Martijn van Raaij

**Biophysical characterization of alpha-synuclein aggregates:
Parkinson's disease at the nanoscale**

BIOPHYSICAL CHARACTERIZATION
OF ALPHA-SYNUCLEIN AGGREGATES:
PARKINSON'S DISEASE AT THE NANOSCALE

M.E. van Raaij

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Cover image:

Three-dimensional rendering of an atomic force microscopy height image of fibrils formed by the protein α -synuclein deposited on mica. The brown-yellow color contrast is taken from the corresponding tapping mode phase image, and indicates that the ends of the fibrils have different material properties than the fibril bulk. The lateral dimensions of the image are approximately $1 \times 2 \mu\text{m}$, and the diameter of the fibrils is around 8 nm. For more information, see section 2.3.4 on page 33.

M.E. van Raaij

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BIOPHYSICAL CHARACTERIZATION
OF ALPHA-SYNUCLEIN AGGREGATES:
PARKINSON'S DISEASE AT THE NANOSCALE

PROEFSCHRIFT

ter verkrijging van
de graad van doctor aan de Universiteit Twente,
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Prof. dr. V. Subramaniam (promotor) en
Dr. G.M.J. Segers-Nolten (assistent-promotor)

...but of what nature that morbid change is; and whether originating in the medulla itself, in its membranes, or in the containing theca, is, at present, the subject of doubt and conjecture...

— James Parkinson (1817), *An Essay on the Shaking Palsy*.

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Introduction

The misfolding and aggregation of proteins in human brain cells is at the heart of many neurodegenerative disorders. Parkinson's disease is one striking example: pathologists find large aggregates consisting of the protein α -synuclein in the brain tissue of patients who suffered from Parkinson's disease.

Although the link between α -synuclein aggregation and Parkinson's disease has been established, we lack understanding of the causes of protein misfolding, the mechanisms of protein aggregation, and the ways in which neuronal damage occurs. A molecular level knowledge of these processes may eventually point to therapies that act directly on the cause, and not on the effects, of the disease.

The etiology of neurodegenerative disorders at the molecular scale presents a problem that may be approached from many angles: there is clinical, pathological, biochemical and biophysical research on the nature and behavior of misfolding proteins in progress in hospitals and research laboratories around the world. In this work we choose a biophysical approach: we mimic the aggregation process *in vitro* and study the morphology of fibrillar structures formed by α -synuclein using advanced microscopic and spectroscopic methods. This allows us to characterize the process of α -synuclein aggregation and identify differences in the aggregates formed by disease-related mutants of the wildtype protein. To evaluate how these properties correlate with the *in vivo* situation, we then compare the *in vitro* results with measurements on Parkinson's disease patient brain tissue sections.

This first chapter introduces the biophysical and pathological context of the research described in this thesis and defines the research questions we seek to answer.

1.1 Protein misfolding and amyloid formation

The vast majority of proteins at work in our bodies possess a very specific secondary and tertiary structure¹. When proteins are not folded in a particular way, they typically are not able to perform their biological function in the organism: the so-called structure-function paradigm. Intrinsically disordered proteins (IDPs), also called natively unfolded proteins, escape this paradigm: they do not have a fixed secondary and tertiary structure and yet perform a biological function in the cell (Wright and Dyson, 1999). One of the biological rationales for the existence of such proteins is that conformational plasticity may be required for binding to many different partner molecules in a cell. Also, cells may be able to control the concentration of IDPs more closely than that of other proteins since IDPs can be produced and degraded more rapidly than well-folded proteins (Wright and Dyson, 1999).²

In a healthy organism, proteins that are not folded properly will be disposed of by cellular waste disposal mechanisms such as the ubiquitin-proteasome system (Ciechanover and Brundin, 2003). However, under certain circumstances, the misfolded proteins will self-assemble into supramolecular structures (Dobson, 2003). These structures are typically thread-like fibrils with a diameter of ~ 10 nm and a length in the micrometer range (fig. 1.1). These so-called amyloid fibrils³ will then cluster together and form large intra- or extracellular proteinaceous masses that may severely disrupt the functioning of the surrounding tissue.(Dobson, 2001; Soto, 2003; Forman *et al.*, 2004; Chiti and Dobson, 2006).

The formation of amyloid fibrils has been observed in many proteins and polypeptides, all displaying strikingly similar aggregation behavior (Uversky and Fink, 2004). Examples include $A\beta$, which is involved in Alzheimer's disease (Hardy and Selkoe, 2002); huntingtin in Huntington's disease (Diaz-Hernandez *et al.*, 2004); prion protein in bovine spongiform encephalopathy (Anderson *et al.*, 2006; Jones and Surewicz, 2005), β_2 -microglobulin in dialysis-related amyloidosis (Radford *et al.*, 2005) and α -synuclein in Parkinson's disease (Goedert, 2001). Not all amyloidogenic proteins are involved in diseases:

¹The secondary structure of a protein is the localized organization of parts of a polypeptide chain, stabilized by hydrogen bonds between specific residues. The tertiary structure is the overall arrangement of all amino acids in the entire protein, as stabilized by hydrophobic interactions between patches of the polypeptide chain (Lodish *et al.*, 2000).

²To be intrinsically disordered is not the same as to be denatured. Denatured proteins have a random coil structure, whereas IDPs may possess an ensemble of conformations where parts of the protein have some order, and other parts are denatured and/or collapsed (Frieden, 2007).

