ-Secretase cuts C99 several times and the cleavage sites are named by ε, ζ and γ where the γ-cleavage can occur between residues 38-42 [23]. This process produces mainly Aβ$_{40}$ and Aβ$_{42}$ and their ratio is believed to be crucial for the rate of amyloid formation [23]. The β-secretase is an aspartyl protease that belongs to the pepsin family and its biological function is still unclear [40, 41]. Interestingly, an enhanced β-secretase expression has been reported for patients with sporadic AD [42], which may be one of the reasons for an increased Aβ generation. The γ-secretase is a membrane enzyme complex and four units, presenilin-1 or -2, nicastrin, anterior pharynx defective-1 and presenilin enhancer-2, are necessary for its activity [40, 43].

2.2.2 Metal ions

The brain of AD patients are characterized by a dyshomeostasis of metal ions [45] and increased metal concentrations have been detected in amyloid plaques [46, 47]. In the plaques metal ions are co-localized with Aβ [48] and, in vivo and in vitro, metal ions have been shown to bind to Aβ and modulate the aggregation process. Several review articles have been, entirely or partly, dedicated to this topic [49–55].

The presence of zinc, copper and iron ions is enhanced in the brain compared to the body [52]. Their concentrations in the cerebrospinal fluid are in the order of a few µM and slightly more abundant in the serum. The metal ion concentrations are significantly increased during synaptic release and, in the synaptic cleft, the zinc ion concentration may be estimated to ~200-300 µM, while the copper ion concentration may be around 15 µM (reviewed in [56, 57]). An about 1.5 to 2.5-fold excess of metal ions was reported in amyloid plaques compared to the surrounding tissue in AD patients’ brain [46, 47]. Notably, the level of metal ions in AD neuropil tissue compared to control neuropil is also elevated by a factor of 2 and 4 for zinc and copper, respectively [46]. Zinc and copper ions were found to be co-localized with a heterogeneous distribution in amyloid plaques, yet, with increased presence in regions with a high β-sheet content [58].

Besides the elevated concentration of metal ions in amyloid plaques, another important issue is the redox activity of certain metal ions, like copper and iron. These ions can lead to production of reactive oxygen species (ROS) and
Figure 2.1: Processing of APP and structure of C99 - (A) Aβ is cleaved from APP by the β- and γ-secretases and is subsequently released to the extracellular space. This APP processing is referred to the amyloidogenic pathway. In contrast, cleavage by the α- and γ-secretases generates non-amyloidogenic material. [28, 85]. (B) The well-defined part of the C99 structure is shown (residues 683 to 728) where the disordered N-terminus and cytosolic domain are omitted (pdb code 2LP1) [44].
oxidative stress (reviewed in [49; 50]). ROS have been suggested to play a cru-
cial role in AD pathology and proposed to be the responsible species that cause
neurotoxicity in AD [49; 50]. Zinc, in contrast, is redox-inert and may even
be neuroprotective [49]. The origin of neurotoxicity in AD remains, however,
unclear and there is a controversial debate about it in literature (see Chapter
2.2.3).

Binding of metal ions to Aβ is characterized by the binding affinity and
ligand coordination, which has been investigated in vitro. The binding affinity
is highly dependent on the used buffer system due to coordination of the metal
ions by salt counter ions and a broad range of dissociation constants, \( K_D \), has
been reported in literature (reviewed in several articles [51–53]). Different
methods have been applied to determine \( K_D \) among them quenching of intrinsic
tyrosine fluorescence, isothermal calorimetry, potentiometry and NMR. For
the Aβ–Cu\(^{2+}\) complex the \( K_D \) value may fall in the wide range of 0.01–1 nM,
while the affinity of Aβ for Zn\(^{2+}\) is lower and \( K_D \) is estimated to be around
1–60 \( \mu \)M [50; 53].

The binding affinities are, thus, in a biologically relevant range but in vivo
several other metal binding proteins, such as chaperones, with possibly higher
metal affinities are abundant [55]. Yet, the elevated presence of metal ions
in amyloid plaques indicates that Aβ efficiently binds metal ions also in vivo.
Where the metal binding takes place remains still to be investigated although
the synaptic cleft, where the metal ion concentration is high, is suggested to
be, at least partly, involved [59].

Different modulation of aggregation in vitro has been reported and both
protective and promoting effects of zinc and copper ions on Aβ self-assembly
have been presented (for reviews see [51; 53; 54]). These seemingly contradic-
tory results may be explained by a metal ion concentration-dependent modula-
tion of Aβ aggregation. At low Zn\(^{2+}\) concentration Aβ fibrillization is retarded
(see Paper IV and [60]), while at high concentration the type of aggregates
is different and amorphous structures have been reported [61; 62]. In Paper
IV we demonstrated that Zn\(^{2+}\) at a sub-stoichiometric ratio provokes retarda-
tion of Aβ\(_{40}\) fibril generation and the aggregation half time is exponentially
dependent on zinc ion concentration (see Chapter 5.3).

Zinc ions bind to the N-terminus of Aβ and the minimal metal binding site
was localized to the first 16 N-terminal residues [63]. The suggested ligands
for Zn\(^{2+}\) are the three histidines H6, H13 and H14, while the fourth ligand
has been more controversial and D1, Y10 and coordinated H\(_2\)O have been
proposed (reviewed in [53; 54]). Chemical shift changes in \(^1\)H–\(^{13}\)C HSQC
experiments provide, however, evidence that the N-terminal D1 is the fourth
ligand [64]. Signal attenuation of \(^1\)H–\(^{15}\)N HSQC cross-peaks including all N-
terminal residues indicate that the whole N-terminus is involved in a chemical
exchange process \cite{64,65}. In Paper IV we investigated this process in detail and showed that the N-terminus encapsulates the Zn\textsuperscript{2+} ion forming a compact dynamic complex with A\textbeta\textsuperscript{5} (see also Chapter 5.3).

Furthermore, a putative second binding site of Zn\textsuperscript{2+} with a much lower metal affinity was proposed, located between residues 23--28 \cite{64}. Also, Cu\textsuperscript{2+} is suggested to have a second binding site in this region \cite{64,66}. This binding site may become more populated at high metal concentrations when formation of amorphous aggregates was reported \cite{61,62,67}. Under these conditions the metal ions might help to bridge different A\textbeta\textsuperscript{5} peptides and thereby stabilize these structures \cite{68,69}.

2.2.3 Neurotoxicity – species and mechanism

The debate about the neurotoxic origin of AD disease has been long and up-to-date there has been no consensus about the neurotoxic mechanism neither \textit{in vivo} nor \textit{in vitro}. In AD both intracellular tau aggregates and extracellular A\textbeta\textsuperscript{5} may cause neurotoxicity \cite{23,71}. In recent years, the hypothesis of A\textbeta\textsuperscript{5} oligomers as the toxic species has become popular and plenty of research studies have concerned this topic (reviewed in \cite{12,23,71}). The cell toxicity mediated by these oligomers may be based on different mechanisms \cite{24,71}. Firstly, oligomers may bind to receptors and thereby hindering their function, which may primarily occur intracellularly. Different potential receptors have been reported, among them the cellular prion protein (PrP\textsuperscript{c}) \cite{72,73,74}. However, PrP\textsuperscript{c} interaction may not necessarily be required to cause toxicity \cite{75}. Secondly, oligomers may lead to pore or channel formation in membranes causing ion dyshomeostasis. And thirdly, the cell membrane may be perturbed leading to general membrane permeabilization. \cite{71}

A large variety of different species has been reported that are generally referred to "toxic oligomers" \cite{24,71,76}. Different sizes and forms were assigned to these species suggesting multiple heterogeneous aggregation pathways (see Chapter 4.1). However, the huge variety may also, at least partly, originate from the different methods used to characterize and define oligomers and from the applied techniques to test or detect neurotoxicity \textit{in vitro} and \textit{in vivo} \cite{71}. An interesting aspect is also whether the oligomers studied \textit{in vitro} are biologically relevant \textit{in vivo} at all.

Toxic species often exhibit an enlarged hydrophobic surface that is evident from strong binding to the fluorescent dye 1-anilino-8-naphthalene sulfonate (ANS) \cite{77}. This property may facilitate interaction with receptors and cell membranes. One recent study presented that cell-toxic proto-fibrils exhibit already a cross-\beta structure \cite{78} that is a common characteristic for mature amyloid fibrils (see Chapter 2.3.3). However, due to the large pool of possibil-
ities of other structural states assigned to "toxic oligomers", the link between structure and toxicity still remains unclear.

Another important aspect is the ratio between $\alpha_{40}$ and $\alpha_{42}$ or $\alpha_{43}$. The longer variant is much more prone to aggregate and neurotoxicity may be enhanced by small changes of the ratio $\alpha_{40}$.

Besides a specific toxic species there is also the hypothesis that the nucleation process pre se may lead to neuronal dysfunction $\alpha_{71} \alpha_{80}$. In a study where the neurotoxicity of fibrils and soluble peptides were tested the authors found that the cointaneous presence of both fibrils and monomers greatly increased cell toxicity and assigned its origin to the polymerization process rather to one specific species $\alpha_{81}$. It may be speculated that the origin can be found in particular folds that occur during the nucleation process and exhibit "cell-toxic surfaces" $\alpha_{80}$.

A crucial issue is whether generation of toxic species can be introduced by "infection" of toxic material as is evident for prion diseases $\alpha_{82}$. A detailed discussion about this question is given in references $\alpha_{71} \alpha_{83}$. In vitro, $\alpha$ aggregation can be efficiently accelerated by addition of seeds $\alpha_{84}$ but experiments in vivo have not provided a clear answer. Animal studies showed that amyloidosis of transgenic, APP-overexpressing mice can be seeded by AD brain extract $\alpha_{85} \alpha_{86}$.

Interestingly, not only injection in the brain but also in the peritoneal cavity could seed amyloid plaque formation, even though with prolonged incubation time $\alpha_{86}$. These results suggest, thus, that transport of amyloidogenic material within the body might be possible. However, the link between seeded aggregation and the neurotoxic mechanism remains open $\alpha_{71} \alpha_{83}$.

Taken together, the aspects of what is/are the toxic species and what is/are the underlying neurodegenerative mechanism(s) remain puzzling up-to-date. A specific target of the toxic species in the cell or in the cell mechanism is unclear. Also, a more generic mechanism is possible where an interplay of multiple effects occurs including, e.g. perturbation of the membrane bilayer, oxidative stress and unbalanced ion homeostasis as well as functional modulation of several receptor proteins.

2.2.4 Therapeutic approaches

Various therapeutic approaches have been suggested and while some of them have only been proposed based on in vitro results, others have even been tested in clinical phase II and III trials. The different strategies involve interference with production of $\alpha$ (see Chapter 2.2.1), preventing or targeting toxic species (see Chapter 2.2.3) and modulation of environmental conditions, such as metal ions (see Chapter 2.2.2).
One potential way to avoid formation of amyloid material is to reduce the amount of aggregation-prone Aβ peptides. This may be achieved by inhibition or modulation of β- and γ-secretases (reviewed in [32]). Targeting γ-secretase is complicated since γ-secretase is, beside APP, also responsible for cleavage of other transmembrane proteins, e.g. the Notch receptor that is crucial for normal embryonic development [23][32][35][87]. A complete inhibition of the γ-secretase is not desired due to these side effects, but a specific inhibition of the APP cleavage. Some potential drug candidates have been reported that modulate γ-secretase such that APP cleavage is reduced or that generation of the most amyloidogenic Aβ42 is shifted toward less aggregation prone variants [23][32]. Inhibition of β-secretase might not be accompanied by the same destructive side effects as deletion of γ-secretase since mice still show the normal phenotype [88]. The large active site of β-secretase requires, yet, large potential drug molecules, which should also penetrate the blood-brain barrier [32].

As some studies propose an aggregation promoting effect of metal ions, chelator compounds that bind metal ions have been discussed as potential AD drug candidates [50]. But the effect of metal ions on Aβ aggregation is dose-dependent (see Chapter 2.2.2) and the interplay between different metal ions in the presence of Aβ is only poorly understood, even in vitro [53]. The desired metal affinities of such chelators should be higher than that of the peptide in order to be able to compete with Aβ and efficiently bind the metal ions [50]. Moreover, it is absolutely crucial that essential metal levels are not disturbed to maintain the natural metal homeostasis.

