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Jon Pallbo Arvidsson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Proteins constitute a major component of living cells and are essential to their function. Sometimes proteins misfold or aggregate in abnormal ways, which can give rise to diseases. Amyloids are a special type of highly ordered protein aggregates made of long intermolecular β-sheets stacked into fibrils. Formation of amyloids is associated with many diseases, especially diseases related to neurodegeneration such as Alzheimer's and Parkinson's disease. That is one major reasons for why there is a strong research interest in amyloids. The physiological environment, and especially neural tissue, is also very rich in lipids. Therefore, it is of special interest to understand more about how amyloids behave in the presence of lipids. For example, to what degree do amyloid forming proteins and peptides co-aggregate with lipids? How does the presence of lipids affect the kinetics of amyloid formation? Can amyloids have disruptive effects on lipid structures such as membranes? In this thesis a short amyloid forming peptide, known as NACore, has been used as a model peptide to study amyloid formation and amyloid-lipid interaction. NACore is an 11 amino acid residue peptide fragment from the protein α-synuclein, which forms amyloid fibrils in Parkinson's disease. More specifically, NACore spans from residue 68 to 78 of α-synuclein and has the sequence GAVVTGVTAVA. It is part of the so-called non-amyloid-β-component of α-synuclein, which is the central region of the protein. To investigate amyloid-lipid interaction, the peptide was studied in the presence of phospholipid vesicles and a fatty acid. We found that NACore readily forms amyloid fibrils *in vitro* and that the process can be controlled using pH. In terms of lipid interaction, we found that NACore and phospholipids co-aggregate on the aggregate level but remain mostly segregated on molecular length scales. The presence of lipids can substantially inhibit the fibrillation of the peptide. That was especially the case in the presence of the fatty acid linoleic acid, where even a very low lipid concentration resulted in substantial inhibition. Co-aggregation of amyloids and lipids could be important in mechanisms of toxicity to cells. For example, trapping of lipid membranes in amyloid fibril networks might disturb cellular processes such as vesicle trafficking. Clarifying the effects of lipids on the kinetics of amyloid formation might help us to better understand how amyloid formation is normally kept under control in the physiological environment. Our results obtained with this model peptide can also shed light on commonalities and differences between different amyloid forming proteins and peptides. Furthermore, they contribute to better understanding of biomolecular self-assembly in general.

Key words
amyloids, peptides, lipids, NACore, aggregation, fibrillation, crystallization, self-assembly, interaction

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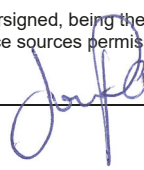
Signature  Date 2020-09-10

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Table of contents

Populärvetenskaplig sammanfattning

Proteiner är inte bara ett näringsämne i mat. De är biologiska makromolekyler som utgör en essentiell del av allt liv vi känner till. Exempelvis styr de många biokemiska processer genom att snabba på vissa reaktioner mellan olika molekyler, och de utgör ett viktigt byggmaterial i och utanför celler såsom i cellskelett och i bindväv. För att allt ska fungera är det viktigt att proteiner inte veckar sig fel och inte klumpar ihop sig med varandra på onormala sätt. Om det händer kan det leda till olika sjukdomstillstånd. Det finns en särskild typ av proteinklumpar som kallas amyloider. De utgörs av mikroskopiska trådar som kan ansamlas i eller utanför celler. Amyloider förekommer exempelvis i samband med sjukdomar som bryter ner hjärnan, såsom Alzheimers och Parkinsons sjukdom. I takt med att folk lever längre blir det viktigare att förstå dessa sjukdomar, eftersom de ofta är kopplade till hög ålder. Den här avhandlingen är ett bidrag till den grundforskning som försöker förstå amyloidbildning och dess möjliga samband med olika sjukdomstillstånd. Snarare än att studera amyloidbildning i levande celler har jag framförallt studerat ett kort proteinfragment, en peptid, i relativt enkla provlösningar som ett modellsystem för amyloider. På det viset kan beteenden och mekanismer lättare förstås från ett fysikaliskt perspektiv. Peptiden är ett 11 aminosyror långt fragment av proteinet alfa-synuklein, som är involverat i Parkinsons sjukdom.

Proteiner är inte den enda typen av biomolekyler. En annan stor grupp är olika typer av fettmolekyler, så kallade lipider, som till exempel bildar membran som omger alla levande celler. I exempelvis nervsystemet är lipider en viktig del i så kallade axoner, dendriter och synapser som utgör grunden för all nervaktivitet. Man tror att bildandet av amyloider, liksom bildandet av lipidstrukturer, till stor del drivs av att undvika kontakt med vatten. Därför är det också sannolikt att amyloider och lipider kan samaggregera med varandra, vilket redan har observerats experimentellt i vissa fall. Samaggregering eller andra processer som stör normala lipidstrukturer är en möjlig mekanism som kan förklara hur bildandet av amyloider kan leda till sjukdomstillstånd. I den här avhandlingen har jag fokuserat just på växelverkan mellan amyloider och lipider.

Sammanfattningsvis kan man säga att provlösningens pH, hur sur eller basisk den är, visade sig vara användbart för att styra modellpeptidens amyloidbildning.

Amyloidtrådarna som bildas av peptiden kan beskrivas som små och väldigt avlånga kristaller. Vad gäller växelverkan med lipider så fann vi att lipiderna har en stark tendens att fastna på amyloidtrådarna, och därmed att de bildar en sorts samaggregat på stora längdskalor. På små längdskalor, i storleksordningen några få nanometer, så verkar det dock som att majoriteten av amyloidtrådarna och lipiderna fortfarande behåller sina normala separata strukturer, i alla fall när lipiderna är så kallade fosfolipider. Vi fann också att närvaron av lipider kraftigt kan hämma modellpeptidens amyloidbildning, även med väldigt små mängder lipid. Att lipider fastnar på amyloidtrådarna kan i sig vara en mekanism som leder till skada på celler, eftersom det exempelvis kan störa det normala flödet och bildandet av olika lipidstrukturer i cellerna. Genom att jämföra resultaten från modellsystemet jag har studerat med resultat för andra proteiner och peptider så kan likheter och skillnader mellan olika amyloidsystem identifieras. Det kan till exempel hjälpa framtida forskare att veta vilka aspekter som är särskilt värdefulla att fokusera på för att förstå just de säregenskaper och mekanismer som karakteriserar en viss typ av amyloidbildning.

List of papers

I. Aggregation behavior of the amyloid model peptide NACore
Jon Pallbo, Emma Sparr, Ulf Olsson
Quarterly Reviews of Biophysics, 52:e4. 2019.

II. Co-aggregation of NACore amyloid fibrils and phospholipids
Jon Pallbo, Masayuki Imai, Yuka Sakuma, Ulf Olsson, Emma Sparr
Manuscript

III. NACore amyloid formation in the presence of phospholipids
Jon Pallbo, Masayuki Imai, Luigi Gentile, Shin-ichi Takata, Ulf Olsson, Emma Sparr
Submitted to Frontiers in Physiology - Membrane Physiology and Membrane Biophysics

IV. Potent inhibition of NACore amyloid formation by a fatty acid
Jon Pallbo, Ulf Olsson, Emma Sparr
Manuscript

V. Ganglioside lipids accelerate α-synuclein amyloid formation
Ricardo Gaspar, Jon Pallbo, Ulrich Weininger, Sara Linse, Emma Sparr
Biochimica et Biophysica Acta - Proteins and Proteomics, 1866:1062-1072. 2018.

Not included in the thesis:

Controlling amyloid fibril formation by partial stirring
Fredrik G. Bäcklund, Jon Pallbo, Niclas Solin
Biopolymers, 105:249–259. 2016.

Contribution to papers

I. I designed the study together with the co-authors. I performed the experiments. I analyzed the data with input from the co-authors. I wrote the manuscript with contributions and input from the co-authors.

II. I designed the study together with UO and ES. I performed the experiments. I analyzed the data with input from co-authors. I wrote the manuscript with contributions and input from co-authors.

III. I designed the study together with UO and ES. I performed the SANS experiments together with MI, LG, and ST, and performed the other experiments by myself. I analyzed the data with input from co-authors. I wrote the manuscript with contributions and input from co-authors.

IV. I designed the study together with the co-authors. I performed the experiments. I analyzed the data with input from the co-authors. I wrote the manuscript with contributions and input from the co-authors.

V. I performed and analyzed the CD measurements. I took part in discussions about the study and during the writing of the manuscript, and I contributed to the writing of the manuscript.

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The Division of Physical Chemistry is a very international and dynamic place to be in, and I have met a lot of different people. From the time I started as a PhD student a bit more than four years ago, almost the entire population of junior staff (PhD students and postdocs) has been turned over. There have also been countless guests from all over the world, staying for a few days, weeks, or months. Furthermore, the division has a lot of shared environments such as the coffee and lunch room, as well as in the form of labs and equipment. I have very much enjoyed my stay during these years. I have been part of two somewhat overlapping research groups, one for each of my supervisors. I would like to thank the people in both. Some have coincided with my stay for a long time, such as Dat, Enamul, Axel, Marija, Veronica, Kasia and Simon. Others I have got to know a bit more recently, such as Xiaoyan, Maria G, Viktoriia, Erika, and Birte. I thank them for discussions, social company, and other activities. I would like to especially thank the people in the "lipid group meeting" and in the "amyloid journal club" for interesting presentations and discussions.

I am particularly grateful for having been given the opportunity to stay in Japan on three occasions during these years. It has been a big source of inspiration for me on many levels. During the last visit, in 2020, I (luckily?) managed to go back to Sweden in early March, shortly before most international travel was closed down due to the COVID-19 pandemic that is still ongoing. I would like to thank my main host at Tohoku University in Japan, Masayuki Imai, as well as the other people in the lab there. I would also like to especially acknowledge some of the Japanese people I have met: Kazuki, Ippei, Akiko, Yoshi, and Yoshimi. I have had a chance to meet all of them both in Sweden and in Japan. They made my stay there even more interesting and enjoyable.

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Finally, I would like to thank my parents, family, and the rest of my relatives for my existence and for supporting me during my entire upbringing.

Prologue

In the work for this thesis a short model peptide (NACore) has been used to study the phenomenon of amyloid formation and amyloid-lipid interaction. The aim has been to contribute to physicochemical knowledge and understanding of amyloids. Below is a summary of the main findings in four short points.

1. The peptide readily forms amyloid fibrils *in vitro* and the fibrillation can be controlled by pH.

2. The fibrillation of the peptide is a crystallization process rather than just aggregation.

3. The amyloid fibrils and phospholipids co-aggregate on the aggregate level but remain mostly segregated on molecular length scales.

4. The presence of even very small amounts of lipids can substantially inhibit the fibrillation of the peptide.

1. Introduction

Amyloids are a very popular research topic nowadays. Much work is focused on the medical and clinical aspects of amyloids because they are associated with many diseases [1,2]. The work for this thesis is not directly applicable in that context. Rather, it is focused on the underlying physical chemistry of amyloids. There are a lot of things we still do not understand about this topic due to the complexity of biology and physiology. The use of model systems, such as a short peptide and simple model lipids as in the work for this thesis, is a tool to provide a better understanding of the amyloid phenomenon. That is because some of the complications in real biological systems are avoided. Once the model systems have been understood, more complexities can be added in a gradual manner until a complete picture has been reached.

Below, some of the background topics for this thesis will be introduced. In chapter 2 the main experimental methods used will be described, and in chapter 3 the main experimental results will be summarized and discussed.

1.1 Proteins and peptides

A typical protein is a small particle with a diameter of a few nanometers. A human cell contains of the order of 10^{10} protein particles [3], roughly the same as the number of people on Earth. A typical bacterium, which is much smaller than a human cell, contains a few million protein particles [3]. The activities of the proteins direct many of the physical and chemical processes of life. From a more chemical perspective, proteins are biopolymers made of peptide backbones on which side groups of various chemical compositions are attached. The units along the backbones are called amino acid residues because they are formed by joining amino acids together. In total there are 20 amino acid from which natural proteins are typically created [4,5]. Which kinds of proteins, as well as where and when they are synthesized, is determined by the genetic code stored in DNA. For protein synthesis to occur, a region of DNA is first transcribed to RNA in the form of so-called messenger RNA (mRNA). The mRNA then comes into contact with a ribosome, which translates it into a string of amino acid residues. As the amino acids are stitched together, the protein is formed [5].

When a protein is present in an aqueous environment such as inside a cell, it usually folds up into a compact shape, due primarily to hydrophobic interactions [6]. However, in the last decades some proteins have been found to remain partially or fully disordered in solution. These are so-called intrinsically disordered proteins [7]. The detailed chemical structure of the side groups along the peptide backbone of a protein determines how it folds, because the side groups of the protein introduce various geometric and chemical constraints. There are only some folds for which various hydrophobic patches and other features are matched up in a thermodynamically stable way [6]. Inside a protein, two kinds of local protein structures are very common. These are α-helices and β-strands. In between these structures the peptide backbone typically adopts more disordered structures. Several β-strands can be stacked next to each other to form β-sheets. The sequence of α-helices, β-strands, and other local folds along the peptide backbone is called the secondary structure of a protein. The overall 3D fold of a protein is called the tertiary structure, whereas the sequence of amino acid residues that makes up the protein is called the primary structure [4,5] (Figure 1.1). The structure and chemical nature of proteins are what give them their properties in the biological environment. In the case of enzymes, which facilitate chemical reactions through catalysis, a so-called active site with special geometric and chemical features has evolved through natural selection to promote a certain chemical reaction [4,5]. Other proteins do not promote chemical reactions, but act as structural components of cells, or for example take part in signaling pathways. If the proteins for some reason do not fold properly, or aggregate with each other in an abnormal way, the biological function might be compromised, and this can lead to diseases in the organism [1].