³The term 'amyloid' (or starch-like) refers to the property of these fibrils to display apple-green birefringence in the same manner as starch does when stained with the dye Congo Red and viewed under polarized light (Sipe and Cohen, 2000).

Figure 1.1. Protein misfolding and aggregation.

Cartoon from Forman (2004), reproduced with permission. Intrinsically disordered proteins may misfold for various reasons. The misfolded state is represented here by trapezoids. In a healthy cell, misfolded proteins are degraded by cellular waste disposal mechanisms, but in certain diseases the misfolded proteins are not disposed of and may aggregate to form fibrils. These fibrils agglomerate in the brain, in the case of PD in the cytoplasm, as Lewy bodies.

many food proteins, such as β -lactoglobulin, bovine serum albumin, and ovalbumin exhibit fibril formation as well (Sagis *et al.*, 2004; Arnaudov *et al.*, 2003). Amyloid fibril formation thus appears to be a generic process available to any polypeptide chain, just as protein folding is (Daggett and Fersht, 2003), when partly denatured under the right conditions (Guijarro *et al.*, 1998). General protein sequence characteristics that promote self-assembly are high hydrophobicity, high β -sheet propensity, and low net charge (Bemporad *et al.*, 2006; Chiti and Dobson, 2006).

It is possible to predict which regions of a protein have a high intrinsic aggregation propensity from the amino acid sequence. These regions are highly protected in the native state both in intrinsically disordered proteins and in globular proteins: the proteins need to be destabilized before they become available to intermolecular interactions (Jahn and Radford, 2005; Tartaglia *et al.*, 2008). These findings are corroborated by *in silico* techniques such as molecular dynamics simulations: a small number of amino acids in a peptide were found to produce hydrophobic interactions that have a large influence on aggregate stability (Lopez de la Paz *et al.*, 2005). The aggregation process of globular proteins differs from that of IDPs in the respect that globular proteins need to partially unfold before being able to form fibrils, whereas IDPs need to

take on a more static secondary structure before aggregating (Bouchard *et al.*, 2000; van der Linden and Venema, 2007).

One intriguing aspect of amyloid fibril formation is the multitude of morphologies that can be observed in an aggregating protein solution: the fibril polymorphism. Kodali and Wetzel (2007) discuss two types of polymorphism of amyloid fibrils. The first kind is the polymorphism observed in an aggregating protein solution as a function of aggregation time. A typical amyloid-forming protein starts as a monomer, several of which combine into oligomers, and then form long filaments that may or may not combine into mature fibrils. The other kind of polymorphism stems from the fact that within one species of protein, morphologically different mature fibrils can be observed. The morphological differences between those fibrils may be so pronounced, that it suggests that different assembly mechanisms are at work.

One rationale for the existence of such a large degree of polymorphism is that it is a consequence of a lack of structural constraints for a conformational state that does not have any function (Pedersen and Otzen, 2008). Not all aggregates are linear fibrils: several proteins have been shown to also form annular aggregates (Zhu *et al.*, 2004) that may permeabilize cellular membranes and so cause cell dysfunction (the ‘amyloid pore’ hypothesis, Lashuel *et al.*, 2002), and amorphous aggregates have also been observed. Finally, under conditions of low net charge, several amyloidogenic proteins including α -synuclein have formed gels of relatively monodisperse spherical particulates with an average radius of 250 nm (Krebs *et al.*, 2007).

The kinetics of amyloid fibril formation are often consistent with nucleation-polymerization mechanisms. Nucleation-polymerization processes have three characteristic features: (1) there is a lag phase before polymerization starts, (2) seeding can reduce that lag phase, (3) there exists a critical concentration below which no fibrils form (Frieden, 2007). The kinetics of α -synuclein fibril formation will be further discussed in chapter 5.

Amyloid fibril formation is not always a dead-end process, interesting only for the damage it does to cells: some proteins, such as the prion proteins, can transmit biological information (namely, their fold) from one molecule to another (Jones and Surewicz, 2005). Conformational changes of proteins thus transmit biological information within the lifetime of an individual organism (Soto, 2003). This allows the individual organism to adapt to changing environments through mechanisms other than genetic transfer of characteristics. Prion proteins are atypical amyloids in other ways as well: the fibrillization process is characterized by a dramatic effect of the reaction volume on the lag phase, with a volume-dependent threshold effect (Baskakov, 2007). These features cannot be explained by a nucleation-polymerization mechanism but instead a branched-chain reaction model can be applied. In branched-chain reactions, the creation of ‘active centers’ produces a very rapid growth of the polymer

(Baskakov, 2007). The branched-chain mechanism postulates that during the lag phase, mature fibrils are already present, but they are fragmenting to form new active centers.

In a non-pathogenic context, amyloid and amyloid-like fibrils have been shown to be functional materials (Chiti and Dobson, 2006): the amyloid fibrils present in the extracellular polymeric substance secreted by an algae have tensile strength in multiple directions perpendicular to the axis of the fibril, so that the alga can withstand environmental forces (Mostaert and Jarvis, 2007). And in bacteria such as *E. coli*, surface fibres known as curli may be assembled in the same way as amyloid fibrils: the harsh extracellular environment requires that fibres are formed without the help of chaperone molecules, use little folding energy, and are resistant to denaturation and chemical disturbances (Epstein and Chapman, 2008).