Another strategy is modulation of the aggregation pathway to prevent aggregation or, at least, generation of neurotoxic species. This may be achieved by various aggregation modulators among them small organic molecules, antibodies and peptide-based inhibitors. Plenty of compounds have been screened and reported to modulate Aβ self-assembly in vitro. A detailed discussion of these compounds and their mechanism of action is provided in Chapter 4.3.

Several potential therapeutics have been run through phase II and III clinical trials [32][89], however, until today the results have been disappointing as all compounds that completed phase III did not meet their intended purpose [90][91]. These six therapeutic programs included inhibition and modulation of γ-secretase, amyloid plaque clearance by antibodies and direct binding to monomeric peptide [90]. The drug candidates were tested on AD patients who showed mild or moderated signs of AD and compared to placebo treatment. None of the six drug candidates could achieve the desired effect [90]. It may, therefore, be questioned whether the search for anti-amyloidogenic drugs is "on the right road" [91] or whether simply more time and trials are needed.

Nevertheless, information from these recently reported clinical failures of
AD therapies can be used to develop new strategies, potentially based on partially successful treatments. It has also been suggested to investigate in particular very early symptoms as AD treatment should be most efficient at the early stage of the disease [89, 92, 93]. Daily new insights and accumulating knowledge in research institutes and clinics around the world provide, nevertheless, the basis for an optimistic hope to find a cure of the disease or, at least, delay its progression.

2.3 Structural polymorphism of Aβ

Aβ may be seen as a "structural chameleon" since, depending on the environmental conditions, it may adopt very different secondary structures including random coil-like, β-structured and α-helical states [54]. The structural state of Aβ is highly dependent on the environmental conditions as well as the stage in the aggregation process.

2.3.1 Aβ exhibits an α-helical structure in a membrane environment

Before γ-secretase cleavage Aβ is part of the transmembrane helix of C99 where residues 16-23 and the complete C-terminus (from residue 29) form an α-helical structure (Figure 2.1B), while the N-terminus and residues 24 to 28 are disordered [44].

Similarly, in the presence of sodium dodecyl sulfate (SDS) micelles, Aβ was shown to form two α-helices involving residues 15–24 and 30–35 [94, 95]. With the help of paramagnetic manganese ions Mn²⁺, the C-terminal helix was found to be positioned inside the micelle, whereas the second helix was suggested to be located at the surface of the micelle [94]. Strikingly, these α-helices are localized to very similar Aβ segments that also form α-helices in the transmembrane part of the C99 fragment of APP (compare Figure 2.1B). Also, in the presence of dodecylphosphocholine (DPC) micelles, which in contrast to the negatively charged SDS micelles do not have any net charge, an α-helical conformation of Aβ was reported [96].

2.3.2 Aβ monomers in solution are predominantly unstructured

Intrinsically disordered proteins (IDPs) feature transient non-random conformations that may be locally formed [3, 97]. This is reflected by secondary structure propensities even though no long-lived conformations are observable. NMR has turned out to be a powerful tool to investigate secondary structure propensities of IDPs [97, 98]. In solution Aβ monomers are predominantly unstructured as evident from NMR and CD spectroscopy [99, 100]. But also
Aβ features some properties of IDPs and was shown to exhibit a distinct β-structure propensity in the two hydrophobic segments as indicated by \(^3J_{HNNH}^\alpha\) couplings [100]. These findings were supported by NMR relaxation measurements that indicated a reduced motional freedom and restricted dynamic motions in these segments compared to the more flexible N-terminus and the more hydrophilic middle region (residues 25–30) [101]. The β-structure propensity is temperature dependent with an increased overall motional freedom at high temperatures [101]. Furthermore, CD experiments showed that Aβ features a spectrum with typical random coil characteristics, while when lowering the temperature an increasing population of Aβ adopts the structure of a left-handed polyproline type 2 helix [100]. \(^1H-^{15}N\)-HSQC signals of Aβ are generally attenuated when increasing the temperature, which may be explained by an increase of NH-exchange rates ([101] and Paper I). At the same time, \(^1H-^{13}C\)-HSQC cross-peak intensities are reduced where the attenuation is most pronounced in the central region (residues 23–30) [102]. The presence of a chemical exchange process can cause signal broadening (see Chapter 3.3.2) and structural exchange including a transient formation of a β-hairpin was suggested to explain the observed results [102].

An NMR solution structure of a β-hairpin in Aβ could be obtained in the presence of an Affibody protein [103]. This protein in a 2:1 Affibody:Aβ complex stabilizes a β-hairpin in Aβ where residues 17–23 & 30–36 adopt a β-structure (Figure 2.2). This β-hairpin can also be created by designing an Aβ variant (A21C and A30C) that exhibits an intramolecular disulfide bridge between C21 and C30 [104]. This variant was shown to easily aggregate and form oligomeric species but generation of mature fibrils was completely inhibited [104]. The S–S bridge prevents a parallel β-sheet arrangement, which is the essential building block of amyloid fibrils (see following section), and thereby counteracts fibril formation.

2.3.3 Mature fibrils feature a cross-β structural motif

Mature amyloid fibrils show a characteristic so-called cross-β X-ray diffraction pattern where the intersheet distance is 4.8 and 10 Å along and perpendicular to the fibril axis, respectively [24]. While there is no crystal structure from full length Aβ available, the Eisenberg group has published several structures of smaller Aβ peptide derivatives [105]. They found that the fiber forming segments exhibit several different steric zipper motives. Both parallel and anti-parallel alignments of β-sheets were revealed, consolidated in the term packing polymorphism [105].

The cross-β structure motif was confirmed by various solid-state NMR studies, both on the 40 and 42 residue variants [106–111] (Figure 2.2). Here,
a parallel alignment of the β-sheets was found for Aβ40 and Aβ42 [106–108], whereas the Iowa substitution (D23N-Aβ), which is associated with early onset familial AD, showed both parallel and anti-parallel forms [112, 113] (Figure 2.2). The anti-parallel form of the Iowa variant is, yet, thermodynamically less favorable compared to parallel alignment [113]. Nonetheless, the anti-parallel arrangement appears to be the major form when this Aβ mutant aggregates spontaneously, i.e. without additional seeding, pointing toward that this variant possesses a greater propensity for spontaneous nucleation [113].

Besides the common feature of a parallel cross-β alignment of Aβ fibrils, different inter-protofilament contacts have been reported [107–111]. Paravastu et al. presented two different arrangements depending on whether the alignment is "striated ribbon" or "twisted", which includes two (striated ribbon) or three (twisted) fiber strings [108, 109] (Figure 2.2). Furthermore, alternative structural models have been suggested that differ from the previous structure by their hydrogen pattern between the fiber filaments (summarized in Refs. [114] and [54]). This may reflect the large polymorphism of Aβ fibrils but it also underlines the distinct dependence of fibril morphology on sample preparation.

The Tycko group demonstrated that well-defined fibril structures can be produced from Aβ samples that were seeded with Aβ40 fibrils extracted from AD patients’ brain tissue [115, 116]. Interestingly, fibrils grown from two different AD patients exhibit different fibril morphologies [116]. It is tempting to speculate whether distinct fibril structures reflect variants in pathology of AD. The fibril structure derived from one of the patients clearly differs from in vitro fibril structures discussed above and shows a three-fold symmetry (Figure 2.2). In contrast to in vitro structural models, also the N-terminus is well-defined and part of the in vivo fibril model [116].
Figure 2.2: Structural states of Aβ in fibrils and in complex with an Affibody protein - (A) Fibril of Aβ_{42} that contains two parallel β-sheets in residues 18–26 and 31–42 (pdb code 2BEG) [107]. (B,C) Morphologies of striated ribbon (B) and twisted (C) Aβ_{40} fibrils from [108] (2LMN) and [109] (2LMP), respectively. (D) The Iowa variant D23N-Aβ_{40} exhibits an anti-parallel alignment (2LNQ) [113]. (E) Structure of a Aβ_{40} fibril derived from human brain tissue (2M4J) [116]. (F) Aβ_{40} forms a β-hairpin in complex with an Affibody protein (2OTK) [103]. All fibril structures in A-E were obtained by solid-state NMR, while the structure of the complex in F was calculated from solution NMR data.
3. Methods

In this chapter different biophysical techniques that have been applied in this thesis, and their underlying theory are introduced. The focus is on spectroscopic methods, including NMR and optical spectroscopy, e.g. CD and fluorescence. Spectroscopy is generally related to the measurement of the interaction between electromagnetic radiation and investigated matter. This typically involves energy transitions between different energy levels of electrons or nuclei, which can be described by the time-dependent Schrödinger equation \[\psi(t) = H \psi(t)\] (3.1)

in which \(\psi\) stands for the time-dependent wave function of a state and \(H\) is the Hamiltonian operator, which generally describes the total energy of the state.

3.1 Secondary structure and conformational changes followed by circular dichroism

To investigate secondary structure features of proteins and peptides and to monitor conformational changes between different secondary structure elements CD can be readily applied. This method measures the difference in absorption of left and right circularly polarized light, which can be related to secondary structure by using empirical reference spectra. Exclusively chiral molecules, \textit{i.e.} molecules that do not superimpose with their mirror images, exhibit a non-zero CD signal. CD spectra of proteins and peptides are typically recorded in the far-UV region (190–260 nm) where the signals originate primarily from the peptide bond of the backbone and, thus, report on secondary structure elements.

CD intensity can be physically described by the difference of absorption coefficients, \(\Delta \varepsilon\), of left and right circularly polarized light, which itself is determined by the transition probability \(w(0 \rightarrow 1)\) of an electron from the ground to an excited state \[119,121\]. This transition probability is proportional to the

\(^1\text{Chiral}\) is derived from the Greek word for \textit{hand}, which is one of the most prominent examples for chiral objects \[118\].
CD signal and can be described with the electric, \textbf{m}, and magnetic, \textbf{\mu}, dipole moments by the Rosenfeld equation \cite{119,122}:

\[
\text{CD signal} \propto \Delta \varepsilon = \varepsilon_L - \varepsilon_R
\]
\[
\propto \Delta \omega (0 \rightarrow 1) = \omega_L (0 \rightarrow 1) - \omega_R (0 \rightarrow 1)
\]
\[
\propto \Im (\langle \Psi_0 | \hat{\mu} | \Psi_1 \rangle \cdot \langle \Psi_1 | \hat{m} | \Psi_0 \rangle)
\]

where \(\Psi_0\) and \(\Psi_1\) are the wave functions of the ground and the first excited state, respectively, and \(\Im\) refers to the imaginary part of the scalar product.

Photon absorption gives rise to energy transitions involving electrons in the peptide bond, \textit{i.e.} \(\pi \rightarrow \pi^*\) (around 190 nm) and \(n \rightarrow \pi^*\) (around 220 nm) transitions. These transitions are specific for the different secondary structure elements and spectral characteristics can be used to obtain secondary structure information of proteins and peptides.

Conformational changes involving two distinct states are easily recognized by an \textit{isodichroic} point, \textit{i.e.} the cross-point in the spectral series. This is illustrated in Figure 3.1, which displays the structural transition of \(\text{A}_{\beta_{40}}\) during the aggregation process from an initial random coil-like structure to a final \(\beta\)-structure. The signals at secondary structure characteristic wavelengths, the random coil minimum at 198 nm and the \(\beta\)-structure minimum at 216 nm, follow approximately a sigmoidal time dependence.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_3.1.png}
\caption{Structural transition of \(\text{A}_{\beta}\) - (A) \(\text{A}_{\beta}\) shows a structural conversion from a random coil-like to a \(\beta\)-structure during the aggregation process. Aggregation kinetics were monitored by CD on 10 mM \(\text{A}_{\beta_{40}}\) in 5 mM sodium-phosphate buffer, pH 7.2, under continuous stirring at 37 \(^\circ\)C. (B) The CD signals of the random coil (198 nm) and \(\beta\)-structure minimum (216 nm) follow an approximately sigmoidal time dependence.}
\end{figure}
3.2 Fluorescence Spectroscopy

Fluorescence is beside phosphorescence associated with the emission of light, which is generally summarized by the term luminescence, and involves an electron transition from an excited singlet state, $S_1$, to the ground state, $S_0$, while phosphorescence implicates a transition from a triplet state, $T_1$ \[123\]. The physical principles of luminescence were schematized by Jablonski in 1933 in the form of a diagram that was named after him, Jablonski diagram \[124\]:

After absorption of light, which causes an electron transition to a higher electronic and vibrational state, the electron "relaxes" back to the lowest vibrational state, $S_1$, giving rise to an absorption spectrum described by: $S_0 + h\nu_{ex} \rightarrow S_1$.