The distinction between proteins and peptides is rather fuzzy. One way to define them is simply by the length of the polymer, where proteins (also referred to as polypeptides) are very long peptides, although no clear cutoff size exists [8]. For this thesis, a short 11 amino acid residue peptide has been used in the experiments. This peptide is named NACore [9,10] and it is a fragment of the so-called non-amyloid-β component (NAC) of the protein α-synuclein [11,12]. α-Synuclein is normally found in neural tissues. The normal function of this protein is still not fully understood, but it is believed to be involved in synaptic membrane functions in neurons [13]. There are several variants of synucleins (α-, β-, and γ-) and this group of proteins was initially named in 1988, when it was found co-localized with the nucleus in neural cells [13-15]. In Parkinson's disease, special aggregates of α-synuclein are found in deposits called Lewy bodies inside neurons [16-18]. The NAC fragment of α-synuclein can also be found aggregated in patients with Alzheimer's disease, together with other proteins called amyloid-β and tau [13]. That is why it is called "NAC".

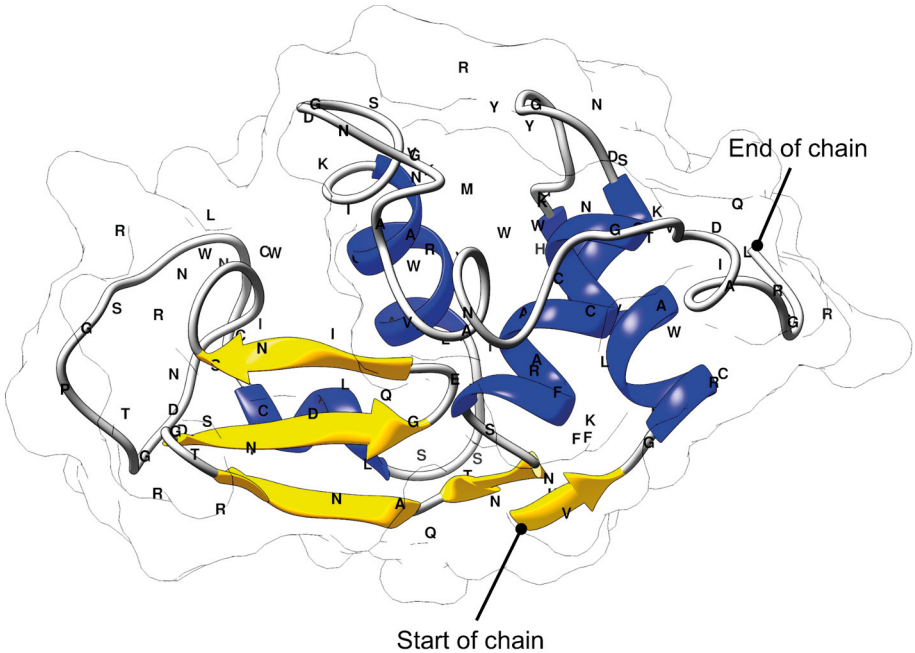

Figure 1.1. Example of a protein structure. The figure shows the structure of the enzyme lysozyme, which was one of the earliest protein structures to be determined in detail. The primary structure is the sequence of amino acid residues along the peptide backbone, represented by letters. The secondary structure is the distribution of local folds such as α-helices (blue coils) and β-sheets made of β-strands (yellow arrows). The tertiary structure is the overall 3D shape of the protein. PDB ID: 2LYZ, rendered with UCSF Chimera [19].

1.2 Amyloids

Amyloids are made up of very long inter-molecular β-sheets that are stacked together, giving rise to fibrils [1,2,20] (Figure 1.2). Both full-length proteins and short peptides can form such fibrils. The fibrils are typically of the order of 10 nm in width and often extend for several μm but can in principle be indefinitely long [1,2,20]. Amyloid formation in fact seems to be a rather ubiquitous feature of proteins. It is not just a few proteins that can form such structures. Many or even most proteins and peptides might be able to do so, even if they are unrelated to diseases [1,2]. In many ways amyloid formation resembles crystallization. Many proteins are known to be able to crystallize in their native fold [21]. That is a key fact that has been used historically to determine protein structure through X-ray crystallography [21,22]. A useful way of viewing amyloids is perhaps as a crystalline state of a non-native fold of proteins and peptides in which each peptide backbone is folded in a plane, rather than in the normal tertiary structure. However, amyloids are usually fibrillar and twisted, rather than extending indefinitely in all directions. Whether that is the true thermodynamically most

stable state, or if an extensive 3D crystalline state might be more favorable but kinetically inaccessible, is an open question. It has been proposed that an extensive 3D crystalline state is in fact the most stable structure [23,24]. This consideration becomes especially relevant in amyloid formation by short peptides, where it has been experimentally observed that the same peptide can form both fibrils and more extensive 3D crystals, with similar molecular packing [24-27]. Analogously, some non-peptide molecules are also known to readily form fibrillar crystals rather than extensive 3D crystals [28,29].

During the 1930s William Astbury (1898-1961) et al. did X-ray scattering experiments on denatured proteins [30-32]. Various types of structures were classified into so-called α- and β- forms [22], which are due to the content of α-helix and β-sheet structures in the proteins, clarified by Linus Pauling et al. in the 1950s [33,34]. Of particular interest in the context of amyloids, Astbury et al. did X-ray scattering on denatured egg albumin for which a structure that would become known as cross-β was inferred [30,35,36]. These experiments could perhaps be considered the birth of the biophysical branch of the amyloid field because the cross-β structure is the structure of amyloids (Figure 1.2). Parallel to the biophysical branch, diseases in which deposits referred to as “amyloid” were found in the human body had been describes already in the 1800s [37]. These deposits were named “amyloid” because they were originally believed to be carbohydrate-like in their chemical nature, but soon they were found to primarily be deposits of aggregated proteins rather than carbohydrates [37,38]. This can be considered the medical or clinical branch of the amyloid field. A connection was formed between the biophysical and clinical branches in the 1950s due to electron microscopy observations that amyloid deposits in diseased tissues are made of bundles of fibrils [39-42]. Based on X-ray scattering, these fibrils were identified to have the cross-β structure [35,36,43-47]. In addition to so-called systemic amyloidosis, which are diseases where amyloid deposits are found in tissues throughout the body [2], connections were also made with neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease, where amyloid deposits can be found in the brain. For example, tentative links were developed between α-synuclein, Parkinson’s disease, and amyloid fibrils [14,15,48-50]. Similarities between amyloids and prions were also noted [51]. The presence of proteins misfolded and aggregated into amyloid fibrils might lead to an auto-catalytic cascade. More and more protein of the same type is promoted into adopting the amyloid structure by the aggregates already present in the diseased tissue. That is one hypothesis for how diseases such as Alzheimer’s and Parkinson’s spread through the brain, once initiated [52].

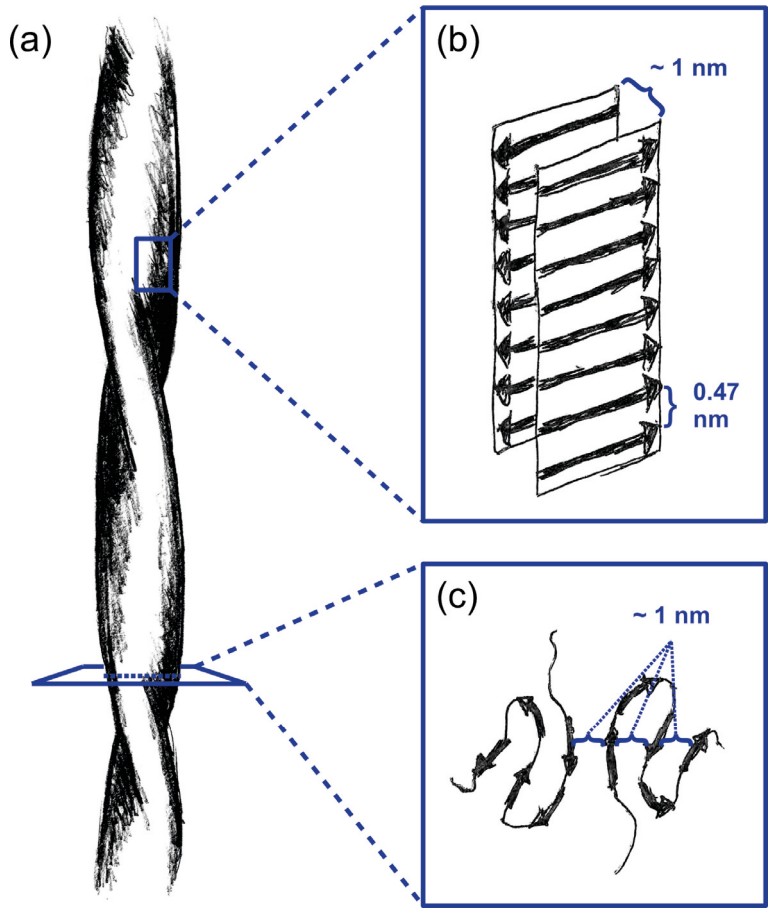

Figure 1.2. Schematic illustration of the typical structure of an amyloid fibril. (a) Amyloid fibrils are typically of the order of 10 nm wide and often extend for several μm. They are usually twisted. (b) Illustration of cross-β structure, which is the defining feature of amyloids. Several long intermolecular β-sheets are stacked next to each other. The structure has two characteristic periodicities. One is the separation between β-strands within each β-sheet and is about 0.47 nm. The other one is the separation between stacked β-sheets and is typically about 1 nm. These periodicities give rise to corresponding diffraction peaks in X-ray scattering. The illustration shows parallel β-sheets, but they could in principle also be anti-parallel. (c) Cross-sectional view of the fibril. In the case of long proteins, the peptide backbone is typically folded in a complex shape in the plane perpendicular to the fibril axis. This also further illustrates the origin of the roughly 1 nm separation between β-sheets.

One prominent research group in the field of amyloid formation by short peptides has been David Eisenberg et al. They have studied crystallization of small amyloidogenic peptides and coined the term “steric-zipper” to describe the structural principle by which amyloids are stabilized [26,27]. The basic feature of a steric-zipper is interdigitation of the side groups of two β-sheets running next to each other. To form a stable amyloid structure, it is necessary that certain geometric constrains are fulfilled. Eisenberg et al. classified amyloid structures formed by short peptides into various categories, depending on their symmetries [20,27]. For example, the β-strands in the β-sheets that make up the amyloids

might be either parallel or anti-parallel, and the orientation of the β-sheets facing each other can also vary [20]. The NACore peptide used for the work in this thesis has been previously characterized to some extent in 2001 in an *in vitro* cellular system [9], and later by the Eisenberg et al. group in 2015 when they determined a 3D crystal structure of the peptide [10].

Another very influential research group in the amyloid field has been Christopher Dobson et al. This group for example highlighted the idea that perhaps most proteins, and not only a few disease-associated ones, can form amyloids [53]. They have also been influential in the proposal that it is oligomeric intermediates during the amyloid formation process that are the primary toxic species for cells, rather than the final mature fibrils [54]. Still, some open questions exist on this issue. The mechanism by which amyloids might lead to neurotoxicity, and thereby play a key role in the associated diseases is still poorly understood. Several lines of thought exist [1]. Some have focused on how amyloids might lead to oxidative stress [55]. Others have focused on if and how interactions with lipid membranes could be a mechanism of toxicity [56,57]. Yet another line of thought is that amyloid formation might be a consequence rather than a cause of disease [58]. In this thesis the main line of investigation is how amyloids interact with lipids and lipid membranes.

1.3 The NACore peptide

As mentioned above, the NACore peptide is a fragment from the protein α-synuclein, which is an intrinsically disordered protein involved in Parkinson's disease. More specifically, the fragment spans from residue 68 to 78 in the protein and has the sequence GAVVTGVTAVA (Figure 1.3). This is part of the NAC region, which is a mostly hydrophobic stretch of amino acid residues in the center of α-synuclein and makes up the central part of α-synuclein amyloid fibrils. In all experiments for this thesis, an uncapped form of the peptide has been used, meaning that the peptide had free amino ($-NH_2/-NH_3^+$) and carboxyl ($-COOH/-COO^-$) ends. These are the so-called N- and C- terminals (or termini) of the peptide, and their charge depends on the pH of the sample (see chapter 3). Before the work for this thesis started, some things were already known about this peptide. In 2001 Bodles et al. [9] did a series of experiments with various fragments of the NAC region with the aim of identifying minimal fragments that still retained properties such as the ability to fibrillate and toxicity to cells. The NACore peptide fragment, referred to as NAC(8-18) in that study, showed an ability to form fibrils as seen by negative stain transmission electron microscopy after incubation in water for several days at 37 °C. It also showed some toxicity to cells *in vitro* based on a diphenyltetrazolium bromide (MTT) reduction assay. Based on circular dichroism spectroscopy, they also reported that the peptide had

a disordered structure in aqueous buffer, but that it adopted increasing amounts of β-sheet structure upon addition of the organic solvent acetonitrile, known to promote β-sheet formation [9].