Protein aggregation is often considered to be the trigger of the cascade of events that result in neurodegenerative disorders: this is the ‘amyloid hypothesis’ (Lansbury and Lashuel, 2006). In the case of Parkinson’s disease, the culprit is α -synuclein, one of three synucleins (George, 2001), a family of proteins that is primarily expressed in brain tissue.

1.2 Aggregation of α -synuclein

α -Synuclein⁴ is a 140 amino acid cytoplasmic protein. It accounts for about 1 % of the total amount of cytosolic protein in nerve cells, and it interacts with many types of molecules in the cell, including lipids, other proteins, and metal cations. The *in vivo* function of α -synuclein is not clear-cut, but it most likely revolves around the many interactions α -synuclein can have with other molecules, and the conformational plasticity it exhibits while doing that. Several functions have been suggested, including the protection of nerve terminals against injury, the regulation of vesicle release and/or turnover, the inhibition of lipid oxidation, and molecular chaperoning of folding and refolding of synaptic proteins (see Uversky (2007) for a review).

α -Synuclein is an intrinsically disordered protein. This lack of static secondary and tertiary structure in α -synuclein allows it to adopt a multitude of conformations, adapting to its environment. When monomeric in solution, α -synuclein may adopt an ensemble of rapidly changing conformations (Maiti *et al.*, 2004). α -Synuclein has been found to assume a horseshoe-like two-helix antiparallel α -helical structure (fig. 1.2) when bound to small unilamellar lipid vesicles (Drescher *et al.*, 2008) or to membranes (Woods *et al.*, 2007), to take β -sheet form, for example when aggregated (Maiti *et al.*, 2004). α -Synuclein can

⁴The name ‘synuclein’ refers to that the protein was originally described as a protein specific to neurons and localized to the pre-synaptic nerve terminals, or SYNapses, and the NUCLeus (recounted in Uversky, 2007).

Figure 1.2. α -Synuclein may assume many secondary and tertiary structures

Ribbon diagram of α -synuclein (protein data bank structure 1XQ8) made using ArgusLab. When bound to membranes, α -synuclein takes α -helical secondary structure. Sites of disease-related mutations A30P, E46K and A53T are indicated.

also adopt an extended state under physiological conditions, and even a molten globule state under extreme environmental conditions (such as low pH, high salt, or high temperature). This flexible conformational behavior has earned α -synuclein the nickname of ‘protein-chameleon’ (Uversky, 2007).

The conformation of proteins inside aggregates can be determined through nuclear magnetic resonance (NMR) techniques. With NMR it is possible to deduce which sections of the protein molecules inside the fibrils are in close proximity to one another. Vilar *et al.* (2008) recently proposed a folding scheme where each α -synuclein molecule forms a five-layered β -strand sandwich perpendicular to the long axis of the fibril. These β -sheets then align parallel and in-register to form five β -sheets in the long axis direction of the fibril. However, solid-state NMR has also shown that the molecular structure in amyloid fibrils is not absolutely universal: both parallel and antiparallel β -sheets have been observed (Tycko, 2004). NMR studies also suggest that the structure of α -synuclein amyloid fibrils is encoded in the conformation of the monomeric protein (Kim *et al.*, 2007).

The propensity of α -synuclein to aggregate depends strongly on its environment. The addition of polyamines (Antony *et al.*, 2003), decreasing the pH, and increasing salt concentrations (Hoyer *et al.*, 2002), enzymes involved in protein folding (Gerard *et al.*, 2006, 2008), among others, are known to increase the rate and extent of fibrillation. There are also compounds that inhibit fibril formation: tissue transglutaminase, an enzyme that catalyzes cross-links within proteins (Andringa *et al.*, 2004), has been found to completely inhibit α -synuclein fibrillization, presumably by imposing structural constraints on α -synuclein (Segers-Nolten *et al.*, 2008). α -Synuclein fibrils can also dissociate, for example by application of high hydrostatic pressure (Foguel *et al.*, 2003). $A\beta$ fibrils have been dissociated by addition of 4,5-dianilinophthalimide (DAPH), a small molecule that reduced β -sheet content of aggregating $A\beta_{1-42}$ (Blanchard *et al.*, 2004). It should be noted that fibril stability *in vitro* does not mean that fibrils cannot be broken down *in vivo*: by shutting down transgene expression in a mouse model of Huntington’s disease, huntingtin- and ubiquitin

Figure 1.3. Several structural models for α -synuclein aggregation

Morphological studies of α -synuclein aggregates have led to the formulation of several structural models for α -synuclein fibril formation. α -Synuclein assembly can be hierarchical, segmented or amorphous in nature. Scheme based on Khurana *et al.* (2003) and Jansen *et al.* (2005).

fibrils disassembled (Diaz-Hernandez *et al.*, 2004).

The misfolding of α -synuclein is a prerequisite to amyloid fibril formation. The actual assembly of the resulting fibrils has been described by several structural models. The *hierarchical assembly model* or HAM defines three stages of aggregation (Ionescu-Zanetti *et al.*, 1999; Khurana *et al.*, 2003): a single sheet of β -sheet folded monomers forms, two of these protofilaments intertwine, and finally two of those intertwined filaments combine into a 'mature fibril' (fig. 1.3). If this model is correct, these mature fibrils consist of four filaments. However, there are also reports in the literature claiming there are other mechanisms at work in protein fibrillization, such as lateral assembly of segments (this was shown for insulin by Jansen *et al.*, 2005). Also, under many *in vitro* solution conditions no fibrils are formed, but the protein clumps together in amorphous aggregates instead. All these structural models of α -synuclein aggregation are summarized in fig. 1.3.