From $S_1$ the electron returns to various vibrational energy levels of the ground state releasing fluorescent light of different wavelengths, which implicates an emission spectrum: $S_1 \rightarrow S_0 + h\nu_{em} + heat$.

Besides organic molecules that can be used for fluorophore labeling, the amino acids tryptophan, tyrosine and phenylalanine are natural fluorophores. Among them tryptophan has the strongest quantum yield followed by tyrosine, while phenylalanine is a very weak fluorophore \[123\]. Aβ lacks tryptophans but has one tyrosine, Y10, which serves as an eligible intrinsic fluorophore (see Paper II).

Furthermore, fluorescence molecules, such as Thioflavin T (ThT), bind to amyloid material and thereby change their fluorescence properties, which can be used to detect amyloid formation \[125\]. ThT consists of a benzylamine and benzathiole ring that can freely rotate around the rings’ C–C bond in solution, which quenches all fluorescence. Binding to amyloid material, in contrast, stabilizes the structure giving rise to a great enhancement in the fluorescence yield \[125; 126\]. ThT has turned out to be suitable for kinetics studies of amyloid formation (see Chapter 4).

3.3 Nuclear Magnetic Resonance

NMR is a spectroscopic method that employs the nuclear spin, $I$, which is an intrinsic quantum-mechanical property of the nucleus characterized by its gyromagnetic ratio $\gamma$ and the magnetic moment $\mu = \gamma I$. The basic principles of an NMR experiment comprise an alignment of the spins to an external static magnetic field $B_0$ (by convention directed along the z-axis) and an alterable electromagnetic field in form of radio-frequency (rf) pulses applied perpendicular to $B_0$. The strength of $B_0$ is usually given with respect to the Larmor frequency $\omega_0 = -\gamma B_0$ of protons, which is associated with the energy transition between two spin states, and typically ranges between 400 MHz to 1 GHz (corresponding to 9.4 to 23.5 T) for protein NMR. A detailed discussion of
NMR theory is presented in the textbooks by Keeler [127], Levitt [128], Cavanagh et al. [129] and Kowalewski & Mäler [130], which provide the basis of the summary given in this thesis.

The rf-pulses are adjusted such that they are on (or close to) resonance on the Larmor frequency, which perturbs the external field and causes a change of the direction of the net magnetization to the xy-plane. The effect of the rf-pulses may be described in terms of the Hamiltonian \( \hat{H} = -\mu \cdot B = \hat{H}_z + \hat{H}_{rf} \).

After applying an rf-pulse the nuclear spins "relax" back to their equilibrium position aligned to the external magnetic field. This time-dependent behavior of the spins, also referred to as the time evolution of the spin system, is one of the fundamental principles of NMR. The evolution in time is governed by the time-dependent Schrödinger equation where the Hamiltonian accounts for the energy in the system (see Eq. 3.1). In order to mathematically handle large spin systems more easily a density operator \( \hat{\rho} \) is introduced and the time-dependent Schrödinger equation can be rewritten in form of a time evolution of the density operator. This is generally referred to the Liouville-von Neumann equation, which uses the concepts of commutators [129, 130]:

\[
\frac{d}{dt} \hat{\rho}(t) = \frac{i}{\hbar} [\hat{\rho}(t), \hat{\mathcal{H}}(t)]
\] (3.5)

The Hamiltonian contains, besides the rf-pulse, information about diverse NMR characteristics as chemical shift, scalar and dipolar coupling, chemical shift anisotropy, etc. A detailed discussion about these features exceeds the scope of this thesis and the interested reader is referred to textbooks [127–130].

NMR is an excellent tool to study a multitude of characteristics of biological macromolecules. This includes three-dimensional structure determination via multi-dimensional NMR experiments [131, 132], diffusion and complex size characterization (see Chapter 3.4) and protein/peptide dynamics on different timescales (see Chapter 3.5).

3.3.1 NMR relaxation mechanisms

Relaxation is a basic phenomenon in NMR that describes the return of the bulk magnetization to the equilibrium position, e.g. after applying a 90° pulse the xy-magnetization decreases to zero and the z-magnetization returns to its equilibrium value. A detailed description of the theory of the relaxation mechanisms is provided by e.g. Kowalewski & Mäler [130] and Palmer [133] and sections of several textbooks are dedicated to this topic [127, 129].

Relaxation mechanisms may be understood as stochastic perturbations by small local magnetic field fluctuations, \( B_{loc}(t) \), that cause the spin ensemble magnetization to reach its steady-state magnetization. The spin ensemble magnetization is the average magnetization of all spins in the system and variations
in the local field can be described by introducing the time-correlation function $G(\tau)$ \cite{127}:

$$G(\tau) = B_{loc}(t)B_{loc}(t+\tau)$$ (3.6)

Fourier transformation of the time-correlation function leads to the spectral density function $J(\omega)$, which depends on the correlation time $\tau_c$. The correlation time can be understood as the timescale of oscillations in a random process or as the average rotation time of the molecule axis for one radian in liquid. \cite{127, 130, 133}

$$J(\omega) = \int_{-\infty}^{\infty} G(\tau) \exp(-i\omega\tau) d\tau$$ (3.7)

$$J(\omega) = \frac{\tau_c}{5 \left(1 + \omega^2 \tau_c^2\right)}$$ (3.8)

The dominant relaxation mechanisms originate from dipole-dipole interactions, chemical shift anisotropy (CSA) and interactions with paramagnetic species. The relaxation of the $^{15}$N spin in the peptide backbone is of primary interest in this thesis where the dominating mechanisms are dipole-dipole relaxation and CSA.

Since the magnetic moment spins act as dipoles, interactions between two spins give rise to dipole-dipole relaxation. This type of relaxation consists of different terms, referred to as longitudinal and transverse relaxation, characterized by their rates $R_1$ and $R_2$, and the NOE originating from magnetization transfer of one spin to another, which is also called cross-relaxation.

CSA, in contrast, involves only one spin and originates from an anisotropic shielding of the magnetic field. This shielding is usually specified by a shielding tensor $\sigma$ that describes the reduction of the external field by $B_{loc} = B_0(1 - \sigma)$ and gives rise to a contribution in the relaxation term.

3.3.2 Chemical exchange processes

When a molecule undergoes exchange between different chemical or conformational states its magnetic environment is modulated, which gives rise to an alternation of the transverse relaxation behavior and is reflected in a dephasing of coherences \cite{133, 134}. In the following discussion the exchange is limited to two states, i.e. the states A and B, and characterized by the first-order rate constants for the forward, $k_1$, and reverse, $k_{-1}$, transition, respectively.

$$A \xrightarrow{k_1} B \xleftarrow{k_{-1}}$$

The two states are characterized by their populations $p_A$ and $p_B$, the difference, $\Delta\omega = |\Omega_A - \Omega_B|$, between their resonance frequencies $\Omega_A$ and $\Omega_B$ and the exchange rate, $k_{ex}$, defined by $k_{ex} = k_1 + k_{-1} = k_1/p_B = k_{-1}/p_A$ \cite{134}.
The presence of chemical exchange gives rise to a contribution to the transverse relaxation constant \[135\]:

\[ R_2 = R_2^0 + R_{\text{ex}} \]  

(3.9)

In theory, the alteration of the time dependence of the magnetization caused by chemical exchange can be described by the *Bloch-McConnell equations* \[136\] or, more generally, by the stochastic *Liouville equation* (compare Eq. \[3.5\]). A detailed theoretical discussion is found in articles by Palmer *et al.* \[134, 135\]. Chemical exchange is generally divided into three categories slow, intermediate and fast exchange, which can be defined by \( k_{\text{ex}} \) and \( \Delta \omega \) \[133, 134\]:

\[
\begin{align*}
  k_{\text{ex}} &\ll \Delta \omega & \text{Slow exchange} \\
  k_{\text{ex}} &\approx \Delta \omega & \text{Intermediate exchange} \\
  k_{\text{ex}} &\gg \Delta \omega & \text{Fast exchange}
\end{align*}
\]

In Figure 3.3 simulated line shapes in the presence of chemical exchange are depicted that visualize the different exchange regimes.

### 3.4 Diffusion and hydrodynamic radius

Diffusion of biomolecules in liquids is on a timescale that is measurable with specially designed NMR experiments. These NMR measurements are focused on translational diffusion and an overview about the experimental setup and theory can be found in several review articles \[137-140\]. The first reported diffusion experiments date back to the 1950s and were conducted by Hahn \[141\] and Carr & Purcell \[142\]. A simple diffusion pulse sequence contains a spin echo sequence with two gradients, which are typically applied along the z-axis \[143\]. The presence of the gradients cause a variation of the magnetic field along the z-axis, *i.e.* the molecules experience different magnetic fields depending on their (longitudinal) location in the sample volume. The time duration of the gradients, \( \delta \), and the separation between the two gradients, \( \Delta \), are two basic parameters of a diffusion experiment. Different gradient strengths give rise to a modulation of the signal attenuation, which follows an exponential decay with increasing gradient strength, described by the *Stejskal-Tanner equation* \[143\]. This means that, besides the decay caused by transverse relaxation, the signal intensity is attenuated as a function of the gradient strength and the diffusion coefficient. Thus, the translational diffusion coefficient, \( D_t \), can be obtained as a fitting parameter. Assuming a spherical complex, the size or, more precisely, the hydrodynamic radius, \( R_H \), of the molecule can be
estimated with the Stokes-Einstein equation by

\[ R_H = \frac{k_B T}{6\pi \eta D_t} \]  

in which \( k_B \) is the Boltzmann’s constant, \( T \) the temperature and \( \eta \) the dynamic viscosity.

### 3.5 Dynamics of biological macromolecules

Proteins and peptides in solution show a high degree of conformational flexibility that involves a broad range of different timescales (Figure 3.2). A substantial state of knowledge about biomolecular dynamics is, therefore, essential for an understanding of the biological function, even though the direct link between protein dynamics and function might be challenging to establish [134;144]. NMR provides a set of unique tools to study these motions, which was already recognized about five decades ago [145], albeit the techniques have continued to be substantially developed until today [133;144]. Figure 3.2 gives an overview about the typical timescales of biological processes and dynamics and suitable NMR techniques to monitor these.

Intramolecular motions, like vibrations and side chain rotations, occur on a timescale of pico- to nanoseconds and the measurements of longitudinal, \( R_1 \), and transverse relaxation rates, \( R_2 \), as well as of the Nuclear Overhauser Effect (NOE) provide insight into these dynamics (see also Chapter 3.3.1 and references therein).

Molecular motions and chemical exchange processes, however, take place on a micro- to millisecond timescale measurable in NMR chemical exchange experiments. These methods were recently put into perspective [135;146] and a characterization of chemical exchange is provided in Chapter 3.3.2. Depending on the exchange rate and population of the system, different experiments are appropriate. ZZ-exchange or magnetization exchange spectroscopy (EXSY) [147] experiments are suited for slow exchange, where the resonances for all chemical states are still observable. For faster exchange or if some chemical state resonances are invisible relaxation dispersion experiments based on Carr-Purcell-Meiboom-Gill (CPMG) [134;148-150] or rotating frame \( R_{1\rho} \) [151;152] pulse sequences can be applied. Typically CPMG experiments are utilized for an exchange rate between 200–2000 s\(^{-1}\) and \( p_B \geq 1\% \), whereas \( R_{1\rho} \) may capture \( k_{ex} \) rates up to 40000 s\(^{-1}\) [146]. More recently, two saturation transfer methods denoted as CEST [153;154] and DEST [155;156] have been developed, which are also applicable to sparsely populated states but with slower exchange rates between 20–300 s\(^{-1}\) [146].
Dynamic binding processes of Aβ with different binding partners are on the μs-ms timescale and 15N-CPMG experiments have turned out to be suitable for investigating these dynamics (Paper II, III and IV). These experiments are discussed in detail in Chapter 3.5.1.