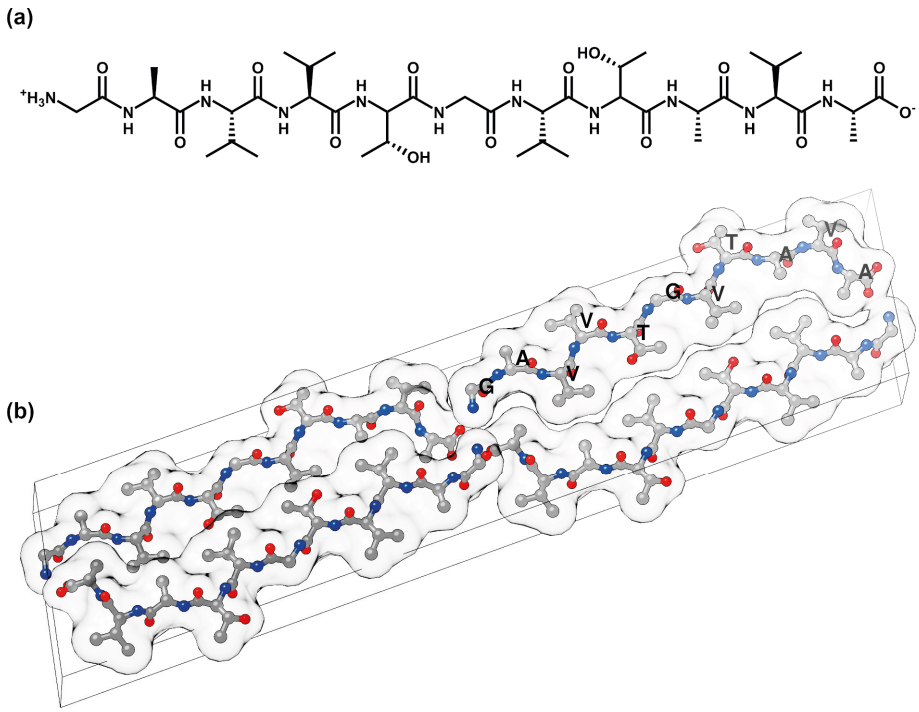

Figure 1.3. The NACore peptide. (a) Chemical structure of the NACore peptide, which has the primary sequence GAVVTGVTAVA. (b) The packing of the NACore peptide in the crystal unit cell determined by Rodriguez et al. in 2015 [10]. PDB ID: 4RIL, rendered with UCSF Chimera [19].

In 2015, Rodriguez et al. in the Eisenberg group coined the name "NACore" for the peptide and confirmed its ability to form fibrils as well as its cytotoxicity *in vitro* [10]. In addition, they could find small 3D crystals of NACore in samples of the peptide dispersed in water (invisible by normal light microscopy). By using electron diffraction, they determined the structure of these crystals with atomic resolution (Figure 1.3b). That NACore crystal structure was then used to propose a molecular structure for amyloid fibrils of full-length α-synuclein, which was unknown at the time. However, soon after that, reports of the structure of full length α-synuclein amyloid fibrils based on nuclear magnetic resonance (NMR) and cryo-TEM appeared, showing that the structure is rather different, and also that it exhibits polymorphism [59-62]. There is perhaps no fundamental difference between fibrils and small crystals of the NACore peptide. Rather, it could be a matter of aspect ratio, which could depend on kinetic factors during

the formation process such as the growth rate of the various crystal faces. As was mentioned in the previous section, fibrillar or "needle-like" crystals are quite common also for non-peptide molecules. In Paper I of this thesis we studied NACore using pH as a tool to control its aggregation and fibrillation. That served to further establish NACore as a useful amyloid model peptide. Following that, we investigated its interaction with lipids in Paper II-IV.

1.4 Lipids and cell membranes

Although proteins play a very prominent role in the biological environment, they are not the only type of biomolecules present. In addition to countless small molecules, there are other macromolecules such as nucleic acids and carbohydrates. Lipids are another very prominent type of biomolecules [3]. One of the major roles of lipids in cells is to make up the basis of the cell membranes in the form of lipid bilayers. These are the "skins" of the cells and are present both on the outer surface and internally (in eukaryotes), where they are part of organelles. These bilayers are primarily made of lipids known as phospholipids, which typically have a polar headgroup on which two hydrocarbon chains are attached (Figure 1.4). In many organisms such as humans, lipids also act as an energy reserve in the form of fatty deposits [5,63]. Especially in neural tissues such as in brains, lipids are an important component. The function of brains is based on "lipid-rich wires" in the form of axons and dendrites. The connections between these wires, the synapses, are also lipid-rich structures and in addition depend on trafficking of synaptic lipid vesicles for signal transduction [64]. Lipid membranes are semipermeable barriers that control the transport of molecules in and out of cells, as well as internally between different compartments within the cells. The semipermeable nature of the lipid membranes arises from a combination of the intrinsic physicochemical properties of the lipids such as their hydrophobicity, and the presence of membrane proteins embedded in the bilayers that often act as channels for specific molecules. Disturbance of membrane integrity is one potential mechanism of toxicity for cells [56,65,66].

In vitro, phospholipids can form vesicles that can be used as model systems to capture some of the properties of real cell membranes [67] (Figure 1.4a). Due to their relative simplicity, their behavior can be more easily understood from a physicochemical perspective than what is the case with real cellular structures in living organisms. Knowledge of how lipids form vesicles *in vitro*, and details about cell membranes, are things that have developed a lot in the latter half of the 1900s. In 1965 a paper was published on swollen phospholipid lamellae, which became important for the usage of giant vesicles as model systems for lipid membranes [68,69]. In the 1970s the so-called lipid mosaic model of biological

cell membranes was introduced [70]. At its core, it is still the main conceptual framework for the structure of cell membranes today.

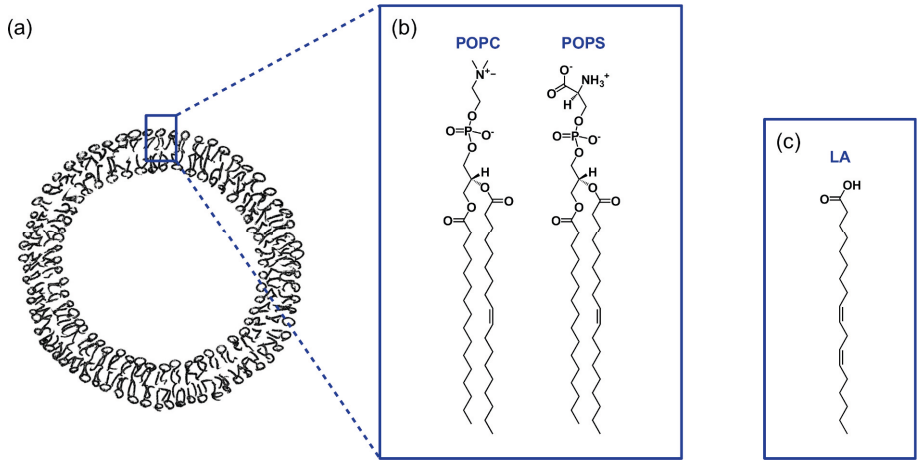

Figure 1.4. Lipid structures. (a) Schematic cross-sectional illustration of a small phospholipid vesicle. The vesicle is made up of a spherical lipid bilayer. Typically, a phospholipid bilayer is about 5 nm thick. (b) Chemical structure of two phospholipids called POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) and POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine). (c) Example of a fatty acid. The chemical structure of linoleic acid (LA) is shown, which was used in experiments for Paper IV of this thesis.

An important part of the self-assembly of lipids in aqueous solutions is hydrophobic interaction [71]. Due to the effective interfacial free energy with surrounding water, non-polar parts of molecules like lipids associate with each other in such a way that the exposure towards water is reduced. The exact structure of the lipid self-assemblies depends on factors such as the shape of the lipids. One approach to rationalize the various structures formed is based on considering the so-called lipid packing parameter [72,73] of the lipid molecules, which can be considered as a measure of how cone-shaped a lipid molecule is. Lipids with a low packing parameter form self-assemblies such as spherical micelles, and with progressively higher packing parameters the lipids self-assemble into structures such as cylindrical micelles, bilayers and bicontinuous structures, and inverted micelles [72,73]. The effective packing parameter of lipid molecules can be influenced by factors such as the temperature, ionic strength, and pH of the surrounding solution. For example, changes in pH alters the chemical structure of some molecules by protonation and deprotonation, which results in variations in the number of charges on the molecules and thereby their interactions. For example, the self-assembly of fatty acids (Figure 1.4c) depends a lot on the pH. At high pH when the carboxyl group on the fatty acid molecules is deprotonated, they form micelles, and at intermediate pH when the molecules are partially protonated, they can assemble into bilayers that form vesicles. At

low pH, when essentially all molecules are protonated, a macroscopic oil-phase is instead formed. This was for example utilized in Paper IV.

1.5 Amyloid-lipid interactions

Disruption of lipid structures in the biological environment is considered as one potential mechanism by which amyloids can cause pathology [65,66]. Amyloid deposits in diseased tissues, such as Lewy bodies in Parkinson's disease, have been found to contain lipids in addition to protein [74]. An early X-ray scattering study from 1969 of amyloid deposits from diseased spleen tissue also revealed evidence of lipids present in the aggregates [43]. During the last decades there has been a substantial amount of *in vitro* studies investigating the interaction between amyloids and lipids, both in terms of structure and kinetics. For example, amyloid forming proteins such as α-synuclein and islet amyloid polypeptide (IAPP), which is involved in type 2 diabetes, have been found to co-localize with giant phospholipid vesicles and either disrupt or distort the membrane structure [75,76]. Distorted vesicles have also been observed with electron microscopy in the presence of amyloid fibrils of proteins such as β_2 microglobulin, IAPP, and α-synuclein, and could also be linked to vesicle disruption [77-79]. Overexpression of α-synuclein in a yeast model system has been reported to induce clustering of cytoplasmic vesicles, although not necessarily due to amyloids [80]. Mixed amyloid-lipid co-assemblies forming on molecular levels might also exist, such as in the case of α-synuclein aggregated in the presence of the phospholipid DMPS (1,2-dimyristoyl-sn-glycero-3-phospho-L-serine) or DLPS (1,2-dilauroyl-sn-glycero-3-phospho-L-serine) [81]. Furthermore, it has been proposed that pre-fibrillar oligomers can form pores that insert in cell membranes and permeabilize them [65]. That is similar to the mechanism of action of some naturally occurring anti-microbial peptides [82].

In terms of kinetics, lipids of various types have been found to either accelerate or inhibit amyloid formation, depending on the conditions [83-86]. For example, anionic phospholipid membranes have been reported to accelerate α-synuclein fibrillation by primary nucleation [85], and amyloid formation by the protein amyloid-β, involved in Alzheimer's disease, has been reported to be inhibited by the presence of zwitterionic membranes in a solid state [86]. At first, the literature can seem quite confusing, with a mixture of both accelerating and inhibiting effects on amyloid formation by lipids. However, when factors such as lipid-protein ratio, lipid composition, and state of the lipid membranes (fluid or solid) are considered more carefully, the results can be rationalized to some extent. For example, some amyloid forming proteins are known to adsorb to lipid membranes of certain compositions, such as α-synuclein which binds to anionic membranes. At intermediate lipid-protein ratios, both bound and unbound protein molecules

are expected to be present, and the interaction between the protein molecules in the two states could induce nucleation at the membrane interface [85,87]. In the case of a very high lipid-to-protein ratio, essentially all protein molecules might be sequestered by adsorption to the membranes so that the result is inhibition rather than acceleration of amyloid formation [83,85]. Furthermore, solid lipid membranes might reduce the flexibility of bound protein molecules, or the propensity of the protein molecules to bind to the membranes, and thereby reduce nucleation relative to the case with a fluid membrane [88,89].

Many of the questions about NACore-lipid interactions addressed in this thesis are inspired by these studies on other amyloid systems. Some questions that are addressed are: Does NACore co-aggregate with lipids (Paper II)? Does the presence of lipids result in formation of novel peptide-lipid mixed structures on a molecular level (Paper III)? How does the presence of lipids influence the kinetics of fibrillation (Paper III and IV)?