These structural models emphasize the formation of fibrillar aggregate species. However, aggregate species existing transiently during early stages of

aggregation may also play an important role in the amyloid-related diseases (Frieden, 2007). Oligomers contain more α -helical structure and less β -sheet structure than the fibrils do (Apetri *et al.*, 2006), and the oligomer population in α -synuclein aggregation peaks at the end of the lag phase, just before the formation of fibrils (Kaylor *et al.*, 2005; Fink, 2006).

α -Synuclein is associated with several neurodegenerative disorders. Among these are Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies, collectively called synucleinopathies (Recchia *et al.*, 2004; Uversky, 2007). Most incidences of Parkinson's disease are sporadic (non-inherited), but there are a few families with a hereditary variant of the disease. They have point mutations (that is, single amino acid substitutions in the primary sequence) at specific sites of the α -synuclein sequence: A30P, E46K, and A53T (Kruger *et al.*, 1998; Zarranz *et al.*, 2004; Polymeropoulos *et al.*, 1997). These point mutations are all in the central part of the amino acid sequence: the part of the protein that is incorporated in the core of the fibril is deemed essential for fibril formation (Giasson *et al.*, 2001). The mutations also cause increased aggregation rates (Li *et al.*, 2001; Choi *et al.*, 2004; Greenbaum *et al.*, 2005), and have different vesicle permeabilization propensities (Fredenburg *et al.*, 2007).

Fluorescence resonance energy transfer (FRET) experiments with Trp- and Tyr- mutated versions of α -synuclein have shown that the N-terminal part of α -synuclein is incorporated in the fibril core, whereas the C-terminus is exposed (Kaylor *et al.*, 2005). The C-terminus also helps to solubilize the monomeric protein, and it stabilizes long-range interactions within the protein to shield the central region and prevent aggregation (Bertoncini *et al.*, 2005). When the C-terminus is truncated, the aggregation proceeds faster (Serpell *et al.*, 2000; Hoyer *et al.*, 2004).

The mechanism by which α -synuclein and/or its various aggregated forms damage neurons has not been identified (Goedert, 2001). Research suggests neurodegeneration is due to either loss-of-function of α -synuclein itself, inflammation of the cell, or to toxicity of any of the β -sheet folded aggregation species (Soto, 2003). Injury to the mitochondria may be involved (Giasson, 2004), or damage to ubiquitination pathways (Giasson and Lee, 2003). Even the questions whether the aggregation of α -synuclein is a cause for or an effect of Parkinson's disease has not been settled (Lansbury and Lashuel, 2006). In toxicity studies on cultured neurons, the toxicity of protofilaments was practically abolished when filaments were broken in smaller aggregates using high hydrostatic pressure, suggesting that breaking or removing of protofilaments could be a strategy for alleviating the symptoms of PD (Follmer *et al.*, 2007).

Figure 1.4. Explosive increase in literature on synuclein and amyloid.

(a) Cover of James Parkinson's original 1817 Essay on the Shaking Palsy.

(b) A search for the topic 'synuclein' on Web of Science yielded 4,439 results in June 2008. In 1997, the first link between a genetic mutation in α -synuclein and Parkinson's disease was found (Polymeropoulos *et al.*, 1997); since 2006, on average 2 new papers on the topic 'synuclein' came out every single day.

(c) The literature on amyloid formation in general has been growing since the early 1980s, and very rapidly since about 1990. A topic search on 'amyloid' yielded 37,961 results in June 2008.

1.3 Parkinson's disease and Lewy pathology

Almost 200 years ago, James Parkinson (1755-1824) described the medical cases of six Londoners who suffered from symptoms such as involuntary tremulous motion and a propensity to bend the trunk forwards and fall (Parkinson, 1817, and fig. 1.4a). This was the first time this 'Shaking Palsy' was carefully described and distinguished from other, more or less similar disorders involving involuntarily shaking limbs. Some 45 years later, the term "Parkinson's disease" was first used (Neylan, 2002).

Despite massive research efforts into the etiology of Parkinson's disease and similar neurodegenerative disorders (fig. 1.4b and c show the increasing growth of literature), a definitive cure for the affliction has not been found. Currently prescribed medication is directed at alleviating the symptoms, not halting or

reversing the progress of the disease, and is accompanied by significant side effects (Schapira *et al.*, 2006). Dopamine-replacement therapies such as L-DOPA, for example, are effective at reducing PD symptoms, but at the cost of cumulative development of motor complications (Schapira *et al.*, 2006).

The main reason for this is that our knowledge of the etiology of the disease at the molecular level is as yet insufficient to develop drugs that target the cause, not the effect, of the neurodegeneration taking place in the brain of a Parkinson's disease patient.

The urgency of finding a cure for Parkinson's disease, as well as for other neurodegenerative disorders, is ever increasing: Parkinson's disease progresses with age, and in an ageing society more and more people will be affected in their quality of life. In the Netherlands alone there are an estimated 50,000 people with symptoms of PD (RIVM, 2006). The diagnosis of PD is made based on clinically observable symptoms, but there is no single set of symptoms that applies to every patient to the same degree, nor is the damage to the brain tissue as observed by a pathologist the same for every 'Parkinson patient'. It is therefore more appropriate to speak of *Parkinsonisms* than of a well-defined 'Parkinson's disease'. Parkinsonism can only be diagnosed by general symptoms; this may be a reason why as many as 25% of patients get an inadequate diagnosis, and subsequent sub-optimal medication (R. de Vos, personal communication). Such an incorrect assessment of the disease becomes apparent only after the patient has died and a pathologist examines the affected brain tissue under the microscope.