Further techniques pictured in Figure 3.2 include residual dipolar coupling (RDC) [157] and H-D exchange experiments [158, 159]. For a detailed presentation of these methods see the given references or Ref. [144] for an overview.

![Figure 3.2: NMR timescales](image)

**Figure 3.2: NMR timescales** - NMR provides various methods to study biological functions and dynamics on different timescales. Figure is based on Refs. [133, 146, 160].

### 3.5.1 CPMG relaxation dispersion

When the exchange dynamics occur on the μs-ms timescale, CPMG pulse schemes [142, 161] can be applied to study this process. The basic element is a block of 180° pulses, which are typically used to refocus signals, with a delay \( \tau_{CP} \) between the 180° pulses in the \( \tau_{CP}/2 - 180° - \tau_{CP}/2 \) spin-echo period. In this thesis a modified pulse sequence was used [150, 162] that consists of two blocks of 180° pulses with a mixing time of \( T_{CP}/2 \) each. At high CPMG frequencies, \( \nu_{CPMG} = 1/(2 \tau_{CP}) \), the signals are predominantly refocused, which is reflected in a larger signal intensity, while at low CPMG frequency the signals are significantly broadened. From the signal amplitudes the observed transverse relaxation rates, \( R_{2}^{obs} \), can be calculated by

\[
R_{2}^{obs} = \frac{1}{T_{CP}} \ln \left( \frac{I_0}{I} \right)
\]
where $I_0$ is the signal intensity obtained from a reference experiment with $T_{CP} = 0$ ms.

The observed transverse relaxation rate is determined by the populations, the chemical shift differences, the exchange rate and intrinsic transverse relaxation rates, $R^0_{2A}$ and $R^0_{2B}$, of the two states. The general relation for a two-state model is given by \[134; 148, 149\]:

$$R_{obs}^2(\tau_{-1}) = \frac{1}{2}(R^0_{2A} + R^0_{2B} + k_{ex} - \tau_{CP}^{-1} \cdot \cosh^{-1}[D_+\cosh(\eta_+) - D_-\cos(\eta_-)]$$

with

$$D_{\pm} = \frac{1}{2}(\pm 1 + \frac{\psi + 2\Delta \omega^2}{(\psi^2 + \xi^2)^{1/2}})$$

$$\eta_{\pm} = \frac{\tau_{CP}}{\sqrt{2}}(\pm \psi + \frac{\psi^2 + \xi^2}{2})^{1/2}$$

$$\psi = (R^0_{2A} - R^0_{2B} - p_ak_{ex} + p bk_{ex})^2 - \Delta \omega^2 + 4p_ap_b k_{ex}^2$$

and

$$\xi = 2\Delta \omega(R^0_{2A} - R^0_{2B} - p_ak_{ex} + p bk_{ex})$$

(3.12a)

Experimentally the chemical exchange parameters can be obtained by a fit of the $R_{obs}^2$ values to equation 3.12. In this thesis a simplified model was applied with $R_{calc}^2 = R^0_{2A} = R^0_{2B}$ to reduce the number of independent parameters (Papers II, III and IV). Furthermore, it was assumed that the exchange process is global, i.e. all residues undergo the same exchange process, which decreases the number of degrees of freedom of the fit. With this approach there are, thus, two global fit parameters, $k_{ex}$ and $p_B$, and two fit parameters, $R_{calc}^2$ and $|\Delta \omega|$, which are specific for each residue. Figure 3.3 visualizes Eq. 3.12 and the effect of the different exchange parameters.

With this fitting routine only the absolute value of $\Delta \omega$ can be obtained. However, protocols have been reported that use additional experiments to determine the sign of $\Delta \omega$, i.e. a combination of HSQC and HMQC (Heteronuclear Single/Multiple Quantum Coherence) experiments, HSQC experiments at different magnetic fields or $R_1$ experiments \[163, 164\]. The H(S/M)QC strategy makes, for instance, use of the slightly different effect of exchange processes on single (HSQC) and zero & double quantum coherences (HMQC), which results in a chemical shift difference between these two experiments \[163\]. The theoretical chemical shift difference is, however, only small and a favorable combination of values for $k_{ex}, p_B$ and $|\Delta \omega|$ is experimentally required, e.g. $p_B$ should be $\gtrsim 3\% \[163\].
Figure 3.3: Simulated chemical exchange line shapes and CPMG relaxation dispersion profiles - (Left panel) In the absence of exchange the signals are located at $\Omega_A$ and $\Omega_B$. An increase of $k_{ex}$ causes broadening of resonance signals until the point of coalescence (intermediate exchange) is reached. At fast exchange only one single line is observed at the population-averaged resonance $\Omega = p_A \Omega_A + p_B \Omega_B$. Lines were simulated using the solution of the Bloch-McConnell equations [134] with $R_A = R_B = 10 \text{s}^{-1}$, $\Delta \omega / 2\pi = 200 \text{s}^{-1}$ and $p_B = 0.25$. (Right panel) Simulated $^{15}$N-CPMG relaxation dispersion profiles at 700 (solid line) and 500 MHz (dashed line) using Eq. 3.12 and parameters $R_0^A = R_0^B = 10 \text{s}^{-1}$, $p_B = 3\%$, $\Delta \delta = 2 \text{ppm}$ and $k_{ex} = 1000 \text{s}^{-1}$ (black). The colored curves visualize the effect of changing one single parameter by a factor of 0.5 for $p_B$ (blue), $\Delta \delta$ (red) and $k_{ex}$ (green).
4. Aggregation pathways

4.1 Aggregation process

The aggregation process describes the formation of mature fibril structures from soluble monomeric peptides via various intermediate states (Figure 4.1). Aβ has a large propensity to self-assemble, and formation of various soluble aggregated species have been detected that are usually assigned to oligomers and protofibrils [12, 23, 77]. The term oligomer describes, according to the Greek origin oligos, the assembly of only "a few" peptides but in literature the term is often used in a much broader meaning and stands for different kinds of pre-fibrillar species that lack a specific structure [77]. In contrast, protofibrils exhibit a more elongated shape with a high β-structure content and appear later in the aggregation pathway [77]. A large variety of these aggregation intermediates has been reported [71], indicating a great heterogeneity of the aggregation pathways. The high dependence of Aβ aggregation on environmental conditions, such as temperature, pH and buffer, may (partly) explain the heterogeneous results reported. Furthermore, different aggregation kinetics and properties were reported for synthetic and recombinant Aβ [165], which also contributes to the heterogeneity of reported in vitro experiments.

An important feature is the formation of β-structure during the aggregation process, which is finalized in the well-ordered cross-β structure of the mature fibrils (see Chapter 2.3.3). The term amyloid is hereby closely associated with aggregates that exhibit a cross-β structure. Both aggregates that are predominantly disordered and those that exhibit a high β-structure content were characterized and found to appear on-pathway, i.e. towards mature fibril formation [71, 77], where highly β-structured aggregates should be late-stage intermediates. As monomeric Aβ has a propensity to transiently form a β-hairpin (discussed in Chapter 2.3.2), this structure might nucleate and initiate oligomerization. But the β-structure induction might also be a consequence of self-assembly and detailed knowledge about the β-structure formation process is lacking.

Considering the aggregation process as a simple polymerization reaction, which is the topic of Chapter 4.2, the different structural intermediates and their β-structure content are greatly generalized and only nucleation reactions
are concerned in this model. This is visualized in the lower part of Figure 4.1.

Figure 4.1: Schematic model of aggregation pathways - Different structural states have been suggested to be involved in the aggregation mechanism including various on- and off-pathway species. The amyloid-characteristic β-structure is adopted at some point during the aggregation process and mature fibrils exhibit a well-defined cross-β structure. Fibrils are formed from monomers and pre-fibrillar aggregates. The lower part shows the mechanism monitored by aggregation kinetics experiment that lack any information about oligomeric states. [12, 87, 166]

4.2 Aggregation kinetics

The self-assembly process of amyloidogenic proteins and peptides can be investigated by optical spectroscopy methods where ThT fluorescence kinetics experiments (see Chapter 3.2) have been established as a suitable tool to monitor fibril formation [125]. The aggregation profiles typically display a concave or sigmoidal shape that can be described by the empirical formula:

\[
F = F_0 + \frac{A}{1 + \exp[r_{max}(\tau_{1/2} - t)]}
\]  

(4.1)

where \(r_{max}\) is the maximum growth rate, \(\tau_{1/2}\) is the time of half completion of aggregation, which is related to the lag time by \(\tau_{lag} = \tau_{1/2} - 2/r_{max}\), and \(F_0\) and \(A\) are related to the initial fluorescence intensity and amplitude of the fluorescence signal, respectively.
4.2.1 Primary and secondary nucleation reactions

The aggregation mechanism can be theoretically described by a polymerization reaction that involves primary and secondary pathways (Figure 4.2) [167-169]. Primary nucleation refers to fibril formation from monomers only and is characterized by the reaction rate constant, \( k_n \), and the reaction order \( n_c \), which in a simplified picture reflects the nucleus size. Elongation describes the association of monomers to fibril-ends with the rate \( k_+ \). Secondary pathways include both monomer-independent, e.g. fibril fragmentation at rate \( k_- \), and monomer-dependent polymerization reactions, e.g. surfaced catalyzed nucleation at rate \( k_2 \), and are characterized by the reaction order \( n_2 \).

Obviously, any aggregation process that starts from a purely monomeric solution must involve primary nucleation. It is, however, an important question whether secondary pathways are present as well and which type(s) of nucleation reactions is (are) the dominant aggregation mechanism(s). To elucidate this issue aggregation experiments with added seeds, i.e. preformed fibrils, can be performed [169]. In the absence of seeds, when aggregation shows a sigmoidal profile, new aggregates are formed at a very slow rate during the lag time. In contrast, in the presence of seeds generation of new aggregates is generally accelerated and the reaction profile approaches its saturation state faster [84]. If saturation is reached within the lag time of the non-seeded kinetic profile the rapid generation of these new aggregates can only stem from secondary nucleation reactions as primary pathways are, per definitionem, not affected by addition of seeds [167; 169].

Notably, at (very) high seed concentration the aggregation profile typically exhibits a concave shape. When fragmentation and nucleation of new aggregates are negligible, such as for highly preseeded \( \text{A}_\beta \) samples under quiescent conditions (Paper IV and [170; 171]) the increase of fibril mass originates only from association of monomers to fibril-ends. Hence, the initial slope of these profiles is proportional to the elongation rate [169] and highly preseeded samples can be used to roughly estimate the fibril-end elongation rate.

In general, the aggregation half time \( \tau_{1/2} \) is dependent on the initial monomer concentration, \( m(0) \), and can be described by a power law [168; 172; 173]:

\[
\tau_{1/2} \propto m(0)^\gamma
\]  

(4.2)

The half-time exponent \( \gamma \) is related to the reaction order and is a distinct feature of the molecular mechanism of aggregation (Table 4.1).
Figure 4.2: Nucleation reactions - Aggregation can be described as a polymerization process that involves primary and secondary nucleation reactions. Primary pathways describe fibril formation generated solely from monomers with a reaction order $n_c$. Elongation refers to the association of monomers to fibril ends. Secondary pathways can be subdivided into monomer-independent, e.g. fibril fragmentation, and monomer-dependent, e.g. surface catalyzed nucleation, polymerization reactions with a reaction order $n_2$.

Table 4.1: Different aggregation pathways and the dependence of their half-time exponent $\gamma$ on the nucleation reaction [168, 169, 171].