2. Methods

2.1 Circular dichroism spectroscopy

Light is electromagnetic fluctuations and molecules absorb light of various wavelengths depending on how the energy levels of their electric structures match up with the oscillations of the light [90]. In normal ultraviolet-visible spectrophotometry, light is passed through a sample and the attenuation of the transmitted light is measured for each wavelength, giving an absorption spectrum for the sample. In circular dichroism (CD) spectroscopy the fact that light can be polarized in various ways is utilized. More specifically, right and left circularly polarized light is used [91]. For non-chiral molecules, the interaction with these two polarizations of light are the same. However, when the molecules are chiral, as is the case of most biomolecules, the two polarizations are not equivalent. This is the key fact utilized in CD spectroscopy. The difference in absorption of right and left circularly polarized light is measured, either directly by alternating the polarization of the light used or indirectly through the so-called ellipticity of the light [91]. In the case of peptides, the difference in absorption between left and right circularly polarized light vary as a function of wavelength depending on the secondary structure of the peptides. The CD spectrum has a characteristic shape for each secondary structure, such as for α-helices, β-sheets, and random coils (Figure 2.1). CD spectroscopy can therefore be used as a method to get information about the structure of peptides and proteins in a sample [91]. If the sample contains molecules with different secondary structures, or if each peptide or protein consists of a mixture of various secondary structures, the CD spectrum is a superposition of these. It is possible to separate the different contributions through a linear decomposition of the spectrum, or through comparison to a reference database with the spectra of various known proteins [92].

This technique is useful for following the formation of amyloid fibrils. In the case of the NACore peptide studied for this thesis, the peptide molecules first have disordered structures. Then, after the fibrillation process has started, more and more of the peptide molecules in the sample adopt β-sheet structures as they become part of fibrils. This can be clearly seen in the transformation of the CD spectrum (see for example Paper I). However, one complication with this technique is that it is not ideal if the sample contains macroscopic aggregates, as it might lead to distortions of the spectrum due to scattering effects, or flattening

of the curve due to heterogeneous absorption [93,94]. For example, the last effect occurs when there is highly absorbing material heterogeneously distributed in the sample so that some of the irradiating light does not hit many of the aggregates, whereas in other parts of the sample it hits much of the highly absorbing material and is substantially attenuated before it has gone through all of it.

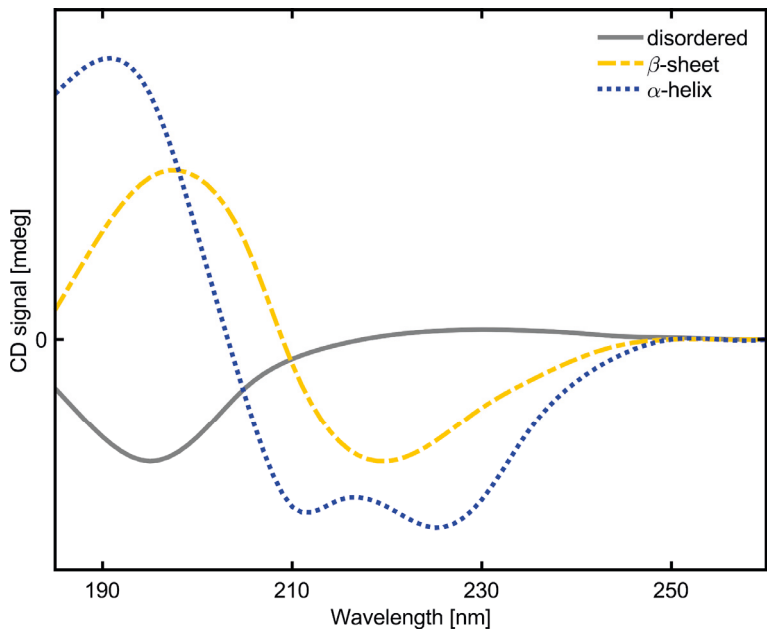

Figure 2.1. Illustration of characteristic CD spectra shapes for different secondary structures of proteins and peptides. The gray line shows a typical spectrum for disordered structures (random coil). The yellow dashed line shows a typical spectrum for β-sheets, and the blue dotted line shows a typical spectrum for α-helices.

2.2 Static scattering

Spatial structure can be represented in “real-space” as we are used to, or alternatively in so-called “inverse-space”. Mathematically, a Fourier transform can be used to convert from real space to inverse space, and this is at the core of how static scattering methods provide structural information about a sample [95]. The measurements are typically based on the scattering of X-rays, visible light or neutrons when passing through a sample. Different parts in the sample have different propensities to interact with the incoming radiation. This is known as scattering length density and gives rise to the contrast. In the case of X-rays, the scattering length density is proportional to the electron density in the sample. In the case of neutrons, it is due to a property of the atomic nuclei and varies in a

seemingly irregular way across elements [95]. Strong scattering at a detector occurs when there are differences in the scattering length density between different parts of the sample [95]. It occurs because re-emitted radiation from different parts of the sample interferes with itself either constructively or by being canceled out as it reaches the detector. Each point on the detector can be represented by a so-called scattering vector (q-vector). The scattering vector is the difference between the wave vector of the scattered and irradiating radiation [95,96] (Figure 2.2). At large angles, the scattering vector is large and gives information about high spatial frequency structure in the sample. At low angles, the scattering vector is small and gives information about low spatial frequency structure. The magnitude of the scattering vector also depends on the wavelength of the radiation used in the scattering experiments (Figure 2.2). The relationship between the real-space structure in the sample and the scattering intensity on the detector at a constant distance from the sample can be described by the equation

$$I(\vec{q}) = c \cdot \left| \int \left(\rho(\vec{r}) \cdot e^{-i \cdot \vec{q} \cdot \vec{r}} \cdot d\vec{r} \right) \right|^2,$$

where $I(\vec{q})$ is the intensity at the detector, $\vec{q}$ is the scattering vector, c is a proportionality coefficient, $\rho(\vec{r})$ is the scattering length density distribution in the sample, $\vec{r}$ is the position in the sample expressed as a vector from an arbitrary origin, and the integral denotes summation over the irradiated sample volume. This means that the scattering intensity is a section of the magnitude squared of the Fourier transform of the real-space sample structure. The scattering vector tells which part of the Fourier transform is probed. As hinted by the equation, one limitation of the structural information obtained with static elastic scattering is that the phase of the scattered radiation is normally lost at the detector (the so-called phase problem) [95]. This means that some structural information from the sample is also lost, and what is obtained is essentially only the auto-correlated real-space structure, rather than the complete structure in the sample.

As mentioned, scattering measurements can be done with several types of radiation, such as visible light, X-rays, neutrons, and electrons. In the case of visible light and electrons, the scattered radiation is easy to steer using lenses (magnetic lenses in the case of electrons). Therefore, structural information with light and electrons can also be obtained through microscopy (see below), which more directly gives a view of the sample structure.

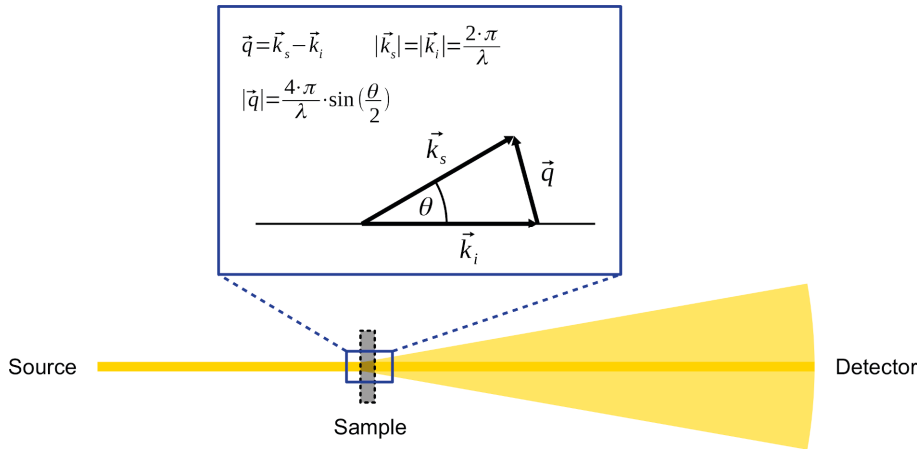

Figure 2.2. Schematic illustration of a typical static scattering experiment. Radiation (such as X-rays or neutrons) is coming from a source as a narrow beam towards the sample. In the sample the radiation interacts with the sample material and is scattered. The transmitted and scattered radiation from the sample then hits a detector that records the intensity at different scattering angles, θ. $\vec{q}$ is the so-called scattering vector, k_s is the wave vector of the scattered radiation, and k_i is the wave vector of the incoming radiation. λ is the wavelength of the radiation.

2.3 Light microscopy and fluorescence

Light microscopy is one of the most traditional techniques used to get structural information on small scales. As the light is shining through the sample, the objects in the sample scatter the light based on differences in refractive index. In addition, different parts of the sample absorb the light to different degrees, especially if the sample has been dyed. A series of optical lenses focuses the light from the sample, which gives rise to a magnified image [97]. With ordinary bright field light microscopy, the contrast in the sample can be rather poor. Fluorescence is one physical phenomenon that can be utilized to enhance the contrast of the image, as well as to specifically label parts of interest in the sample. Molecules consist of atomic nuclei surrounded by electrons. If the frequencies are right, incoming electromagnetic waves that make up light can excite the electrons into higher energy states in the molecules [90,97]. For organic molecules that contain electrons dislocated over many atoms, the resonance frequencies are often in the range of visible light. When such electrons have been excited, they then eventually relax back to a lower energy state and might emit light in the process. This normally happens in several steps through a series of energy states, which results in that the emitted radiation is of a lower frequency than the radiation that exited the electrons, which in turn leads to a red-shift of the emitted light relative to the exciting light. This is the basis of fluorescence [90,97]. By using a set of filters in the microscope, the exciting light can be filtered away so that only the

fluorescently emitted light is detected [97]. The labeled components in the sample then stand out against a dark background in the microscopy images.

There is a fluorescent dye called thioflavin T that can be used as a probe for amyloids (Figure 2.3). It is not fully understood in detail what the mechanism is by which thioflavin T can be used as a probe for amyloids, but a common explanation is as follows. Thioflavin T is a molecule with two parts that can rotate relative to each other when the molecule is free in solution. This rotation can dissipate energy when the molecule has been excited by incoming light, and thereby prevent strong fluorescence. When the molecule binds to an amyloid fibril by intercalating among the stacked β-sheets, the rotation in the thioflavin T molecule becomes hindered and the fluorescence greatly increased [98]. Fibrils of NACore can for example be visualized in fluorescence microscopy by the presence of thioflavin T, which binds to the fibrils (Paper II).

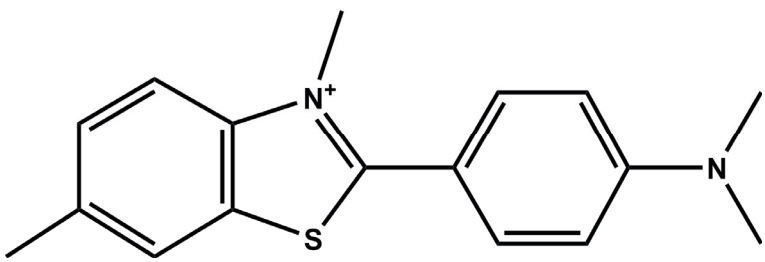

Figure 2.3. Chemical structure of thioflavin T, which is a fluorescent dye often used as a probe for amyloids. In the presence of amyloids, the fluorescence of the dye usually becomes greatly enhanced. The typical excitation peak of thioflavin T in the presence of amyloids is about 450 nm and the emission peak about 485 nm.

One drawback of light microscopy is that the spatial resolution that can be achieved is limited by the wavelength of the light used (fundamentally by the same principle as how the size of the scattering vector determines the resolution in scattering methods). In practice this means that the highest resolution that can typically be achieved with an ordinary light microscope is about 0.2 μm, a little bit larger than the size of a typical virus particle [3]. This is far from the atomic and molecular scales we are often interested in. For example, the size of a stretched out NACore peptide molecule is about 3.6 nm, or 0.0036 μm.

2.4 Confocal laser scanning microscopy

Confocal laser scanning microscopy is a special kind of light microscopy. Rather than illuminating the entire sample, a laser is focused to a single focal point and is then moved along the sample to “scan” the entire sample area [97]. In the microscope there are pinholes which are arranged in such a way that they exclude

light from regions that are outside of the focal plane. This leads to that only a single focal plane is imaged at a time [97]. This is different from ordinary light microscopy where the focused light from the focal plane is mixed with unfocused light from other planes in the sample. A confocal laser scanning microscope typically uses fluorescently labeled samples to visualize the structure. The way a confocal microscope works also enables somewhat higher spatial resolution than an ordinary light microscope [97], although in principle the same limitation of spatial resolution due to the wavelength of the light used still remains. However, it should be mentioned that there are special tricks, based on fluorescence, that can be used to enhance resolution by up to about an order of magnitude. These are the so-called super-resolution microscopy techniques [99] and they were awarded a Nobel prize in 2014. One drawback of confocal microscopy is that the scanning of a sample point-by-point is often relatively slow, which means that the temporal resolution is typically rather poor. In other words, it is difficult to image objects that move around, because it leads to motion blur.