The pathological hallmark of Parkinsonism is so-called Lewy pathology, along with a loss of dopaminergic neurons in the *pars compacta* of the substantia nigra⁵ (Robinson, 2005). Lewy pathology includes two related types of inclusions: Lewy bodies and Lewy neurites (fig. 1.5b). Lewy bodies are relatively large inclusions (around 10 μm) in the cell body of neuronal cells, whereas Lewy neurites are thread-like structures found in cellular processes. Lewy pathology is found in various cell types of the central nervous system, but mainly — and first — in parts of the brainstem (Braak *et al.*, 2003). As the disease progresses, Lewy pathology develops in nerve cells and glial cells⁶ located in other parts of the brain as well (fig. 1.5a).

Over 50 different molecular species have been identified in Lewy inclusions, but the major component of Lewy pathology is α -synuclein. Whether the Lewy bodies and neurites themselves are toxic or harmless is unclear (Goldberg and Lansbury, 2000; Shults, 2006). The formation of Lewy inclusions may well be a protective mechanism employed by neurons: neurotoxic intermediate aggregate

⁵The substantia nigra (SN) is a part of the midbrain, which in turn is a part of the brainstem. The substantia nigra *pars compacta* (SNc) consists of $\sim 400,000$ nerve cells (Uversky, 2007) and is responsible for dopamine production. Disruption of dopamine biosynthesis leads to motor and cognition deficits.

⁶Glial cells give structural and metabolic support to neurons.

Figure 1.5. Lewy pathology characterizes Parkinson's patient's substantia nigra brain tissue

(a) Progression of PD-related pathology. Lesions occur initially in the brainstem (dark) and as the disease progresses, other parts of the brain become involved as well (arrows). Reprinted with permission from Braak *et al.* (2003).

(b) Photomicrograph of a section of substantia nigra brain tissue, stained with hematoxylin (blue, cell nuclei) and eosin (pink, cytoplasm) and an α -synuclein-specific labeled antibody (brown, Lewy bodies (LB) and Lewy neurites (LN)). The typical diameter of the LBs is $\sim 10 \mu\text{m}$. Sample courtesy of Rob de Vos, Laboratorium Pathologie Oost Nederland.

species are converted to inert mature aggregates and 'stored' in Lewy bodies (Uversky, 2007).

1.4 Research questions

The preceding sections introduced the processes of protein misfolding, amyloid formation, and aggregation of α -synuclein, and the relevance of these processes to the molecular-level understanding of Parkinson's disease. We now turn to the open questions we will seek to answer.

One of the most puzzling aspects of the process of amyloid formation is the structural diversity of the protein aggregates. Why are there so many different aggregate species? How did they come to grow in that particular way? Which form (if any) is the toxic species? Questions like these can only be answered if we have a thorough and quantitative understanding of the morphology and composition of these aggregates (Lyubchenko *et al.*, 2006). As a next step, information on structural characteristics of the aggregates *in situ*, that is, in the

human brain, is currently unavailable but much needed for a realistic understanding of the structure and development of amyloids (Kisilevsky, 2000). The morphologies found in brain tissue should also help distinguish which *in vitro* morphologies are relevant to brain diseases, and which should be considered experimental artifacts (Lundvig *et al.*, 2005).

Therefore, the general aim of the research described in this thesis is to contribute to the understanding of the etiology of Parkinson’s disease through quantitative, molecular-level morphological and structural characterization of α -synuclein aggregates, both *in vitro* and *ex vivo*.

Specifically, we seek answers to the following questions:

- a. How can the nanometer-scale morphological properties of protein fibrils be measured accurately? To what extent do α -synuclein fibrils display polymorphism?
- b. Are fibrils formed by disease-related α -synuclein mutants morphologically different from those formed by wildtype α -synuclein? Does that yield insight into the mechanism of aggregation?
- c. How can α -synuclein fibril formation be described biophysically?
- d. How does the *in vitro* morphology and structure of α -synuclein aggregates compare to α -synuclein aggregates in Lewy pathology in Parkinson patient’s brain tissue?

To answer these questions we need an experimental modality that allows quantitative nanometer-scale morphological characterization of protein aggregates. That modality is atomic force microscopy. The operation principles of atomic force microscopy and its application to the study of amyloid formation will be introduced and reviewed in chapter 2.

The research questions formulated above are in fundamental biophysics. When we understand the fundamental molecular biophysical processes at work in α -synuclein aggregation, we may begin a targeted search for drugs and therapies to combat the neurodegeneration that takes place in Parkinson’s disease and the other synucleinopathies.

Knowledge of the structure, function and aggregation mechanisms of amyloid fibrils is also of interest outside the context of human disease. Amyloid fibrils can be used as structuring agents in foods (van der Linden and Venema, 2007) and as new nanomaterials for potential use in nanotechnology applications (Knowles *et al.*, 2007). Nature itself uses amyloid fibrils as a building material as well, as evidenced by the presence of amyloid fibrils in an adhesive secreted by an alga (Mostaert *et al.*, 2006). The strength that non-pathogenic amyloid fibrils provide to composite materials makes them nature’s version of the carbon nanotube (Mostaert and Jarvis, 2007). Atomic force microscopy

and spectroscopy play a major role in the characterization and manipulation of amyloid fibrils in these fields as well.