<table>
<thead>
<tr>
<th>aggregation pathways</th>
<th>exponent $\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>primary pathways</strong></td>
<td>$-n_c/2$</td>
</tr>
<tr>
<td><strong>secondary pathways</strong></td>
<td></td>
</tr>
<tr>
<td>monomer-independent for $n_2 = 0$</td>
<td>$-0.5$</td>
</tr>
<tr>
<td>monomer-dependent for $n_2 &gt; 0$</td>
<td>$-(1 + n_2)/2$</td>
</tr>
<tr>
<td>including saturation of 2nd nucleation</td>
<td>$-0.5 \left( \frac{n_2}{1 + m(0)^n_2/K_M} + 1 \right)$</td>
</tr>
</tbody>
</table>

$^a$ $n_c$ and $n_2$ denote the reaction order for primary and secondary nucleation reactions, respectively, and $K_M$ is the Michaelis equilibrium constant.
4.2.2 Microscopic kinetic rate constants

To obtain further insight into the microscopic reaction mechanism(s) a global fit analysis can be applied using kinetic traces at different initial monomer concentrations [167] [169]. Analytical expressions for nucleated polymerization \( (k_n, k_+ \text{ and } n_c \text{ as parameters}), \) fragmentation \( (k_n, n_c, k_+, k_-, n_2=0) \) and monomer-dependent secondary nucleation \( (k_n, n_c, k_+, k_2, n_2) \) have been reported [167][168][174][175] and explicitly depend on the rate constants and the reaction orders. In practice, it is essential to restrict the number of degrees of freedom by using combined rates as fitting parameters, e.g. \( \sqrt{k_n k_+} \& \sqrt{k_+ k_2} \) for monomer-dependent secondary nucleation. The individual rate constants can be determined only with additional experiments, e.g. highly preseeded aggregation kinetics (\textit{vide supra}), where the elongation rate is obtained, although in general with a much larger uncertainty compared to the value for the combined rates.

For \( \alpha_{\beta}40 \) aggregation it has been shown that saturation of secondary nucleation has to be included in the nucleation model that follows a Michaelis-Menten-type reaction [171]. In this model the time dependence of the normalized fibril mass fraction, \( M(t)/M(\infty) \) can be described by [171]:

\[
\frac{M(t)}{M(\infty)} = 1 - \left( \frac{B_+ + C_+}{B_+ + C_{+e^{\kappa_2}}} \right) e^{-k_{\infty}t}
\]

(4.3)

with the global fit parameters \( \lambda, \kappa \) and \( K_M \), which relate the microscopic rate constants to primary nucleation,

\[
\lambda = \sqrt{2k_+k_n m(0)^{n_c}}
\]

(4.4)

and secondary nucleation

\[
\kappa = \sqrt{2k_+k_2 m(0)^{n_2+1}} \frac{m(0)^{n_2+1}}{1 + m(0)^{n_2}/K_M}
\]

(4.5)

where \( K_M \) is the Michaelis equilibrium constant. All other parameters are related to \( \lambda \) and \( \kappa \) by \( B_\pm = (k_\infty \pm \tilde{k}_\infty)/(2\kappa), C_\pm = \pm \lambda^2/(2\kappa^2), k_\infty = 2k_+P(\infty), \tilde{k}_\infty = \sqrt{k_\infty^2 - 2C_{+}C_{-}\kappa^2}. \)

The Michaelis constant for a two step secondary reaction is defined by \( K_M = (k_b + \tilde{k}_2)/k_f \), which is related to \( k_2 \) by \( k_2 = \tilde{k}_2/K_M \) [171]. The reaction

\[
2k_+P(\infty) = \sqrt{\left| -A(0) - 2k_+k_2 m(0) K_M \log(K_M/m(0)^{n_2}) \right|} \text{ with } A(0) = -\frac{2k_+ k_2 m(0)^{n_2} K_M \log(K_M + m(0)^{n_2})}{n_c} - 2k_+k_2K_M m(0) \left( \sqrt{K_M \arctan(m(0)/\sqrt{K_M})} - 1 \right)
\]

The long time limit of the aggregation number \( P(\infty) \) for \( n_2 = 2 \) is given by [171]:
scheme includes an attachment/detachment of $n_2$ monomers to/from the fibril surface, which is described by $k_f$ and $k_b$ respectively, and a subsequent release of the formed aggregate from the fibril surface with the rate $k_2$.

Here, $\sqrt{k_n k_+}$ and $\sqrt{k_+ k_2}$ as well as $K_M$ are global fit parameters, *i.e.* they share the same value for all kinetic traces at different initial monomer concentrations, $m(0)$. Simulated aggregation kinetics following this model are illustrated in Figure 4.3.

**Figure 4.3: Secondary nucleation reaction including saturation** - Simulated aggregation traces from equation 4.3 using $\sqrt{k_n k_+} = 2.3 \cdot 10^{-2} \text{M}^{-1}\text{s}^{-1}$, $\sqrt{k_+ k_2} = 3.85 \cdot 10^3 \text{M}^{-3/2}\text{s}^{-1}$ and $\sqrt{K_M} = 12.5 \mu\text{M}$ (values from Paper IV) for initial monomer concentrations from 10 to 20 $\mu\text{M}$ (color code from blue to red). For a small range of initial monomer concentrations the aggregation half times, $\tau_{1/2}$, can be fitted to Eq. 4.2 to determine the half-time exponent $\gamma$. 

![Graph](image-url)
4.3 Aggregation modulators

In Chapter 2.2.4 different strategies to establish therapeutics against neurogenerative diseases were introduced. In this chapter, one potential strategy, namely modulation and interference of/with the aggregation pathway of amyloidogenic proteins and peptides, is discussed in more detail [176, 177]. Modulation of protein/peptide self-assembly is a rather broad term and interference with the protein/peptide can occur on various levels, which means that the aggregation modulators may interact with a protein/peptide at (several) different aggregation states [177, 178]. Various types of aggregation modulators have been reported including small organic molecules, peptide-based inhibitors, antibodies, metal ions, organic & inorganic nanoparticles and natural chaperones [177, 178]. In particular, chemical and biochemical design of potential inhibitors has become a dynamic field of research. As a molecular understanding of the modulator binding/interaction is beneficial for specific drug design, biophysical methods have turned out to be suitable tools to contribute essential insights.

4.3.1 Potential drug targets

Potential inhibitors may interact with different aggregation states, i.e., with monomers, oligomeric/prefibrillar species or and mature fibrils, and consequently affect kinetic rate constants associated with theses states. An analysis of the inhibition mechanism that focuses on the kinetic aspects is discussed in further detail in Chapter 4.3.2.

Interaction between the modulator and protein/peptide may take place on a monomer level, i.e., already the very beginning as well as later stages of the aggregation process may be affected. Interaction with monomeric Aβ causing an inhibition of Aβ self-assembly was shown in the presence of an Affibody protein [179]. This engineered protein encapsulates monomeric Aβ and stabilizes a β-hairpin structure in the peptide [103]. Also later states in the kinetic aggregation profile are influenced by the Affibody protein, i.e., states where a significant increase in ThT fluorescence is observed, as monomeric peptide is transiently present even at these states [180]. In addition, the organic compounds, lacmoid and Congo red interact with monomeric Aβ where peptide monomers transiently bind to co-aggregates (Paper I and II). Similarly, interactions on a monomer level have been shown for αSN and Congo red micelles [181]. Interaction studies of Aβ with these compounds are subject of Paper I and II and are discussed in detail in Chapter 5.

In fact, a large variety of different organic molecules have been reported to inhibit/modulate Aβ aggregation [182–184]. These compounds can be subdi-
vided into different classes depending on whether they inhibit A\(_\beta\) oligomer and/or fibril formation \[182\]. In this scheme inhibition of oligomerization was defined by an anti-oligomer specific antibody A11 \[185\] and fibrillization was characterized by turbidity at 400 nm and TEM images. Following this classification lacmoid was assigned to inhibition of both oligomerization and fibrillization, while Congo red was found to inhibit oligomer formation only \[182\]. Interestingly, also ThT (see Chapters 3.2 and 4.2) was found to affect oligomer generation by itself \[182\]. A comparison between CD and ThT fluorescence experiments suggests, however, that ThT does not significantly alter \(\beta\)-structure formation \[171\] and should thus still be a good agent to study kinetics of fibril formation.

Instead of aiming for a complete inhibition of aggregate formation an alternative strategy might be to redirect the aggregation pathways such that only non-toxic species are generated. One potential agent for this strategy is epigallocatechin-3-gallate (ECGC), a substance that is found in green tea, which was reported to be beneficial against A\(_\beta\)-induced neurotoxicity in cultured hippocampal neurons \[186\] and, also, to reduce A\(_\beta\) levels and amyloid plaque formation in transgenic mice \[187; 188\]. ECGC was shown to prevent fibril formation of A\(_\beta\) and \(\alpha\)SN, which then form non-toxic oligomeric species that do not convert into amyloid fibrils \[189\]. It was further demonstrated that ECGC has the potential to convert even mature fibrils into non-toxic globular amorphous aggregates \[190\]. The authors found that the re-assembly process occurs directly from fibrillar to amorphous species, \textit{i.e.} not via re-assembly into monomers or small oligomers and a subsequent build-up of these amorphous aggregates \[190\]. Another study showed that the formed aggregates possess, yet, some defined structure as evident from ssNMR spectra \[191\]. NMR chemical shift changes indicate that ECGC primarily binds to the hydrophobic regions in the peptide sequence and A\(_\beta\) adopts a \(\beta\)-structure in the globular, not fibrillar, end-state aggregates \[191\].

Another strategy may be to avoid generation of highly toxic intermediates and, therefore, accelerate formation of end-state fibrils, which presumably are not, or less, toxic than the pre-fibrillar intermediates (see Chapter 2.2.3). Potential candidates might be found in the class of inhibitors that prevent oligomer but not fibril formation. The lacmoid-like molecule O4 could be a candidate for this inhibition type. This molecule was reported to promote formation of \(\beta\)-sheet rich fibrils and protofibrils, while disfavoring the appearance of toxic aggregation intermediates \[192\]. Also Congo red was shown to favor \(\beta\)-structure conformation in A\(_\beta\) \[193\], yet its effect on fibril formation is controversial and seemingly diverging results have been reported \[182; 183; 193; 194\]. The different findings might, however, be explained by a two-step association of the compound including a lower and higher binding
affinity that may promote or decelerate fibril formation \[194\]. Nevertheless, several studies indicate a beneficial toxicity reducing effect in in vivo system \[195–197\].

![Lacmoid and Congo red](image)

**Figure 4.4:** Small molecule aggregation modulators - Small organic compounds, like lacmoid and Congo red, as well as surfactants, like SDS & LiDS, modulate Aβ aggregation.

### 4.3.2 Microscopic kinetics of inhibition mechanism

Interactions of aggregation modulators with amyloidogenic proteins/peptides may occur at various structural states that these proteins/peptides adopt during the aggregation process, i.e. monomers, oligomers/protofibrils and/or fibrils (vide supra). Hence, different nucleation reactions can be the target of aggregation inhibitors (reviewed in Ref. \[178\]).

All nucleation reactions except fibril fragmentation, i.e. primary and monomer-dependent secondary nucleation as well as fibril elongation, are dependent on monomer concentration. An aggregation modulator that interacts with monomers affects consequently all these nucleation events. In contrast, inhibitors that stabilize or modify oligomer structures alter both primary and surface-catalyzed secondary nucleation as these reactions involve formation of oligomeric aggregates. Furthermore, when an aggregation inhibitor interacts with amyloid fibrils the molecular mechanism is modulated depending on whether specifically fibril ends are targeted, which results in a modulation of the elongation rate, or whether the inhibitor blocks fibril surface reducing the number of surface-catalyzed secondary nucleation events. \[178\]

To elucidate the molecular mechanism of aggregation inhibition a global analysis of kinetic profiles can be performed (Paper IV and \[198; 199\]). Aggregation traces of the protein/peptide in the presence of different inhibitor concentrations are used in the global fit routine. Applying the model described in Eq. 4.3 there are two free fitting parameters \(\sqrt{k_+k_0} \) & \(\sqrt{k_+k_2}\) or \(\sqrt{k_0/k_2}\).
& $\sqrt{k_{+}k_{2}}$ ($K_{M}$ can be fixed to a previously obtained value). To investigate, for instance, the inhibition effect on surface-catalyzed secondary nucleation, $k_{2}$, a fit can be performed fixing $\sqrt{k_{n}k_{+}}$ to one single constant value, while allowing $\sqrt{k_{+}k_{2}}$ to vary across different inhibitor concentrations. The effect on $k_{+}$ and $k_{n}$ can be tested with similar constraints in the global fit analysis (see Paper IV, Supporting Information). A reduction of the specific microscopic rate constants and its effect on the aggregation profile is illustrated in Figure 4.5.