2.5 Cryogenic electron microscopy

As mentioned above, light microscopy has a limited spatial resolution due to the relatively large wavelengths of visible light. However, due to the wave-particle duality of matter, many other types of radiation with shorter wavelength can be utilized to obtain much higher spatial resolutions. One such form of radiation is a stream of electrons. Their wavelength depends on their kinetic energy and can easily be made to be fractions of a nanometer. This is what is utilized in electron microscopy. There are two main types of electron microscopy. One is so-called scanning electron microscopy (SEM) and the other one is so-called transmission electron microscopy (TEM). In the work for this thesis, TEM is the type of electron microscopy that has been utilized. In principle, TEM is very similar to normal light microscopy except that the light has been replaced with a stream of electrons accelerated by an applied voltage, and that the normal optical lenses have been replaced by magnetic lenses which are capable of steering the electrically charged electrons. The electrons hit the sample and are scattered or absorbed by different parts of the specimen. The electrons coming thought the sample are then focused by the magnetic lenses onto a detector to create a magnified real-space image of the structure in the sample [100].

One special type of TEM is so-called cryogenic transmission electron microscopy (cryo-TEM). In cryo-TEM a thin film of a sample that has been placed on a sample grid is very quickly frozen down to typically less than -150 °C. This quick freezing, or vitrification, is so fast that the sample solidifies without having time to substantially rearrange its structure (such as by crystallization) [101]. The vitrified sample is then transferred to the electron microscope and imaged at low

temperature. This means that a snapshot of the otherwise dynamic structure in the sample can be obtained with cryo-TEM. This technique was awarded a Nobel prize in 2017. With cryo-TEM, spatial resolutions down to a few nanometers can easily be achieved. If a sample contains a lot of identical objects, the spatial resolution can be brought down to an atomic level by calculating the average of many images [102]. One drawback of cryo-TEM is that the electron radiation rather quickly damages the sample, so that there is a limit to how many electrons that can be used to image it [101,102]. Another limitation is that it only gives gray-scale images. The selective labeling of different components of a sample is not as straightforward as in fluorescence microscopy, for example.

3. Summary and discussion of results

3.1 Controlling self-assembly through pH

The pH of a solution is an indirect measure of the chemical potential of hydrogen ions (protons). In dilute aqueous solutions relatively close to neutral pH, this is very closely related to the hydrogen ion concentration (or rather H_3O^+ concentration) by the equation

$$pH = -\log_{10}\left(\frac{[H^+]}{1M}\right),$$

where $[H^+]$ is the hydrogen ion molar concentration.

Many molecules have sites with loose protons, and pH affects the degree to which such protons are dissociated. In the case of the NACore peptide, the N- and C-terminal sites can be either protonated or deprotonated depending on pH (Figure 3.1). Close to neutral pH the peptide is zwitterionic with a net charge of zero. If the pH is increased the N-terminus becomes deprotonated, and the peptide becomes net negatively charged. That net charge is also associated with an increased water solubility. This can be utilized to control the self-assembly of the NACore peptide (Paper I) (Figure 3.1). NACore can be dissolved at a high pH, after which fibrillation can be induced by lowering the pH towards neutral conditions where the peptide solubility is lower. This leads to a fibrillation process that typically extends over several days, and the progress can be followed by CD spectroscopy. The formed fibrils can be visualized by cryo-TEM.

The NACore peptide is far from the only molecule for which the self-assembly can be controlled by pH. Another example is fatty acids, which can adopt structures varying from micelles, through vesicles, to oil droplets. This was for example utilized in Paper IV.

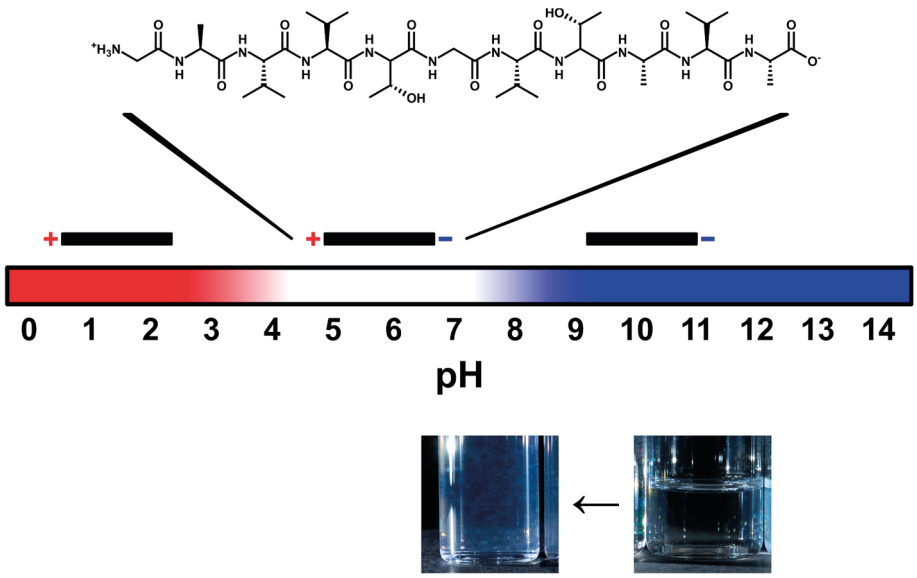

Figure 3.1. Charge of the NACore peptide as a function of pH. The protonation state of the N- and C-termini of NACore depend on the pH of the sample. At around neutral pH the peptide is zwitterionic (expected pI about 5.5). At high pH, the N-terminus becomes deprotonated and the peptide aquires a net negative charge. At low pH, the C-terminus becomes protonated and the peptide acquires a net positive charge. NACore can be dissolved at high pH in aquous solutions. By then lowering the pH towards the isoelectric point fibrillation can be induced.

3.2 NACore fibril structure

The self-assembly of NACore through the procedure described above leads to the formation of fibrils that are typically several micrometers long, and typically ten or a few tens of nanometers wide (Figure 3.2). Through X-ray scattering it can be observed that two prominent diffraction peaks are present, corresponding to periodicities of about 0.47 and 0.85 nm, which is consistent with a cross-β packing of the peptide molecules in the fibrils as is expected for amyloids [10,20] (Paper I). However, when the fibrils have not been aligned the diffraction peaks appear as isotropic circles, rather than as equatorial and meridian arcs as would have been the case with oriented fibrils. Several weaker diffraction peaks can also be detected which are consistent with the NACore 3D-crystal structure that has been reported by Rodriguez et al. [10], suggesting that the organization in the fibrils is similar to that structure (Figure 3.3). From electrostatic arguments and from comparison with literature [10,20], we assume the basic building block of the fibrils to be a “sandwich” of two long parallel β-sheets facing each other. Interestingly, very wide fibrils can sometimes be observed and a striped pattern across the fibrils resolved (Figure 3.4). The separation between those stripes is the same as a stretched out NACore peptide molecules (3.6 nm) and suggests that a prominent feature in the fibril structure is terminus-to-terminus packing of the

peptide (Paper I) (Figure 3.5). The terminus-to-terminus packing is likely enabled by the short nature of the NACore peptide, which makes the terminal sites a major feature of the peptide and also keeps them exposed. Larger amyloid forming proteins and peptides often fold onto themselves in complex ways in the amyloid fibrils, which might make the terminal sites less accessible.

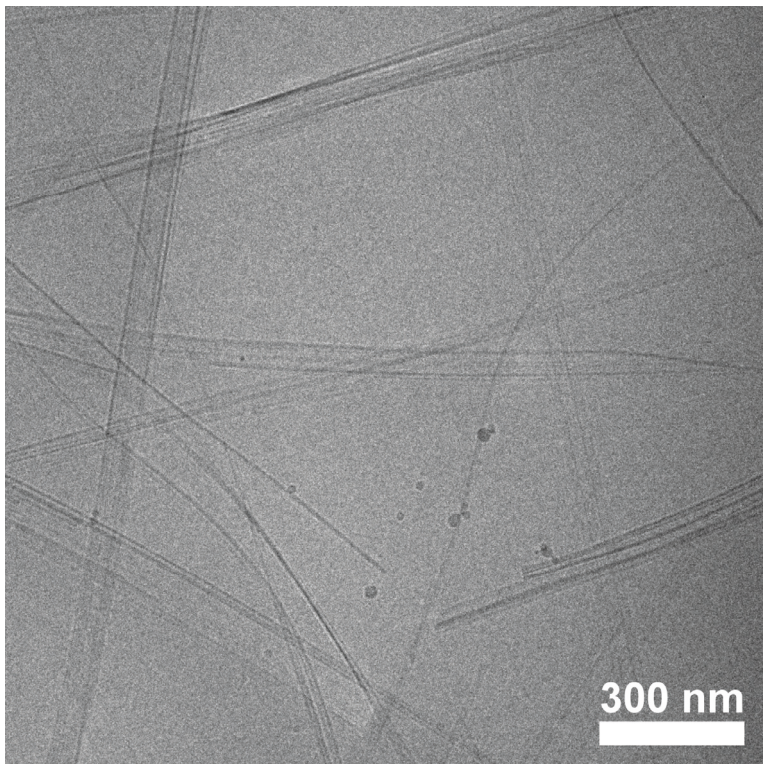

Figure 3.2. Cryo-TEM image of NACore fibrils. The fibrils are typically ten to a few tens of nanometers wide and several μm long. Occasionally, a few twists can be observed but they do not occur regularly. (Paper I.)

Another morphological feature of the NACore amyloid fibrils is their apparent lack of regular twisting. Normally, amyloid fibrils are twisted, presumably due to the chiral nature of the building blocks [103]. A twist to some degree is expected for long fibrillar objects in the generic case and is observed in many other systems (such as actin fibrils, microtubules, and DNA). It is also a possible explanation for why amyloids do not extend indefinitely in width [104,105]. In Paper I we suggest that the lack of twisting of the NACore fibrils could be due to stringent geometric constraints that have to be fulfilled when several β-sheet sandwiches interact with terminus-to-terminus peptide contact. A similar result can be found in a recent simulation study of a short amyloid forming peptide [106].

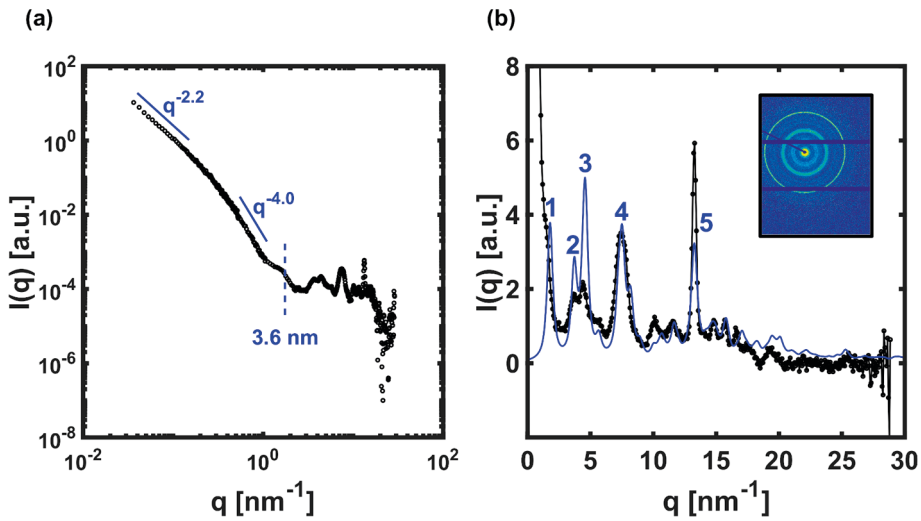

Figure 3.3. Small- and wide-angle X-ray scattering (SAXS and WAXS) of fibrillated NACore. (a) The SAXS pattern shows a characteristic slope at low q-values and a transition to a steeper slope around 0.3 nm^{-1}, which gives information about the cross-sectional dimension of the structures. A small broad peak can be observed at a q-value corresponding to a periodicity of 3.6 nm, which is the same as the length of a stretched out NACore peptide molecule. (b) The WAXS pattern shows several diffraction peaks that are consistent with the crystal structure of NACore determined by Rodriguez et al. [10]. The inset shows the pattern on the detector (solvent subtracted). (Paper I.)

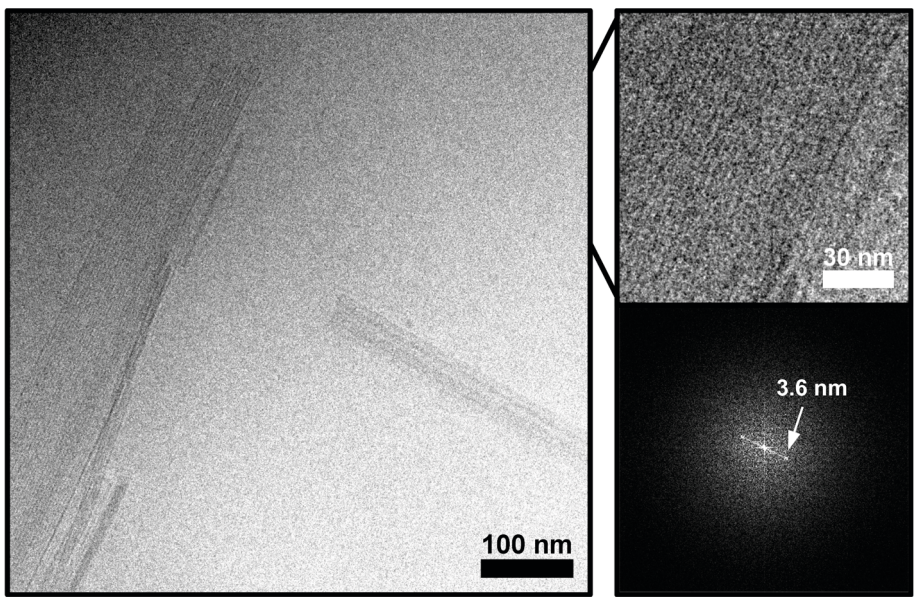

Figure 3.4. Cryo-TEM image of a very wide NACore fibril. A striped pattern can be observed along the width of the structure. The separation between the stripes is 3.6 nm, as revealed by the position of the bright spots in a 2D Fourier transform of the image. This separation is consistent with the small marked diffraction peak in Figure 3.3a and matches the length of a stretched out NACore peptide molecule. (Paper I.)