1.5 Scope of this thesis

This thesis will detail the experiments we performed to answer the biophysical questions posed above. Chapter 2 will discuss the operational principles of atomic force microscopy, imaging artifacts, and the interpretation of AFM data in the context of amyloid fibrils. Then we will review the scientific literature on the use of atomic force microscopy in the study of amyloid fibrils. In chapter 3 we analyze the effect of various AFM imaging parameters, such as the imaging environment and the AFM probe, on the apparent morphology of protein aggregates.

We are then in a position to perform a quantitative analysis of the nanometer-scale morphological characteristics of fibrils formed by wild-type and disease-related α -synuclein mutants (chapter 4). Chapter 5 continues the biophysical characterization of fibrillar aggregates by comparing α -synuclein fibril length distributions to a statistical-mechanical model of fibrillization. With the model, we deduce the free energies of the interactions between the protein molecules in an α -synuclein fibril.

Chapter 6 attempts to bridge the gap between laboratory-based *in vitro* investigations of α -synuclein aggregation and the clinical occurrence of Lewy pathology. We explore the morphology and the protein secondary structure characteristics of α -synuclein aggregates found in brain tissue of Parkinson's disease patients using atomic force microscopy and Raman spectroscopy, and compare these results to the *in vitro* results described in the previous chapters.

The summarizing discussion in chapter 7 interprets the results obtained in chapters 2–6 in the context of the molecular biophysics of Parkinson's disease. We end by suggesting future directions for research that may eventually bring effective treatment of the amyloid diseases one step closer.

1.6 References

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Understanding amyloid fibril formation through atomic force microscopy

Amyloid fibrils are self-assembled protein aggregates which are involved in many human diseases, and which are of increasing interest as a biomaterial in nanotechnology. This chapter reviews which properties of amyloid fibrils can be visualized and which processes related to amyloid fibril formation can be understood through the use of various modes of atomic force microscopy (AFM). We start with a brief technical overview of AFM, with special reference to the measuring environment. After discussing the interpretation of height images, amplitude/error images, phase contrast AFM images, and AFM force spectroscopy data, we review what the judicious application of AFM can add to understanding the formation, morphology and mechanical properties of amyloid fibrils. We give examples from amyloid formation of Parkinson's disease-related α -synuclein, and discuss pathological and materials science applications of amyloid fibril formation.

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2.1 Introduction

The self-assembly of proteins into fibrillar aggregates known as amyloid fibrils is a topic of considerable interest, both in relation to the pathology of several human disorders (Chiti and Dobson, 2006) as in the development of novel biomaterials in nanotechnology (Knowles *et al.*, 2007a). As discussed in chapter 1, the multitude of morphologies these fibrils exhibit, also known as polymorphism, is a particularly important aspect (Kodali and Wetzel, 2007).

The morphology of protein aggregates may be studied to some extent with light microscopy, but at much higher resolution with electron microscopy (EM) and atomic force microscopy (AFM). AFM has the advantages that it requires little sample preparation, makes protein fibrils accessible to imaging under liquid. Furthermore, AFM allows for measurement of material properties through the tip-sample interaction, and manipulation of the sample with the tip. Recent reviews of the application of AFM to biological and supramolecular systems include Cohen and Bitler (2008); Muller and Dufrene (2008); Gosal *et al.* (2006); Samori (2005).

In order to be able to characterize amyloid fibrils, or indeed any biological system, through data obtained by AFM, it is imperative to understand the interactions between the measurement system and the sample. Also it is important to be aware of any artifacts that may be introduced by the imaging method. In this chapter we discuss the possibilities and limitations of AFM imaging of amyloid aggregates, and we review recent progress (roughly July 2004 – July 2008) in the understanding of amyloid fibril formation through the use of atomic force microscopy.

2.2 Atomic force microscopy

2.2.1 Working principle of AFM

The atomic force microscope (Binnig *et al.*, 1986) is a member of the scanning probe microscopes family. AFM descends from the scanning tunneling microscope (STM), where a sharp metal tip is scanned over a conducting surface detecting minute changes in sample topography through the strong distance dependence of the tunneling current. Atomic force microscopy relies on the tip-sample interaction forces for topography contrast. These forces are non-specific and do not require conductive samples, a major limitation of STM in the study of biomaterials.

The working principle of AFM is that a small cantilever (typical length $\sim 100\ \mu\text{m}$) with a sharp tip ($1 - 10\ \text{nm}$) scans a sample surface. As the tip encounters height differences or experiences changing tip-sample interaction forces, the cantilever bends (fig. 2.1). This deflection is detected and a feedback

Figure 2.1. Working principle of tapping mode atomic force microscopy

This simplified schematic gives an overview of the main components of a beam-deflection type AFM in amplitude-modulation tapping mode and explains the origins of the signals that will be referred to in the text. Comparing the amplitude signal with a pre-set setpoint value yields the *error* signal. The feedback electronics uses this signal to adjust the voltage to the *z*-piezo, changing the *height* of the cantilever relative to the surface. Meanwhile, the phase detector compares the phase of the amplitude signal with that of the tapping drive signal to yield the *phase* signal.

system moves the probe to keep the deflection at a set value. In tapping mode AFM, the cantilever is oscillated at its resonance frequency, and feedback is usually done on either the tapping amplitude or frequency signal. In this way, an AFM maps the nanometer to micrometer scale topography and surface properties of the sample. When AFM is performed in liquid, the cantilever and the sample are immersed in a small volume of liquid. This working principle and the signals that we will refer to in the rest of this chapter are displayed schematically in fig. 2.1.