Figure 4.5: Inhibition effect on microscopic kinetics - Schematic inhibition of primary nucleation (left), elongation (middle) and monomer-dependent secondary nucleation (right) assuming a reduction of the individual rate constants by a factor of 5 (red curves). The aggregation profiles were simulated from Eq. 4.3 using $\sqrt{k_{n}k_{+}} = 2.3 \cdot 10^{-2} \text{M}^{-1}\text{s}^{-1}$, $\sqrt{k_{+}k_{2}} = 3.85 \cdot 10^{3} \text{M}^{-3/2}\text{s}^{-1}$ and $\sqrt{K_{M}} = 12.5 \mu\text{M}$, $m(0) = 20 \mu\text{M}$ (values from Paper IV) and an estimated elongation rate of $k_{+} = 3 \cdot 10^{5} \text{M}^{-1}\text{s}^{-1}$ [171].

In addition to a global fit analysis highly preseeded aggregation kinetics can be conducted where, due to the high number of available aggregates, the initial rate is proportional to the fibril-end elongation rate (see Chapter 4.2.1 and [169]). These experiments, thus, directly report on the inhibition effect on elongation reactions.

This kinetic profile analysis approach was applied to investigate the inhibition effect of the chaperone Ssa1p on the prion protein Ure2p self-assembly in yeast cells [198]. In this study the authors found a reduction in elongation rate that accounts for the observed change of kinetic profiles. Notably, in another study it was shown that the molecular chaperone DNAJB6 efficiently inhibits $\beta_{42}$ aggregation by affecting all nucleation events, i.e. besides primary nucleation also elongation and secondary nucleation [199]. In contrast, amyloid fibril formation of $\beta_{40}$ is retarded by sub-stoichiometric amounts of Zn$^{2+}$ ions where in a simplistic model the observed inhibition effect can be explained by a reduction in the fibril-end elongation rate (see Chapter 5.3 and Paper IV).
5. Results and Discussion

This thesis includes four papers that are all centered around Aβ and its aggregation mechanism(s). Different biophysical methods were applied to investigate interactions between Aβ and various aggregation modulators including the small organic molecules lacmoid and Congo red (Paper I and II), the surfactants SDS & LiDS (Paper III) and zinc ions (Paper IV). The goal of these studies has been to obtain a detailed understanding of the aggregation mechanism by accumulating knowledge about how to modulate peptide self-assembly. Insights into the molecular mechanism of action are potentially helpful for development and design of therapeutics against neurodegenerative diseases.

5.1 Small organic molecules (Paper I and II)

Lacmoid and Congo red were chosen as representatives of the class of small organic molecule amyloid inhibitors and their interactions with Aβ were subject of Paper I and II. A long list of small molecules was published that inhibit oligomerization and/or fibrillization [182; 183]. Lacmoid was assigned to the class that inhibits both oligomer and fibril formation, whereas Congo red was found to prevent only oligomerization [182]. Both molecules share the property to form colloidal aggregates ([194; 200] and Paper I), which was proposed to be a common feature of many small molecule aggregation modulators [184].

5.1.1 Nonspecific modulation of Aβ self-assembly (Paper I)

In Paper I we found that lacmoid efficiently inhibits the formation of β-structure as evident from time-dependent CD experiments. While Aβ alone showed a typical transition from an initial random coil-like state to a β-structure, the presence of lacmoid retarded this conformational change and, at high concentrations, even completely prevented β-structure formation. Notably, titration of lacmoid onto a monomeric Aβ solution caused a partial loss of the signal around 198 nm (the random coil minimum), which was accompanied by an impairment of the CD signal-to-noise ratio. This indicates an increased absorbance, which might be caused by lacmoid (aggregates) binding a small population of soluble Aβ. Congo red, in contrast, promotes the formation of
\( \beta \)-structure in A\( \beta \) as evident from previously reported CD experiments [193].

The amyloid inhibition effect of lacmoid was further demonstrated with the help of TEM images, which showed fibril structures for A\( \beta \) alone after a suitable incubation time, while TEM images of A\( \beta \) incubated with lacmoid displayed a substantially reduced amount of aggregated material.

An interesting aspect is the mechanism of interaction between A\( \beta \) and these compounds on a molecular level. To shed more light on the origin of the aggregation modulation properties we performed NMR experiments. At near-physiological conditions A\( \beta \) exhibits a well-resolved \( ^1\text{H}^{15}\text{N}\)-HSQC spectrum. Titration of lacmoid caused an overall NMR signal attenuation along the whole peptide sequence. At the same time chemical shift changes were only very small and slightly increased with increasing lacmoid concentration. A similar NMR signal attenuation was observed in the presence of Congo red [193]. In general, NMR signal attenuation can be caused by formation of larger species that feature short transverse relaxation times and, hence, broaden line widths. Alternatively, chemical exchange on an NMR intermediate time scale can be the origin of line broadening (see Chapter [3.3.2]). To elucidate the reasons for the observed effect we applied various additional experiments whose results are discussed below.

NMR signals applying \( ^1\text{H}^{15}\text{N} \) transverse relaxation-optimized spectroscopy experiments (TROSY), which were developed to improve cross-peak intensities for large molecules [201], showed the same pattern as observed with \( ^1\text{H}^{15}\text{N} \)-HSQC pulse sequences. Additionally, we conducted pulse field gradient diffusion and saturation transfer difference experiments, which revealed no significant change of the observed monomeric A\( \beta \) signals in the presence of lacmoid. Also, A\( \beta \) samples with and without lacmoid showed similar autocorrelation curves in dynamic light scattering experiments. All these findings indicate that no major population of large A\( \beta \)-lacmoid co-aggregates is present.

Clean chemical exchange experiments (CLEANEX) showed that the exchange rates of A\( \beta \) amide protons and water differ along the peptide sequence \( k_{\text{NH,H}_2\text{O}}^{\text{int}} \sim 2 \text{ to } 10 \text{ s}^{-1} \) but do not exhibit any significant differences in the presence of lacmoid. However, firm conclusions about structural changes based on these experiments are not possible when the chemical exchange rate is on a different time scale than the intrinsic amide exchange rate as it is shown in Paper II.

Taken together, these experiments indicate that NMR signal attenuation can be predominantly assigned to a chemical exchange process, not to a major population of co-aggregated A\( \beta \) bound in large complexes. Analyzing the whole class of amyloid inhibitors [182,183] revealed a frequent generic hydrophobic nature of these compounds. Thus, hydrophobic attraction and
conformational preferences of Aβ are suggested to be key determinants of
the modulation effect of small molecules on the aggregation pathway. While
Congo red favors a β-structure in Aβ promoting fibril formation, Aβ in the
presence of lacmoid is mainly unstructured, which presumably counteracts
fibril generation. Moreover, colloidal or surfactant-like properties appear to
be a common characteristic among many aggregation modulating compounds
[184], including lacmoid and Congo red.

5.1.2 Transient interactions and formation of dynamic co-aggregates
(Paper II)
Exchange dynamics of Aβ in the presence of lacmoid and Congo red are on
a favorable time scale for NMR relaxation dispersion studies and a detailed
characterization of the exchange process was subject of Paper II. 15N-CPMG
relaxation dispersion experiments [150], a suitable tool to detect chemical ex-
change in the micro- to millisecond time scale (see Chapter [3.5.1]), were ap-
plied on an Aβ–lacmoid sample where only about half of the initial cross-peak
intensity was left. Significant relaxation dispersion amplitudes were found for
residues located in the two hydrophobic parts as well as in the N-terminus.
The profiles could be reliably described by a two-state exchange model, where
one state was assigned to free monomeric peptide and the second one to Aβ
bound to a co-aggregate. The fit analysis was performed with two global fit
parameters, the exchange rate and the population, while the chemical shift dif-
ference and the intrinsic relaxation rates were residue-specific fit parameters
(see Chapter [3.5.1]). The exchange rate was determined to be 1000 ± 100 s−1
where only 1.0 ± 0.1 % of peptides are bound to a dynamic co-aggregate at
any given time. Additionally, we recorded exchange dynamics at a lower lac-
moid concentration, i.e. half of the previous one, and we found an exchange
rate of 850 ± 200 s−1 and a bound population of 0.3 %. Whereas the exchange
rate declined only slightly, the population bound to a co-aggregate decreased
to less than half of the original value. Such a response is expected when the
morphology of the formed co-aggregate is the same and the bound population
reflects, almost proportionally, the lacmoid concentration.

We also found that an Aβ–Congo red sample exhibited distinctive relax-
ation dispersion profiles under similar conditions, i.e. when the signals were
broadened to about half of the initial intensities. A global fit routine revealed
very similar exchange parameters with an exchange rate of 1050 ± 150 s−1
where the bound population that participates in the exchange dynamics is
3.2 ± 0.4 %. Also, very similar parts of the peptide are involved in the ex-
change process at this time scale. Importantly, Aβ alone did not show such
exchange dynamics within the examined time frame, which is evident from
constant $^{15}$N-CPMG relaxation dispersion profiles.

The co-aggregates may be pictured as generalized micelles. From the fitted relaxation rates and the bound population, when assuming a spherical shape of the complex, the size of the complex can be roughly estimated. We evaluated the hydrodynamic radius to be around 2.5 to 4.5 nm, which corresponds to an estimated number of 10 to 50 $\alpha$-molecules bound in the co-aggregate.

In addition to NMR, we conducted fluorescence stopped-flow experiments and recorded the change of the intrinsic tyrosine fluorescence ($\gamma 10$) while mixing $\alpha$- with lacmoid. Here, we used the observation that the static tyrosine fluorescence intensity is slightly, but significantly, enhanced in the presence of lacmoid. And indeed, we could follow an increase of fluorescence intensity after mixing that fits to a rate constant of $11 \pm 2$ s$^{-1}$. This much slower rate, compared to relaxation dispersion results, may describe the initial formation of an $\alpha$–lacmoid co-aggregate.

Furthermore, we tested the toxicity of $\alpha$–lacmoid co-aggregates on cultured human cells. Two different techniques using resazurin [202] or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) [203] were applied to quantify the cell toxicity. A combined analysis of these methods revealed that neither soluble $\alpha$ alone nor $\alpha$ in the presence of lacmoid had any strong effect on cell viability. Also, lacmoid alone was found to be non-toxic to cells. Hence, we concluded that $\alpha$-lacmoid co-aggregates are not toxic, in stark contrast to various neurotoxic oligomers that have been published in literature (reviewed in Chapter 2.2.3).

In conclusion, we showed that lacmoid efficiently inhibits a $\beta$-structure formation and, thereby, prevents generation of amyloid fibrils. $\alpha$ forms dynamic co-aggregates with lacmoid to which monomeric peptide transiently binds. A similar dynamic process is observed in the presence of Congo red. The exchange rate is $\sim 1000$ s$^{-1}$ and only a small population of $\alpha$ is bound to a co-aggregate and involved in chemical exchange. $\alpha$ alone does not exhibit any exchange dynamics in this time frame but was previously shown, with the help of NMR saturation transfer relaxation methods, that it transiently binds to aggregates with an exchange rate of $73$ s$^{-1}$ [155; 204]. This exchange rate is, hence, much slower than the exchange rate in the presence of the aggregation modulators. Thus, the faster exchange dynamics in the presence of lacmoid could influence $\alpha$ self-assembly and may kinetically modulate aggregation pathways. The aspects that lacmoid and Congo red promote different structural states in $\alpha$, but still show similar exchange dynamics, and the very low population of peptide that undergoes exchange suggest, however, that the aggregation modulation effect is determined by other mechanisms as well. Also, an interaction at a later aggregation state, i.e. involving pre-fibrillar and/or fibrillar structures, is likely to significantly influence the aggregation pathway.
Interestingly, also αSN in the presence of Congo red was shown to transiently bind to Congo red micelles with a similar exchange rate of $\sim 3000 \, \text{s}^{-1}$ and a bound population of $\sim 2\%$ [181]. Dynamic exchange seems, therefore, to be a common property of amyloidogenic peptides in the presence of small molecule aggregation modulators.