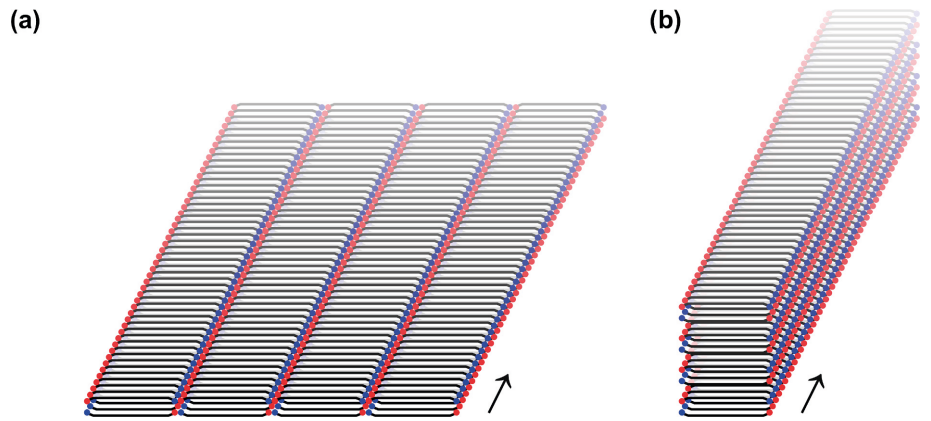

Figure 3.5. Different ways of packing sandwiched β-sheets. The blue and red dots represent the N- and C-termini of the NACore peptide. The arrows show the long axis of fibrils (a) Terminus-to-terminus packing, which seems to be what typically makes up the widths of NACore fibrils based on cryo-TEM (see for example Figure 3.4 and 3.7). (b) Face-to-face packing. (Paper I.)

3.3 Aggregation versus fibrillation

In the case of condensation of a gas (or dilute solution) into a dense liquid, as well as in the case of crystallization, the concept of nucleation applies. For example, when a gas such as steam is condensing into liquid water in response to a change in external conditions, small initial clusters of the liquid are usually thermodynamically unstable due to a relatively large surface-to-volume ratio compared to an infinite bulk-phase. It is necessary that a certain minimum size is exceeded before the clusters become thermodynamically stable and can grow. Such minimal clusters are referred to as nuclei, and overcoming this initial barrier is a nucleation event [107,108]. In the case of crystallization such as the freezing of liquid water, the situation is in many ways analogous. However, in that case the system is already condensed, so the critical step that needs to take place is the formation of a sufficiently large cluster of ordered molecules, rather than an increase in density. When a system is brought directly from conditions in which a dilute state is the most stable to conditions in which a crystalline state is the most stable, the two types of events described above, condensation and ordering, might occur in sequence (and perhaps always does to some extent). This is known as two-step nucleation, which might be a common phenomenon and is found in a range of systems [107,109,110].

Since amyloid fibrils are highly ordered structures, principles like those of two-step nucleation might apply to the fibrillation process [110] (Figure 3.6). If it is assumed that the initial state is a solution of peptide monomers, the first thing that needs to happen for a fibril to form is that several molecules come together in the solution. However, just coming together is not enough, because fibrils are highly

ordered. That means that the peptide molecules that have come together also need to adopt an order that is compatible with fibril formation and subsequent elongation. In principle, the initial association, or the aggregation step, and the adoption of an ordered structure could be separate processes. With the NACore peptide we found some evidence for such behavior in the sense that lowering the pH closer to the isoelectric point of the peptide led to slightly slower rather than faster fibril formation. If fibrillation only involved association into a dense structure, lowering the pH closer to the isoelectric point of the peptide might have been expected to result in faster fibrillation, due to a reduced peptide solubility (higher supersaturation) and thereby a larger overall tendency to aggregate. In addition, we observed small clusters attached to fibrils in the lower pH condition (Paper I) (Figure 3.7). Close to the isoelectric point, conversion to highly ordered structures might be slower than at higher pH, counteracting the otherwise expected faster fibrillation based on solubility and supersaturation alone. The observed clusters could be kinetically transient peptide aggregates that have not yet adopted the molecular order found in the fibrils. Conceptually similar behavior has also been reported in other amyloid systems [111-113].

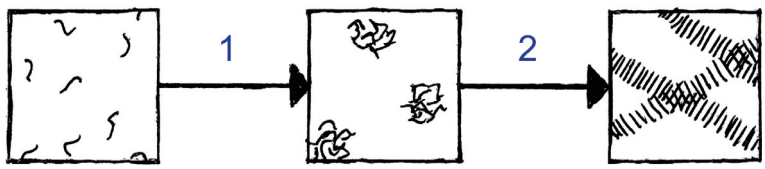

Figure 3.6. Schematic illustration of a two-step fibrillation process. In step 1 peptide molecules come together and form dense clusters. In step 2 the peptide clusters obtain an ordered structure and grow into fibrils.

Clusters attached along the sides of fibrils could also be relevant to mechanisms of secondary nucleation [114]. Secondary nucleation in the context of amyloids is the catalysis of new fibril nuclei by the presence of fibrils that have already formed and leads to an auto-catalytic cascade of fibrillation [115]. Catalysis of nucleation could for example occur through local enrichment of monomers on the fibril surfaces by adsorption from solution and/or through catalyzed structural conversion of non-fibrillar clusters upon contact with the fibrils.

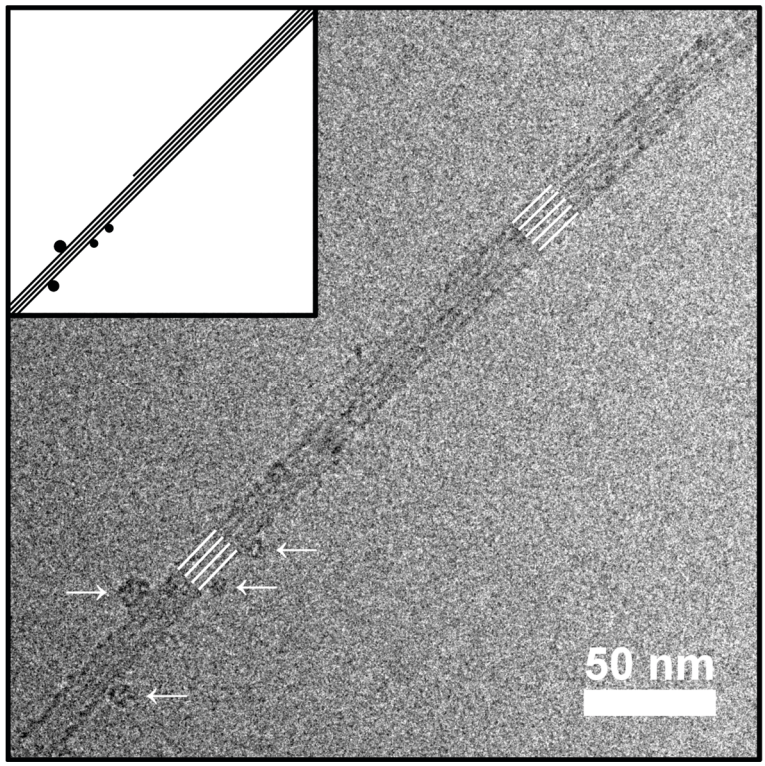

Figure 3.7. Example of clusters seen along the side of an NACore fibril (arrows) after unfibrillated peptide was added to a small amount of preformed fibrils at pH 6. A striped pattern is also seen across the fibril (like the one in Figure 3.4). The width of the fibril changes in the middle of the images, which might give clues about growth mechanisms. The inset shows a schematic interpretation of the structure in the cryo-TEM image. (Paper I.)

3.4 NACore fibrils as colloidal objects

As mentioned above, the NACore fibrils are typically only about ten or a few tens of nanometers wide. In other words, they have colloidal-scale cross-sections and can be considered as colloidal objects [73]. This is important to consider when investigating the interaction of NACore fibrils with other molecules such as lipids because the colloidal dimensions of the fibrils open up for a new type of interaction mode to become dominant, namely interfacial interactions. For example, a cubical structure that is 1 mm on each side has a volume of 1 mm^3 and the total surface area is 6 mm^2. If the same total volume is divided up into 10^{13} fibrils that have a 10-by-10 nm square cross-section and are 1 µm long, the total surface area becomes 402 000 mm^2. In other words, the extent of potential interfacial interactions is very much larger for colloidal objects compared to macroscopic ones of the same total volume. Instead of interactions with other molecules mainly in the bulk parts of the structures, interactions through

interfacial affinities become significant, as might be the case for NACore-lipid interactions (see below, and Paper II-IV).

3.5 Co-aggregation with phospholipid vesicles

One potential mechanism by which amyloid formation might induce cell toxicity is by disruption of lipid membrane structures and functions [65,66]. That is one motivation for why it is interesting to know how amyloid fibrils interact with lipids. By using fluorescence microscopy, we found that when NACore peptide is fibrillated in the presence of giant phospholipid vesicles, the vesicles become captured within the gel-like NACore amyloid fibril networks (Paper II) (Figure 3.8). In these experiments, NACore was incubated in the presence of the fluorescent thioflavin T probe for amyloid fibrils and fluorescently labeled giant phospolipid vesicles, so that the different components of the sample could be visualized. Also, when NACore had first been fibrillated in the absence of lipids and was then mixed with vesicles, they attached to the gel-like NACore fibril networks. This shows that the affinity between the vesicles and the fibrils is not crucially dependent on the fibrillation process itself. However, the fibrillation process might perhaps enhance disruption of vesicles, for example by free energy dissipation during the thermodynamically down-hill fibrillation.

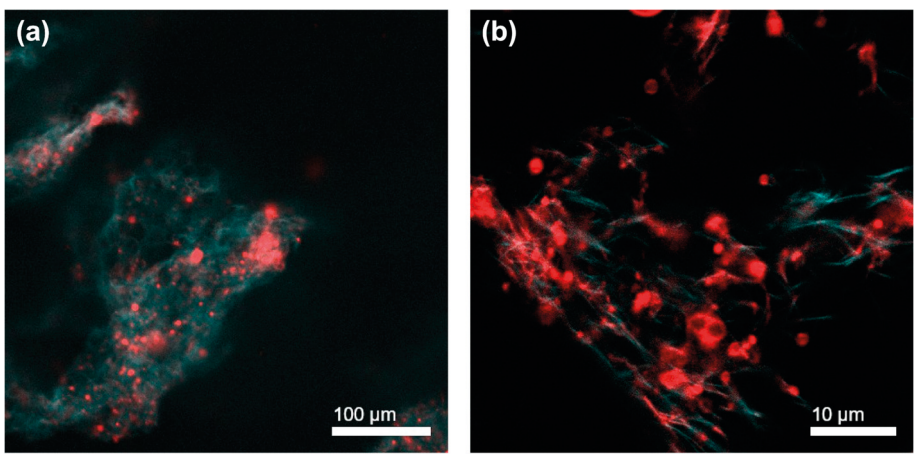

Figure 3.8. Co-aggregates of NACore fibrils (blue) and phospholipids (red) as seen with confocal fluorescence microscopy. NACore was fibrillated overnight in the presence of thioflavin T and fluorescently labeled phospholipid vesicles composed of a mixture of POPC and POPS. (a) Low magnification image of a co-aggregate. (b) High magnification image (not the same sample as in “a”). (Paper II.)

Biological membranes in cells have different lipid compositions depending on where they are [116]. In addition, they have different types of membrane proteins

that confer particular functions to the membranes. One interesting thing to know is whether amyloid formation can scramble the composition of such different membranes. Disruption of their separate compositions, and consequently their distinct functions in the cellular environment, could be a potential mode of toxicity. We did experiments in which there were two populations of phospholipid vesicles. One population was labeled with a red fluorescent dye, and the other one was labeled with a green dye. Apart from the fluorescent dyes, the composition was the same. An equimolar mixture of these differently colored vesicles was co-incubated with NACore to see if the fibril formation would lead to scrambling of the membranes. It was found that NACore fibrillation did not induce total scrambling of the membranes (at least not after one day of incubation), because different regions of the co-aggregates retained their separate colors, rather than forming large areas of mixed (yellow) color as would be the case if total mixing of the bilayers had occurred (Figure 3.9). However, since most of the vesicles were multilamellar this might also reflect an inability of the NACore fibrils to penetrate beyond the outermost bilayer. Some of the single-color regions were spread out into non-spherical shapes. That is an indication of distortion or disruption of the original vesicles (Paper II).