There are several ways to setup an AFM, but the scheme shown in fig. 2.1

is a commonly used one¹. There are several methods of cantilever deflection detection (optically: with quadrant detectors or interferometry, or mechanically using bimetals, to name a few), and there are several ways of controlling the motion of tip relative to sample (the piezo elements or other actuators can be in the piezo tube attached to the cantilever, or they can be in the sample stage), but the general working principle remains the same.

Because AFM can also be used in liquid (Putman *et al.*, 1994a; Hansma *et al.*, 1994), it has proven a very useful technique for imaging biological samples such as DNA, proteins and cells, since these typically reside in an aqueous milieu. Sample preparation for AFM imaging of biological samples is relatively uncomplicated, and a multitude of modes of AFM operation has been developed to investigate various morphological, mechanical and even functional aspects of biomolecules, cells and tissues (Gosal *et al.*, 2006; Parot *et al.*, 2007). Figure 2.2 exemplifies the variety of biological systems whose morphology can be characterized with various modes of AFM.

2.2.2 Modes of AFM

Contact mode

In contact mode atomic force microscopy (CM AFM), the tip is in constant contact with the surface. The tip is effectively ‘dragged along’ the sample while the feedback loop keeps the interaction force constant by maintaining the deflection at a setpoint value. This mode allows for very high lateral resolution on periodic samples with low corrugation, but is typically too damaging for protein aggregates, especially in liquid.

The damaging nature of CM AFM can be used as a tool in studies of controlled damage to fibrillar aggregates. Fig. 2.3 shows that α -synuclein fibrils have ‘weak spots’ at intervals corresponding to their periodicity (Segers-Nolten *et al.*, 2007). This same effect was found for $A\beta_{1-42}$ fibrils (Arimon *et al.*, 2005). Fibrils may be damaged by forces as low as 100 pN during imaging in contact mode in air (Fukuma *et al.*, 2006). Other authors indicate fibrils are damaged by indentation forces ranging from 40 – 100 nN (Mesquida *et al.*, 2007).

Tapping mode

In tapping mode atomic force microscopy (TM AFM)², the cantilever oscillates at or near its resonance frequency. Feedback can be performed on the measured tapping amplitude or frequency (Garcia and Perez, 2002; Higgins *et al.*, 2005),

¹This type of AFM is used for example in the Veeco/Digital Instruments Bioscope series used in chapter 6.

²Tapping mode AFM is sometimes also called dynamic mode AFM.

Figure 2.2. Atomic force microscopy can image the topography of many types of biological specimen

Height images and representative line profiles as indicated by the white lines on the height images are given.

(a). Mitochondrial DNA, tapping mode AFM in liquid. Sample courtesy Henk Garritsen. Scale bar 200 nm.

(b). Membrane fragments containing light harvesting complexes. Tapping mode in liquid. In collaboration with Jaimey Tucker. Scale bar 200 nm.

(c). Red blood cells. Contact mode in air. Sample courtesy Hetty ten Hoopen. Scale bar 20 μm.

Figure 2.3. Contact mode AFM imaging reveals mechanical weak spots in α -synuclein fibrils

(a) Composite tapping mode AFM height image of A30P α -synuclein fibrils. Prior to imaging, the section between the *dotted lines* was subjected to contact mode imaging. The horizontal track in the middle results from repeated line scanning for optimization of imaging parameters. Scale bar 500 nm.

(b) and (c) Comparison of morphology of one fibril before (b) and after (c) contact mode imaging. The height profiles show that least damage was done on those sections that were lowest before scanning (*gray dashed lines*, scan direction was vertical). Horizontal scale bar 200 nm, vertical scale bars 2 nm. Images by K. van der Werf, figure adapted from Segers-Nolten *et al.* (2007).

giving rise to the terms amplitude modulated AFM and frequency modulated AFM respectively.

The intermittent tip-sample contact reduces lateral forces being exerted on the sample. The resonance frequency f_{res} is typically in the 100 kHz range in air, and in the 30 kHz range in liquid, and tapping amplitudes range from several nm in liquid to 100's of nm in air. The minimum tapping amplitude that is necessary in air depends on the force needed to escape the thin water layer due to air humidity that is present on any surface exposed to air.

TM AFM enables imaging of soft biological samples with minimal damage.

This allows one to measure protein aggregate dimensions and material properties in a state as close as possible to the native state. In liquid, high frequency tapping may lead to apparent stiffening of soft biological samples, reducing tip-induced damage even further (Putman *et al.*, 1994b, on cells). TM AFM is the mode most commonly used for imaging amyloid fibrils, and the amplitude of the tapping is one important factor that determines the extent of tip and sample wear. A second critical parameter is the setpoint ratio ($s = A_{set}/A_{free}$): the ratio of the tapping amplitude setpoint (to which the feedback loop regulates the measured amplitude, see fig. 2.1), to the tapping amplitude when the cantilever is suspended free in air or water.