### 5.2 Surfactants (Paper III)

In Paper III we investigated the interaction of the anionic surfactants SDS/LiDS with A\textsubscript{β}. These interactions and their influence on the aggregation pathway are interesting for several reasons. Firstly, as described above small molecule aggregation modulators often feature colloidal or surfactant-like properties [181; 184; 193; 200]. A detailed study of the influence of surfactants themselves appears therefore obvious. Also, a similar NMR signal broadening was reported for this system [205] as observed in the presence of lacmoid and Congo red (Paper I and II). This phenomenon might have its origin in a similar dynamic mechanism. Secondly, intermediate aggregation states with some β-structure content have been proposed to be neurotoxic (see Chapter 2.2.3). It is, yet, difficult to characterize these states and using surfactants might be a good method to model and study β-structure-rich aggregation intermediates. Thirdly, the applicability of surfactants as a membrane-mimicking model system may be questioned, but they may still be a reasonable model for naturally occurring free lipids. Lipids were localized around and within \textit{in vivo} amyloid plaques [206; 207]. Additionally, lipids were reported to revert A\textsubscript{β\textsubscript{42}} aggregates to neurotoxic protofibrils [208] and are suggested to play a significant role in the aggregation pathway.

The use of SDS and LiDS is an excellent tool to induce different secondary structures in A\textsubscript{β} (see Chapter 2.3). In a monomeric peptide solution at physiological pH A\textsubscript{β} adopts a predominantly random coil-like structure [99; 100]. At submicellar surfactant concentrations β-structure is induced in the peptide, which is accompanied by a general loss of NMR signals [205]. In contrast, at concentrations above the cmc, A\textsubscript{β} forms an α-helical structure including residues 15–24 and 29–35 [94].

We found with the help of the fluorophore pyrene, which is commonly used to probe micelle-like structures [209; 210], that A\textsubscript{β} is involved in SDS concentration-dependent formation of hydrophobic clusters. Comparing these observations with CD results, it turned out that the appearance of hydrophobic micelle-like assemblies correlates well with the induction of a β-structure in A\textsubscript{β}.

ThT fluorescence kinetics experiments (see Chapter 4.2) evidenced that the β-structured state (as characterized by CD) is the most aggregation prone
state. In contrast, an $\alpha$-helical structure in $\text{A}_\beta$ at high SDS concentrations efficiently prevents fibril formation. Measuring the CD spectra of the aggregation end-points revealed two different $\beta$-structure minima at 222 and 215 nm for aggregated $\text{A}_\beta$ alone and in the presence of SDS, respectively. This indicates that the final $\beta$-structure arrangement for $\text{A}_\beta$-surfactant co-aggregates may be different from mature fibrils.

To gain some insight into the structure of $\text{A}_\beta$-surfactant co-aggregates we applied time-dependent SAXS experiments. At high SDS concentrations, \textit{i.e.} when $\text{A}_\beta$ adopts an $\alpha$-helix structure, the SAXS results are in agreement with previous NMR work \cite{94} and SAXS studies of protein-surfactant systems \cite{181,211,212} and showed that the peptide is attached to the SDS micelle surface. Scattering data of a $\beta$-structured sample (as characterized by CD) could be fitted to a model consisting of cylinders (with fitted radius and length $\geq 350$ Å) and spheres (with fixed radius of 37 Å). The initial fraction of globular objects (about 80% at the start) completely vanished during the measurement and only cylindrical shapes were present in the final state. The cylinder radius showed a slight time-dependent increase to a final value of ca. 32 Å. These results indicate an initial formation of globular co-aggregates that convert to fibrillar co-aggregates. Data from another sample with a slightly lower SDS concentration revealed that also other arrangements are possible where two co-aggregated fibrils are located side-by-side.

TEM images supported the findings from SAXS and ThT fluorescence kinetics experiments and showed fast fibril formation under $\beta$-structure inducing conditions. Both single fibril strands as well as fibrils arranged side-by-side were visible on the images. Also, the estimated diameters of 50-70 Å fitted well to the determined sizes by SAXS.

As mentioned previously, the induction of a $\beta$-structure state by SDS/LiDS is accompanied by a uniform loss of NMR cross-peaks. Thus, similarly as described for small organic molecules (Paper II and \cite{181}), conditions can be found that enable investigation of the exchange kinetics by NMR relaxation dispersion methods. During the CPMG measurements we observed significant signal loss due to aggregation. This aggregation kinetics is much slower than the exchange dynamics detected by CPMG experiments. Mainly residues in the hydrophobic parts of the peptide showed distinct relaxation dispersion profiles. But also residues R5 and D7 in the N-terminus exhibit exchange dynamics. A global fit yielded an exchange rate of 1100 $\pm$ 150 s$^{-1}$ where about 1.0 $\pm$ 0.1 % are bound in the co-aggregate. These results suggest a dynamic exchange between monomeric peptide and co-aggregate where the hydrophobic parts of $\text{A}_\beta$ bind to hydrophobic parts of the co-aggregate. An estimate of the size of the co-aggregate, assuming a spherical shape, indicated a hydrodynamic radius of 60 Å. Considering the slightly different conditions for NMR
and SAXS experiments and taking into account that the hydrodynamic radius measured by NMR is typically 30 % larger due to a hydration layer [213], the initial globular fraction of co-aggregates determined by SAXS coincides well with the co-aggregates characterized by NMR.

In summary, we show that Aβ and surfactant co-aggregate and form globular and fibrillar structures. Co-aggregated fibril formation and gradual conversion from globular to elongated structures occurs on a slow time scale of minutes-to-hours. In addition, we found that the globular co-aggregates are highly dynamic and exhibit exchange rates around 1100 s⁻¹. The structural preference of a β-structure in Aβ at intermediate surfactant concentrations greatly promotes aggregation. It is interesting to speculate whether the surfactant-induced aggregates could be a model system for in vivo neurotoxic oligomers where natural lipids are co-aggregates with Aβ.

5.3 Zinc ions (Paper IV)

The aim of Paper IV was to obtain more detailed knowledge about the molecular interaction of Aβ and zinc ions and its effect on the aggregation pathway. Metal ions, in particular zinc and copper, have been found to participate in and modulate Aβ aggregation and cytotoxicity (see Chapter 2.2.2 for a detailed discussion). Zn²⁺ has been reported to both inhibit and promote Aβ aggregation and its modulation effect is, thus, suggested to be concentration-dependent (reviewed in [51, 53, 54]). At high zinc ion concentrations formation of amorphous aggregates was presented [61, 62]. We found that at sub-stoichiometric concentrations Aβ fibril generation is efficiently retarded. ThT fluorescence kinetics experiments at different peptide concentrations were conducted to gain insights into the molecular mechanism of nucleation reactions (see Chapter 4.2). When plotting the aggregation half times against the Aβ concentrations on a double logarithmic scale, Aβ in the presence and absence of Zn²⁺ exhibited the same slope, which corresponds to the same half-time coefficient γ. Also, the half times plotted against the maximal growth rate cluster separately with and without zinc ions, but fall on the same line (on a double logarithmic scale). These results indicate that the basic aggregation mechanism determined by the nucleation reactions does not change in the presence of zinc ions.

Kinetics experiments at different sub-stoichiometric zinc ion concentration (with constant Aβ concentration) revealed an exponential dependence of the aggregation half time on the Zn²⁺ concentration. The end-point fluorescence intensities at different zinc ion concentrations reached about the same level, indicating that the amount of amyloid material is the same and only its formation is prolonged. In addition, these samples exhibited almost identical infrared spectra, which also suggest that the fibril morphology is similar.
Kinetic measurements with preseeded samples can be used to determine whether primary and/or secondary pathways dominate the aggregation mechanism \cite{169}. Preseeded samples reached their saturation phase within the lag time of unseeded samples, which points to that secondary nucleation reactions govern the aggregation behavior. In order to characterize which microscopic rate constants are affected, we performed a global fit analysis including all kinetic traces (see Chapter 4.2). A model including primary ($k_n$), secondary ($k_2$) and elongation ($k_+$) nucleation reactions with reaction orders of $n_c = n_2 = 2$ was applied (see Chapter 4.2). In addition, saturation of secondary nucleation, which is characterized by the Michaelis constant $K_M$, was included and a global fit with this model described well the kinetic profiles. This fit analysis delivers combined global fit parameters, $\sqrt{k_n k_+}$ & $\sqrt{k_+ k_2}$, which decreased by a factor of about 2 in the presence of Zn$^{2+}$, while the $K_M$ value remained basically unchanged. The effect of one single rate constant can be estimated by fixing one global fit parameter and allowing the other to vary across all zinc concentrations (see Chapter 4.2). This approach revealed that both $k_+$ and $k_2$ as single effective fit parameters result in equally good fits, while $k_n$ cannot account for the observed effect. To distinguish whether $k_+$ or $k_2$ is the primary cause for the inhibition effect, we performed highly preseeded kinetic experiments. The initial growth rate is then directly proportional to the elongation rate assuming that secondary nucleation is negligible under these conditions (see Chapter 4.2 and \cite{169}). We found that the initial rates obtained from highly preseeded experiments follow the same dependence on Zn$^{2+}$ concentration as revealed from the global fit analysis. Also, the fact that the fitted $K_M$ value is unchanged in the presence of Zn$^{2+}$ indicates that attachment of monomers and subsequent formation and release of aggregates are equally influenced. The fundamentally different nature of these processes suggests that $k_2$ is less likely to be affected by Zn$^{2+}$. Taken together, we concluded that in a simplistic model a primary reduction in fibril-end elongation could entirely explain the observed retardation effect on aggregation kinetics by the presence of zinc ions, which does not exclude an additional effect on secondary nucleation events.

To gain further insight into the molecular mechanism of Aβ-Zn$^{2+}$ interaction, we applied NMR, in particular diffusion and $^{15}$N-CPMG relaxation dispersion methods. Addition of zinc ions gave rise to a distinct NMR signal attenuation of $^1$H-$^{15}$N-HSQC cross-peaks in the N-terminus, as also reported previously \cite{64} \cite{65}. NMR diffusion experiments showed an increased translational diffusion coefficient in the presence of zinc ions, indicating that the Zn$^{2+}$ bound state is more compact. This result can be pictured by a Zn$^{2+}$ ion that is enclosed by the peptide’s N-terminus, resulting in a higher compactness of the zinc-bound state.
Applying NMR relaxation dispersion experiments revealed the presence of chemical exchange involving exclusively N-terminal residues, which is the reason for distinct NMR signal broadening. The experiments were repeated at different temperatures and a global fit showed that the exchange rate increases linearly with temperature from around 350 to 720 s\(^{-1}\) in a temperature interval of 278 to 290 K. The population of the bound state followed a non-linear temperature dependence and ranged between 6.7 to 3.5 \%. Comparing the here determined apparent dissociation constant, \(K_{D}^{app} \sim 210 \mu M\), with a previously reported value of \(K_D \sim 7 \mu M\) from fluorescence experiments [64], we assigned the observed exchange dynamics to folding of the N-terminus around the Zn\(^{2+}\) ion, rather than to Zn\(^{2+}\) binding alone, which occurs rapidly prior to the folding process.

The temperature-dependent population provides information about the thermodynamics of the zinc-bound folded state. To describe the non-linear character, we introduced a heat capacity, which accounts for an explicit temperature dependence of the enthalpy and entropy (see Chapter 1.1.1). The obtained thermodynamic parameters suggest that the zinc-bound folded state is favored by enthalpy, yet disfavored by entropy, and in total is only marginally stable. The heat capacity features a negative sign, indicating that less peptide surface is exposed to water, which is expected upon folding.