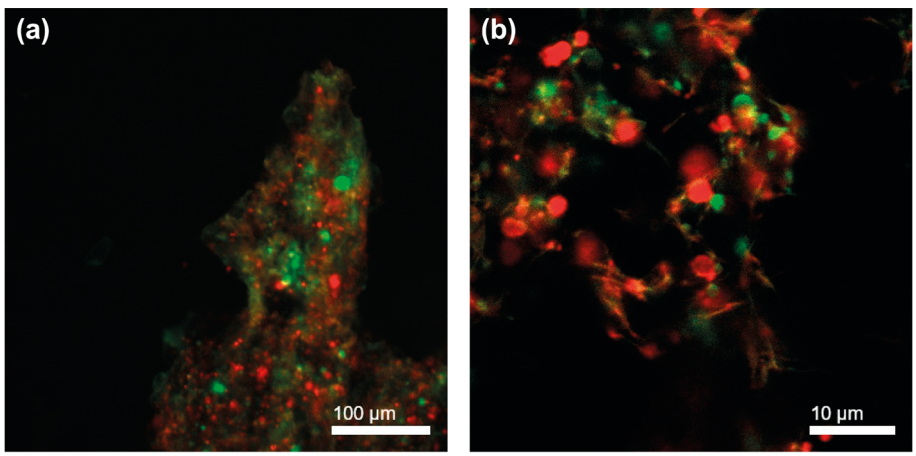

Figure 3.9. Co-aggregates of NACore fibrils formed in the presence of two populations of phospholipid vesicles (red and green) seen with confocal fluorescent microscopy. Separate red and green regions can be seen in the aggregates, showing that the fibrillation process did not lead to extensive mixing of separate lipid bilayers. (a) Low magnification image. (b) High magnification (not the same sample as in “a”). (Paper II.)

3.6 Nanostructure of NACore-phospholipid co-aggregates

In the fluorescence microscopy experiments it was found that phospholipid vesicles co-aggregate with NACore fibrils on optical length-scales. However, light microscopy is insufficient for getting detailed structural information at nanometer length scales, which is the size domain of the cross-section of individual fibrils and of the phospholipid bilayers. To get information about how intimately the peptide and lipids are co-aggregated it is necessary to use additional techniques. One such technique is cryo-TEM. When NACore is fibrillated in the presence of phospholipid vesicles and imaged with cryo-TEM, trapped and distorted vesicles can be found attached to and interspersed in the fibrillar network (Paper III) (Figure 3.10). Especially in the case of zwitterionic vesicles with no net charge there were a lot of distorted bilayer structures (Paper III). Structurally distinct fibrillar and bilayer structures could, however, still be observed with cryo-TEM, suggesting that the fibrils and bilayers to a large extent retain their separate identities on nanometer length scales even after they have been co-aggregated.

To get more structural information about this, X-ray scattering was used. X-ray scattering easily probes structures down to fractions of a nanometer. However, the structures detected with X-ray scattering are based on periodicities in the sample, which means that ordered structures are the ones primarily detected. When several independent structures exist in a sample, the X-ray scattering pattern is a linear combination (a sum) of the scattering from the individual components. One way to test how intimately the peptide and lipid components co-assemble is thus to test whether their X-ray scattering can be fitted as a linear combination of the separate components. If the X-ray scattering profile cannot be fitted as a linear combination, it is an indication of new co-assembled structures on molecular length scales. We found that the X-ray scattering from peptide fibrillated in the presence of phospholipid bilayers could be well fitted by a linear combination of the scattering from fibrils and bilayers, consistent with the conclusion that the fibrils and bilayers retain their separate structures on molecular length-scales when co-aggregated (Paper III) (Figure 3.11). However, the peptide fibrils and vesicles clearly have an affinity for each other, leading to their co-aggregation on large length scales as discussed before and as seen in cryo-TEM. The association between vesicles and fibrils is likely due to interfacial attractions, enhanced by the colloidal scale of the NACore amyloid fibrils which leads to a lot of exposed interfacial area (see Section 3.4).

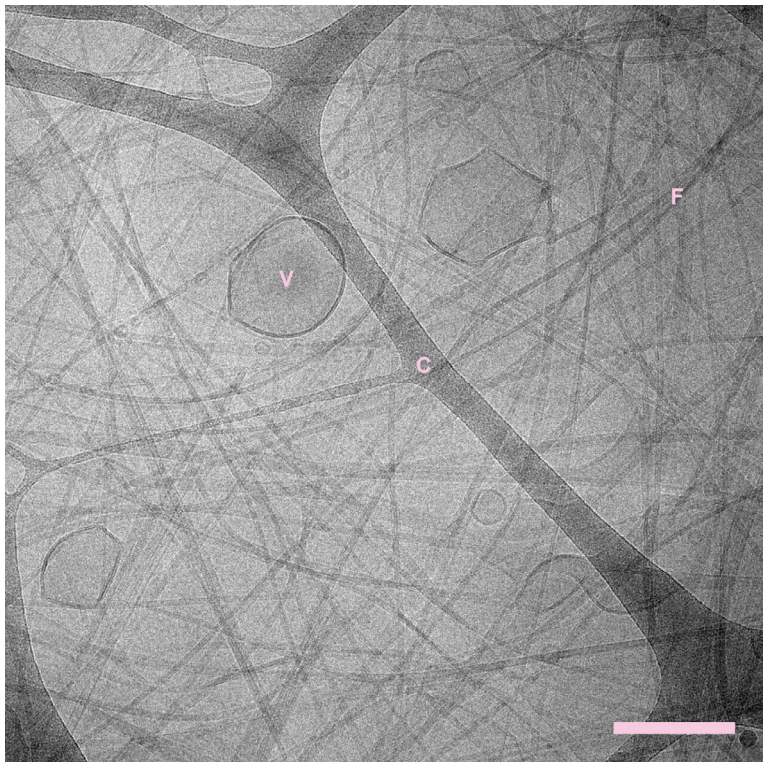

Figure 3.10. Cryo-TEM image of NACore fibrillated in the presence of small unilamellar phospholipid vesicles composed of POPC and POPS. “F” is for fibrils and “V” is for vesicles. “C” denotes the cryo-TEM carbon grid. Trapped and highly distorted vesicles can be seen among the fibrils. The scale bar shows 200 nm. (Paper III.)

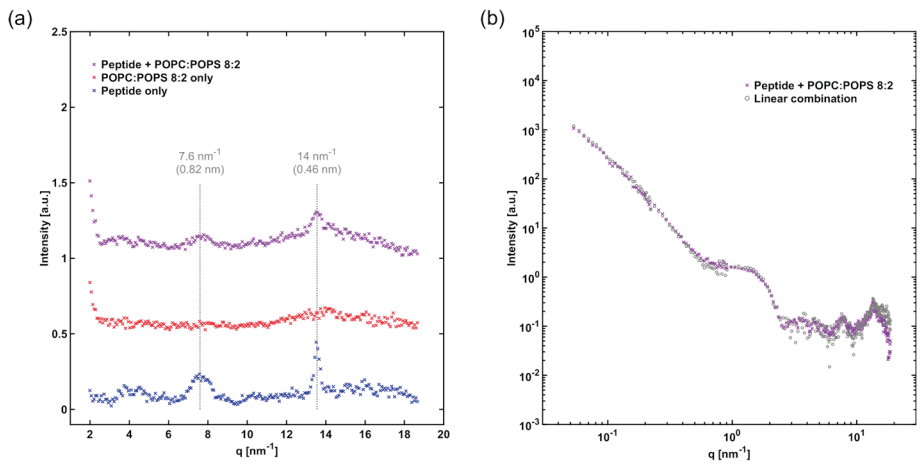

Figure 3.11. SAXS and WAXS data for NACore co-incubated with phospholipid vesicles (POPC:POPS). (a) The main NACore WAXS diffraction peaks are unaffected by co-incubation with the lipids. (b) Co-incubated NACore and POPC:POPS can be fitted as a linear combination of fibrils alone and POPC:POPS alone. The data in “a” and “b” indicate that the nanometer-scale structure is mostly unaffected by co-incubation. (Paper III.)

In this and the previous section, interaction between NACore and phospholipids has been addressed. Instead of phospholipids, a fatty acid, linoleic acid, was used as an interaction partner in one of the studies for this thesis (Paper IV). There, the focus was mainly on fibrillation kinetics. In contrast, the focus in the papers on NACore-phospholipid interaction was mainly on structure, although Paper III includes some experiments and discussion about the effect of phospholipid vesicles on the kinetics of fibrillation as well. In future research it would be interesting to investigate structural aspects of the interaction between fatty acids and the NACore peptide (or other amyloid forming peptides and proteins) in more detail. Conversely, it would be interesting to further investigate the effects of phospholipids on the fibrillation kinetics, especially to find out if the mechanism of action is the same for both phospholipids and fatty acids.

3.7 Inhibition of fibrillation by the presence of lipids

We found that the presence of lipids inhibits NACore fibril formation. This was the case with phospholipids (Paper III) but was especially so with a fatty acid (linoleic acid) (Paper IV). Already at about 1 % of fatty acid by mass relative to the peptide mass in the sample, a substantial inhibitory effect could be observed (Paper IV). This inhibitory effect mainly manifested itself through a prolongation of the lag-phase of the fibrillation reaction (Figure 3.12). One hypothetical way in which the presence of lipids could inhibit fibrillation is by sequestering peptide monomers in the solution, which would essentially lead to a lower free peptide monomer concentration and thereby slower fibrillation due to a lower degree of supersaturation [108]. This was for example suggested in Paper III as an explanation for how phospholipid vesicles can inhibit NACore fibrillation. However, based on the experiments in Paper IV it is hard to imagine this as the main mechanism of action in the case with linoleic acid. That is because an inhibitory effect was observed already at a very low amount of the fatty acid (1 % relative to peptide mass, or 0.00014 % relative to the total sample mass). Consequently, for the free peptide concentration to be substantially reduced it would be required that a lot of peptide monomers are sequestered for every lipid molecule and that the partition coefficient for the peptide between the lipid and aqueous phase is extremely large. Another, and probably more likely explanation, is that interaction mainly occurs between larger particles of each species. That makes sense from an interparticle interaction-potential point of view because the interaction-potential is generally amplified by increasing particle sizes [117]. As a simple example, the van der Waals interaction between two spherical particles at close distance to each other can be approximated by

$$W = \frac{-A}{6 \cdot D} \cdot \frac{R_1 \cdot R_2}{R_1 + R_2},$$

where W is the potential free energy, R_1 and R_2 are the radii of the two particles, D is the separation between them, and A is the so-called Hamaker constant [117]. As the radii increase, the interaction-potential also increases in amplitude by this relation. In the case of NACore-lipid interaction, the main driving force is likely hydrophobic rather than van der Waals forces, but also in that case the attractive interaction would become stronger the larger the particles are (due to increased interfacial areas for larger particles). In cryo-TEM images of NACore in the presence of linoleic acid, we could see rather large non-fibrillar structures among the fibrils (Figure 3.13). Those structures might consist of peptide clusters and fatty acid that have co-associated with each other.

The explanation described above assumes that lipid-associated peptide clusters (non-fibrillar oligomers) are prevented from forming mature fibrils. If the fatty acid molecules are unable to fit in the highly ordered crystalline structure of the mature fibrils, they need to be excluded from the non-fibrillar peptide clusters before they can form the fibrils. This constitutes an entropic barrier and could potentially explain how lipid-associated peptide clusters are stabilized. The population of initial pre-fibrillar clusters might be very small relative to the total peptide concentration in the sample, but it is potentially an essential population in terms of what eventually gives rise to the fibrils. It could consequently be sufficient to sequester this small population of peptide clusters to hinder fibrillation. This could explain how a very small amount of additives, such as the fatty acid in Paper IV, can substantially inhibit or modulate fibrillation. In principle, a mechanism like the one outlined above could also explain the inhibitory effect of phospholipid vesicles on fibrillation of NACore, but that remains to be investigated in more detail.

When comparing the inhibitory effect of linoleic acid on NACore fibrillation at two different pH, we found that it was much more potent at pH 6 than at pH 8 (Paper IV). At pH 6, the net charge and solubilities of both the NACore peptide and linoleic acid are expected to be lower. From an argument based purely on solubility, we might consequently have expected aggregation to be faster at the lower pH. But as has been emphasized, amyloid formation is also about obtaining order. A lower pH likely leads to stronger attraction between the peptide and fatty acid due to reduced electrostatic repulsion, which thereby could result in more inhibition of fibrillation due to more peptide-lipid co-association.

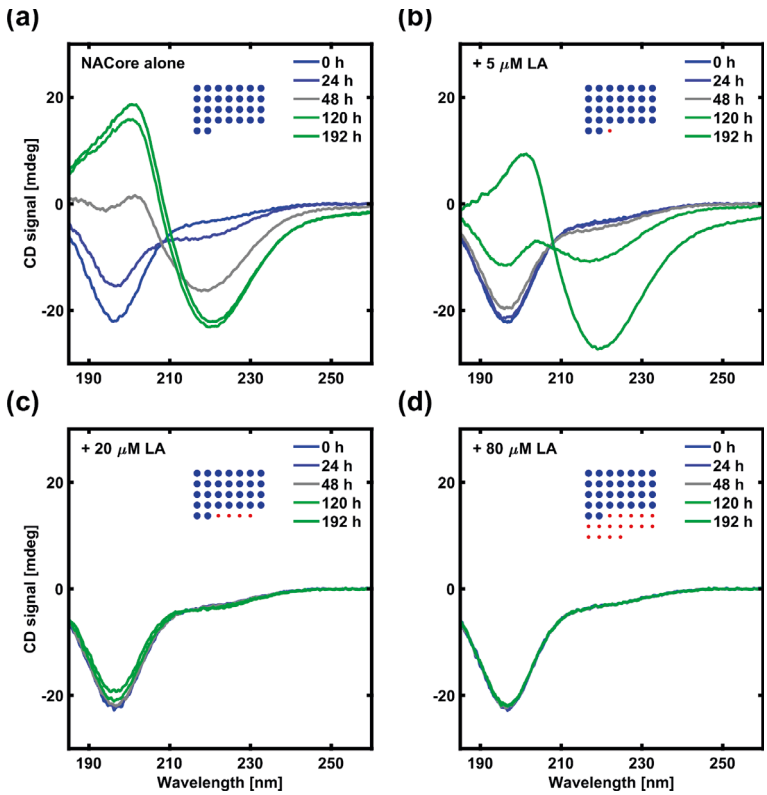

Figure 3.12. Inhibitory effect of linoleic acid (LA) on NACore fibrillation, as seen with CD spectroscopy. The peptide concentration was 150 μM in all samples. (a) NACore without LA. A gradual transition from a disordered structure to a β-sheet-rich structure can be seen over time, which indicates fibrillation. (b) 5 μM LA present in the sample. (c) 20 μM LA present. (d) 80 μM LA present. With increasing concentrations of LA the fibrillation is inhibited. An effect is seen already with 5 μM LA. The insets show the composition of NACore (blue) and LA (red). The numbers and areas of the dots are proportional to the molar concentration and mass, respectively. (Paper IV.)

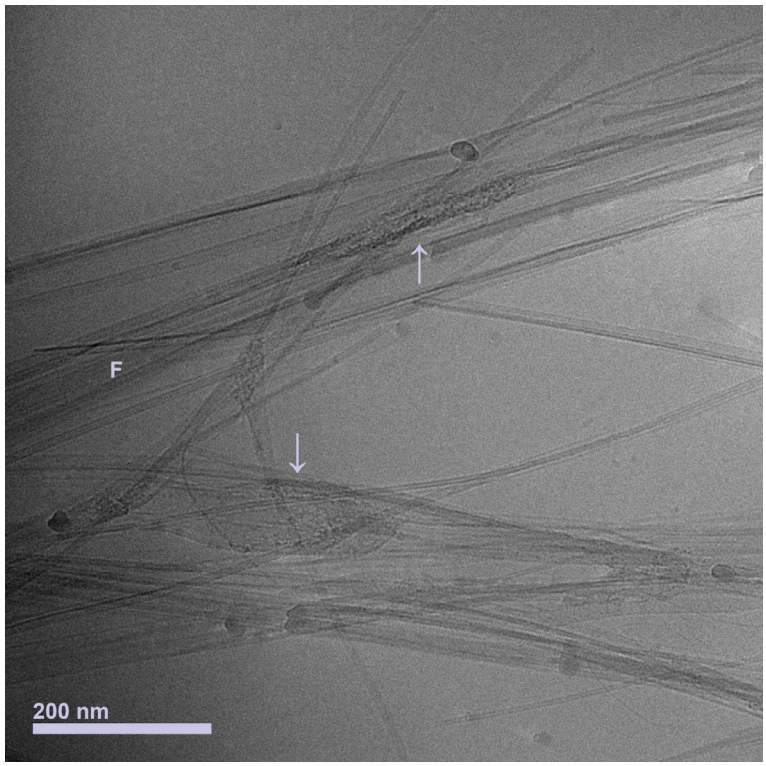

Figure 3.13. Cryo-TEM image of NACore fibrillated in the presence of linoleic acid (same sample composition as in Figure 3.12b). “F” denotes fibrils. In addition to fibrils, non-fibrillar structures can be seen attached to the fibrils (arrows). This might be clusters composed of a mixture of peptide and fatty acid. (Paper IV.)

3.8 Full-length α-synuclein

The investigation of the NACore amyloid model system and its interaction with lipids has been the main focus of the work for this thesis. However, some work on full-length α-synuclein has also been performed, with a focus on how it interacts with lipids (Paper V). There are some qualitative differences in the behavior of full-length α-synuclein and the NACore peptide fragment. The N-terminal region of α-synuclein has a net positive charge at physiological pH and contains seven imperfect KXKEGV repeats (where X is a variable amino acid residue). This leads to adsorption of monomeric α-synuclein to anionic phospholipid bilayers and the adoption of an amphipathic α-helix in the N-terminal part of the protein [118] (Paper V) (Figure 3.14). This is different from the behavior of NACore. For example, the CD spectrum of unfibrillated NACore is essentially identical in the presence and absence of phospholipid vesicles (Paper III). If monomeric NACore adsorbs to the phospholipid bilayers, it does

so in a disordered form similar to that in solution and leaves no trace of it in the CD spectrum.

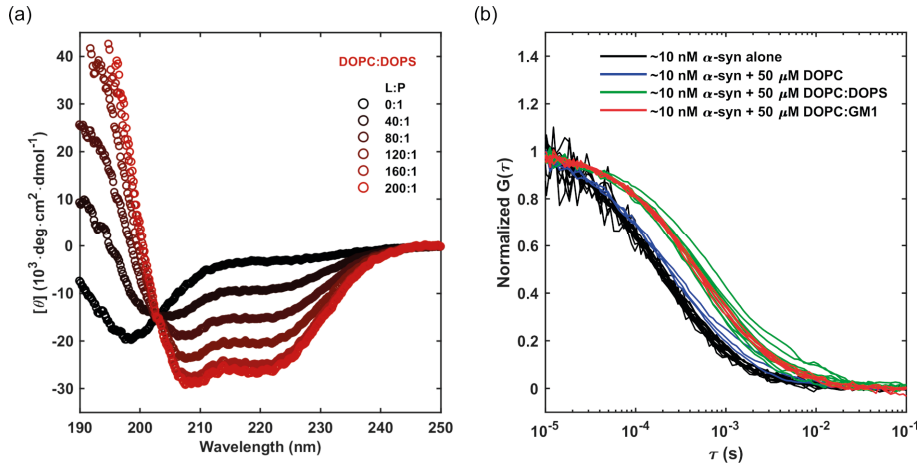

Figure 3.14. Monomeric α-synuclein in the presence of phospholipid vesicles. (a) CD-spectra of α-synuclein with increasing amounts of DOPC:DOPS anionic vesicles present. As the lipid-to-protein ratio increases, more and more α-helix content is seen in the CD-spectra. (From Paper V.) (b) Fluorescence correlation spectroscopy (FCS) measurements of fluorescently labeled α-synuclein in the presence of lipid vesicles with different compositions. FCS gives information about the diffusion rate of fluorescently labeled molecules (α-synuclein in this case). Slower diffusion leads to curves shifted to the right, which happens in the presence of the anionic vesicles made of DOPC:DOPS and DOPC:GM1 (ganglioside). This indicates adsorption to the vesicles. With zwitterionic DOPC vesicles no major shift in the correlation curve is seen relative to α-synuclein alone. (Unpublished data.)

In Paper V we showed that anionic lipid vesicles can accelerate α-synuclein amyloid formation and that the accelerating effect is slightly stronger for so-called ganglioside lipids, which have large carbohydrate headgroups, compared to other anionic lipids. All anionic membranes led to partial α-helical structure of α-synuclein, presumably due to adsorption to the membranes (see for example Figure 3.14b). We also found that uncharged lipids with large headgroups might accelerate fibrillation to some degree, but much less so than anionic lipids. Furthermore, the large anionic headgroup of one of the ganglioside lipids could induce amyloid formation by itself, even without the rest of the lipid molecule, and without inducing α-helical structure of α-synuclein. Although ganglioside lipids led to slightly stronger acceleration, the results imply that the most important factor for acceleration is membrane charge. The fact that some species such as the free ganglioside headgroup also effectively induce fibrillation without inducing α-helical structure of α-synuclein implies that it is not strictly required as an intermediate state to promote α-synuclein amyloid formation.

The accelerating effect of some lipid membranes on α-synuclein fibrillation differs qualitatively from the inhibitory effect on NACore fibrillation. One reason for this could be differences in adsorption behavior of monomeric α-synuclein

and NACore to phospholipid bilayers, as discussed above. In addition, under quiescent conditions and with monomeric α-synuclein alone, amyloid formation often does not spontaneously occur (see for example Paper V). This is different from the case with NACore under the conditions used in the experiments for this thesis, which readily lead to spontaneous fibrillation of the peptide. The presence of anionic lipid membranes is thought to induce α-synuclein amyloid formation by heterogeneous primary nucleation on the membrane surface [85]. Such an effect would not be very noticeable with NACore since nucleation happens anyways under the conditions used. That could also be a reason why an accelerating effect was not observed. Three main factors that I think are important to consider when comparing the effect of lipids on fibrillation kinetics for various amyloid forming proteins and peptides are as follow. (1) Whether fibrillation occurs spontaneously in the absence of the lipids. (2) Whether the protein or peptide monomers adsorb to the lipid structures. (3) Whether adsorption of the protein or peptide to the lipid structures is coupled to a conformational change of the protein or peptide (see also the discussion in Paper IV).

Epilogue

The biological environment is extremely complex, and we are still far from fully understanding it from a physicochemical perspective. In the work for this thesis a short peptide was studied to contribute to our understanding of amyloids, which are involved in many diseases related to old age. That topic is becoming increasingly relevant as people are living longer. Another reason for studying amyloids is simply that they are interesting from a basic science perspective. Furthermore, there could be technological applications. In terms of understanding biology in a broad context, the philosophy underlying this thesis is perhaps a bit like the one in the book "On Growth and Form" by D'Arcy Wentworth Thompson [119] but applied on a molecular level. By becoming familiar with the physical stage on which biology happens, features that have specific biological functions can be more easily recognized among non-evolved features that arise due to generic principles of physical chemistry. Some non-living things can indeed appear rather life-like on first sight (such as self-assemblies of lipids), even before they have been evolved by natural selection.

Fundamentally, I think science is about creating models of the world. Many of the well-established "textbook" theoretical models in physical chemistry are about systems in equilibrium. However, many of the most interesting phenomena in the world occur out of equilibrium (such as living processes). The often very low solubilities of amyloids also often lead to very long equilibration times and consequently make kinetic aspects of the systems important. For example, the fibrillation of NACore typically extends over several days, and even then, the system might not reach its true thermodynamic equilibrium. In the absence of comprehensive theories, one way to test if the initial models you form in your mind really work as you think they do is to do computer simulations. This could be simple coarse-grained simulations, and I think it would be very useful to do this parallel to experiments as an intermediate step towards complete understanding. That is one of my suggestions for future research. In the long history of science, the possibility to easily do such simulations is only a few decades old, due to the quite recent rise of modern computers.

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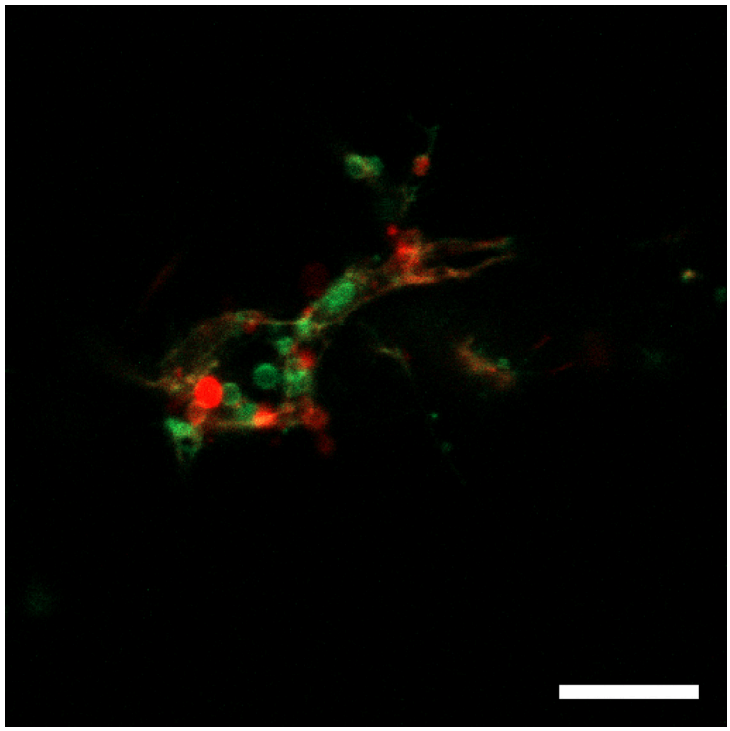

Fluorescent phospholipid vesicles trapped in a network of NACore amyloid fibrils. The scale bar shows 10 µm.

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