Taken together, in a reductionist model the reduction in elongation rate is assigned to be the primary cause of retardation of amyloid fibril generation. An effect on secondary nucleation may additionally be present. We found that the N-terminus of monomeric A\(_\beta\) encapsulates a Zn\(^{2+}\) ion forming a dynamic short-lived complex. The exchange dynamics are on a time scale of milliseconds. The fast exchange process could kinetically redirect A\(_\beta\) from aggregation. However, the low population of bound A\(_\beta\) suggests that the observed effect cannot entirely be assigned to a reduction of the available peptide pool for aggregation. Thus, this hypothesis would imply that A\(_\beta\) adopts less-aggregation prone states after Zn\(^{2+}\) release, \textit{i.e.} that Zn\(^{2+}\) modulates the re-configuration time, which has been suggested to be related to protein aggregation [214]. This mechanism should, however, equally affect all rate constants, which stands in contrast to the simplistic reduction of the elongation rate. An alternative mechanism where the fibril ends are modulated by a folded A\(_\beta\)–Zn\(^{2+}\) complex, may instead account for the specific effect on the elongation rate. The number of free ends is much smaller than the number of peptides, which can explain the large effect on aggregation kinetics already at low Zn\(^{2+}\) concentrations. With these findings Zn\(^{2+}\) at sub-stoichiometric amounts could be assigned to adopt the role of a minimal chaperone mimicking agent that retards A\(_\beta\) fibril formation.
Figure 5.1: Schematic mechanism of $\text{A}_{\beta40}$ aggregation modulation by small molecules and $\text{Zn}^{2+}$ ions - $\text{A}_{\beta40}$ aggregation follows a monomer-dependent secondary nucleation pathway which is affected by the presence of aggregation modulators. The small organic molecules lacmoid & Congo red, the surfactants SDS & LiDS and the $\text{Zn}^{2+}$ ion form dynamic complexes with $\text{A}_{\beta40}$, which are in fast exchange with monomeric peptide ($k_{\text{ex}} \sim ms^{-1}$). This exchange process, the structural preference of $\text{A}_{\beta40}$ in the presence of aggregation modulating compounds as well as interactions of these modulators with fibrillar and pre-fibrillar species may promote or inhibit/retard fibril formation. In a reductionist model $\text{Zn}^{2+}$ reduced the fibril-end elongation rate by modulation of the fibril ends by an $\text{A}_{\beta}-\text{Zn}^{2+}$ complex.
Advances in the understanding of the underlying molecular mechanism of Aβ self-assembly has been a central goal of this thesis. Furthermore, my efforts have been directed into deepening the knowledge about the modulated aggregation behavior in the presence of various compounds and agents, which may be considered as models for environmental conditions (e.g. metal ions, natural lipids, etc.) and therapeutics. This chapter recapitulates the main conclusions from my thesis with the aim to put them into perspective.

A combined approach using different biophysical techniques in this thesis has provided deeper insight into the kinetics and dynamics of Aβ behavior in vitro. We found that Aβ exhibits dynamic exchange when transiently binding to aggregation modulators or co-aggregates thereof. The life-time of these dynamic complexes lies in the order of \( \sim 1 \text{ ms} \). Despite the similar exchange dynamics, Aβ’s aggregation behavior is modulated differently in the presence of the studied modulators. On the one hand, lacmoid greatly inhibits generation of amyloid fibrils and zinc ions efficiently retard fibril formation. On the other hand, Congo red and surfactants at intermediate concentrations (below the cmc) promote formation of a \( \beta \)-structure in Aβ, which in turn promotes fibril formation. In particular, in the presence of SDS at sub-cmc concentrations fibril generation is extensively accelerated leading to fibrils in which Aβ and SDS molecules are co-located. The molecular mechanism of interaction between Aβ and various compounds and their distinctive modulation effect on the aggregation behavior of the peptide is summarized in Figure 5.1.

Notably, lacmoid and Congo red form colloidal structures by themselves, similarly to surfactants, and several other aggregation modulators have been suggested to possess this property [184]. The observed exchange dynamics was assigned to transient binding of monomeric peptide to larger co-aggregates. A similar exchange process was found in the presence of surfactants at sub-cmc concentration. This behavior seems to be a feature shared with other amyloidogenic proteins. In line with that, \( \alpha \)SN was found to exhibit dynamic exchange in the presence of Congo red [181]. In contrast to exchange of monomeric Aβ with larger co-aggregates, the dynamics observed in the presence of Zn\(^{2+} \) apparently stems from interactions with solely monomeric peptide. Here, only the N-terminus is involved in dynamic exchange, which may be assigned to a folding process where the zinc ion is being enclosed by the N-terminus.
Interestingly, also Aβ in the absence of additional compounds was found to undergo transient binding to prefibrillar aggregates, although on a slower timescale with an exchange rate in the order of $\sim 70 \text{ s}^{-1}$ \cite{156,204}. Hence, the faster exchange dynamics in the presence of aggregation modulators may interfere with this slower dynamics of Aβ alone. The self-assembly process may be kinetically modulated when the reconfiguration time of the peptide is significantly altered, which has been suggested to be linked to aggregation kinetics \cite{214}.

Importantly, despite the similar exchange dynamics, the observed modulation of the aggregation behavior is evidently different comparing the investigated compounds. Thus, the structural preference of Aβ in the presence of the modulator, may largely determine the aggregation behavior. Promotion of secondary structure (propensity) in Aβ by the compounds should significantly contribute to the modulation effect on aggregation, where the $\beta$-structured state has the highest aggregation propensity and formation of $\beta$-structured co-aggregates typically accelerates aggregation.

Furthermore, interactions of modulators with fibrillar and pre-fibrillar species are very likely to be decisive determinants of the aggregation behavior. The number of fibrils and fibril ends is usually much lower than the number of monomeric peptides, which can explain large effects on aggregation kinetics at already low modulator concentrations. Specific modulation of the fibril ends by either strong binding to the $\beta$-structured end peptide or perturbation of the folding process into a $\beta$-structure may, for instance, cause an efficient reduction in the fibril-end elongation rate.

Taken together, several mechanisms and interactions on different levels are present in an Aβ–modulator system, which may contribute, individually or combined, to the modulation of Aβ’s aggregation. Which mechanism(s) is (are) the decisive one(s) may be dependent on the specific modulator system.

How these findings in the reductionist \textit{in vitro}-systems translate to the complex environment of a living system remains to be investigated. Nevertheless, also \textit{in vivo}, peptide aggregation is governed by the underlying microscopic kinetics and dynamics and the thermodynamic drift toward a low free energy state.

För att komma detta mål lite närmare, undersökte vi effekten av olika ämnen på Aβ och dess aggregeringsbeteende och använde t.ex. små organiska molekyler (med de två exemplen Lacmoid och Kongo rött), tensider (tvättmedel), och zinkjoner. Dessa ämnen kan påverka aggregering genom att påskynda, fördröja eller ändra riktning på processen, vilket kan förhindra skador på nervceller. För att studera samspelet i detalj använde vi olika optiska och magnetiska spektroskopimetoder. Med kärnmagnetisk resonansspektroskopi (NMR – från engelskans "Nuclear Magnetic Resonance") är det möjligt att undersöka molekylära processer med atomär upplösning, vilket ger en mycket detaljerad förståelse av processer.

Våra studier visade att Lacmoid förhindrar bildning av ett β-flak i Aβ. β-Flaket är den grundläggande byggstenen i Alzheimersfibrillen, vilket leder till att Lacmoid förhindrar bildning av fibriller. Kongo rött, liksom tensider vid en viss koncentration, påskyndar i motsats bildningen av β-flak, som för tensider starkt främjar aggregering. Med hjälp av speciella NMR-metoder kom vi fram till att i närvaro av alla dessa ämnen bildas små aggregat som består av dessa ämnen och Aβ peptider. Aβ peptider binder en kort stund till aggregatet och
lossnar sedan igen. Denna dynamiska process är mycket snabb och i genomsnitt är en peptid bunden till aggregatet i cirka en millisekund. Samtidigt är bara en liten del, dvs. några procent, av Aβ molekylerna bundna.


Sammanfattningsvis kan vi konstatera att Alzheimersfibrillbildning bestäms av dynamiska och kinetiska processer på molekylär nivå. Dessaprocesser kan påverkas av olika ämnen och en omfattande kunskap med detaljerad förståelse av dessa processer är ytterst viktigt för att förstå alla sjukdomar som orsakas av proteinfelveckning, inklusive Alzheimers sjukdom.

Unsere Untersuchungen zeigten, dass Lacmoid die Formation eines β-Faltblattes in Aβ hemmt. Das β-Faltblatt ist der Grundbaustein einer Alzheimer-Fibrille und Lacmoid verhindert somit deren Bildung. Kongorot, ebenso wie Tenside bei einer bestimmten Konzentration, begünstigen dagegen die Formation eines β-Faltblattes, was besonders im Falle von Tensiden die Aggregation stark fördert. Mit speziellen NMR-Methoden fanden wir heraus, dass sich in der Gegenwart all dieser Stoffe kleine Aggregate, bestehend aus diesen Stoffen und Aβ Peptiden, bilden und sich Aβ Peptide daran kurzzeitig anlagern und wieder lösen. Dieser dynamische Vorgang des Bindens und Lösens ist sehr schnell und im Mittel ist ein Peptid, welches diesen Prozess durchläuft, nur für circa 1 Millisekunde an dem Aggregat gebunden. Gleichzeitig ist immer nur ein kleiner Bruchteil, d.h. wenige Prozent, der Aβ Molekülen gebunden.

Auch in der Gegenwart von Zinkionen ist ein dynamischer Austauschprozess beobachtbar. Allerdings sind hier keine Aggregate involviert, sondern der N-Terminus (das Peptidende mit einer Aminogruppe, $-\text{NH}_2$) von Aβ legt sich (faltet sich) kurzzeitig um ein Zinkion. Die Faltung ist nur marginal stabil und die Verweildauer in diesem gefalteten Zustand bewegt sich abhängig von der Temperatur in der Größenordnung 1 bis 3 Millisekunden. Wir fanden heraus, dass Zinkionen die Fibrillenbildung verlangsamen speziell für Zinkionenkonzentrationen kleiner als die Peptidkonzentration. Wir konnten den Aggregationsverlauf mit Hilfe eines bestimmten Farbstoffes genau charakterisieren. Wenn dieser Farbstoff sich an Fibrillen anlagert, fluoresziert er (d.h. er leuchtet, wenn man ihn mit dem Licht einer bestimmten Wellenlänge bestrahlt). Wir stellten fest, dass der Anlagerungsprozess von freien Peptiden an die Fibrillenenden von Zinkionen stark beeinflusst wird und sich dadurch die Bildung von Fibrillen verlangsamt.

I would like to warmly acknowledge a number of persons I have had the great pleasure to work with and with whose support this thesis was brought into existence. This includes many inspiring co-workers, fantastic colleagues and friends as well as my family. In particular, I would like to thank:

**Astrid Gräslund**, for supervising me through the past years with fruitful inspiration and encouraging support. I greatly appreciate your scientific optimism and your sight of the overall picture.

**Jens Danielsson**, for teaching and greatly supporting me with experimental design, set-up and analysis, but also for conveying your attitude to always analyzing the underlying mechanisms in greatest possible detail, which has been very influential for our projects.

**Lena Mäler and Andreas Barth**, for most valuable advice, criticism and discussions as well as teaching me NMR relaxation and infrared, respectively.

**Christofer Lendel**, for introducing me to small molecule aggregation modulators and co-supervising my first project with great motivation.

**Jüri Jarvet**, for your wise advice and helpful discussions as well as being a great teacher for NMR and CD spectroscopy.

**Torbjörn and Haidi Astlind as well as Britt-Marie Olsson**, for your fantastic technical and administrative support!

**All my co-workers participating in my projects**, for your contributions and efforts to the studies and introducing me to new methodology.

**My present and past colleagues in the NMR biophysics group, Johannes, Jobst, Weihua, Pontus, Scarlett, Sofia, Fatemeh, Anna, Göran, Sebastian, Ann, Maurizio, Chenge, Nadja, Marta, Lisa, Xin, Sarah, and others**, for your amazing help and support with all experimental set-up, tricky analysis, assistance in the lab as well as discussions and debates about plenty of scientific and less scientific issues. With you the atmosphere has always been wonderful and it has been a pleasure and great fun to spend my time with you!

**My friends and, in particular, my family**, who have always helped and greatly supported with all issues and decisions around and outside work. I am extremely glad that you have been there!
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