Because both attractive and repulsive forces act on the cantilever, and because they depend on the tip-sample separation in a nonlinear way (as will be discussed in section 3.1.2), two stable oscillation states coexist in amplitude modulated AFM (reviewed in Garcia and Perez (2002), section 2.4.2). The equation of motion for the cantilever has two solutions: a high and a low amplitude branch. This means that a given setpoint tapping amplitude can be achieved at two distinct tip-sample separations. If the driving amplitude is large enough, both branches will merge into one, and only one stable solution to the equation of motion will exist (Garcia and Perez, 2002). For low driving amplitudes however, the AFM operator should be aware that branch hopping (and related height artifacts) may occur.

It is useful to qualitatively define tapping regimes. If s is lower than 30 %, we consider the tapping ‘hard’, if $s > 70\%$, tapping is ‘soft’. In tapping mode experiments on TTR_{105–115} fibrils in air, Mesquida *et al.* (2007) found that fibrils were not damaged by setpoint ratios between 10 – 80 %. This indicates the range of setpoint ratios used in amyloid research: the appropriateness of ‘hard’ or ‘soft’ tapping depends on the sample and the probe utilized.

Force spectroscopy and adhesion imaging

To measure the interaction forces between the AFM tip and the sample, force-distance curves may be measured. Force spectroscopy has evolved into a field of its own (for an exhaustive review, see Butt *et al.* (2005)). The cantilever is moved to the surface and retracted again in a sawtooth motion, while the cantilever deflection is recorded (see fig. 2.8 on page 38 for an example). The adhesion force can then be determined from the deflection before the tip snaps off the surface by applying the Hooke spring law $F = kx$, with k the spring constant of the cantilever and x the deflection. It is also possible use force spectroscopy to measure the forces needed to unfold domains of modular proteins such as titin (Higgins *et al.*, 2006).

Non-specific adhesion of the tip to the fibril may ‘mechanically unzip’ filaments from the fibrils (Fukuma *et al.*, 2006; Kellermayer *et al.*, 2005). When force-distance curves are recorded on every pixel in a raster pattern, a so-called

force-volume image or adhesion image is made (van der Werf *et al.*, 1994). These images show the strength of tip-sample interaction as a function of the location on the sample. When the AFM tip is coated with a ligand, strength and localization of specific interactions with a biological sample may be probed (Kienberger *et al.*, 2006), even at the single molecule level (Hinterdorfer and Dufrene, 2006).

2.2.3 Imaging conditions

AFM on biomaterials can be performed in air or in a liquid, such as an aqueous pH-buffering solution. The following paragraphs highlight the most important differences between imaging in air and in liquid; chapter 3 will describe the effect of the imaging conditions on measured fibril morphology in more detail.

Imaging in air

The main advantages of measuring in air are (i) that sample preparation is easier, since the sample does not have to be kept under liquid, and (ii) the sample can be used for months after preparation without degradation. A disadvantage of AFM in air compared to in liquid is that a tapping amplitude in the order of 100 nm is required (with associated high tapping force) to overcome the strong capillary force that is due to a thin water layer that tends to form on the substrate surface.

Imaging in liquid

One compelling reason for imaging in an aqueous pH-buffered solution is that it is an environment that resembles physiological conditions much more closely for proteins and other biological systems. Also, in an aqueous environment it is possible to study the interactions between biomolecules which are adsorbed to either the substrate or to the tip.

A disadvantage of imaging amyloid fibrils under liquid is that amyloid fibrils appear to be more fragile when imaged in liquid than in air (see chapter 3). Also, it is sometimes hard to bind the sample to the substrate (although that seems to be more of a problem for DNA than for protein aggregates). Another disadvantage is that the samples cannot be kept after use, since the evaporation of the water droplet leads to buffer salt deposition, while continued immersion in a large quantity of buffer would presumably lead to desorption of the aggregates.

A major application for AFM imaging in buffer is that aggregations can be monitored in real-time through *in situ* imaging: when the buffer contains monomers of the protein of interest and the buffer conditions are right, surface templated aggregation may occur (Hoyer *et al.*, 2004). Also, the directionality

of fibril growth becomes accessible (Blackley *et al.*, 2000; Goldsbury *et al.*, 1999; Zhu *et al.*, 2002).

Choice of probe and substrate

An AFM probe consists of a cantilever with a tip, and its properties are critical to the results of the imaging of biomolecular samples. The lateral resolution of AFM images depends on the sharpness of the tip that is used, and the cantilever stiffness and resonance frequency determine the force exerted on the sample, and any resulting damage (section 3.3.3).

The substrate used for imaging of amyloid fibrils is a relevant factor in *in situ* aggregation studies: the fibrils may form a pattern based on the atomic structure of the substrate (Yang *et al.*, 2002). The properties of the surface on which the sample is deposited also influence the kinetics and morphology of amyloid fibril formation. In an *in situ* TM-AFM study in liquid, the morphology of A β aggregates was different on hydrophilic mica than on hydrophobic graphite: on mica, particulate aggregates formed, whereas on graphite, linear assemblies similar to protofilaments formed (Kowalewski and Holtzman, 1999). This dependence of fibril morphology on substrate hydrophobicity implies that fibril formation may be expedited on interfaces between aqueous solutions and lipid surfaces, as would occur at cellular membranes and lipoprotein particles in a living cell. A β_{1-42} early aggregates deposit with a higher yield on hydrophilic mica than on hydrophobic graphite, and the opposite is true for mature fibrils (Arimon *et al.*, 2005). Finally, hydrophobic surface favor the formation of surface nanobubbles (Yang *et al.*, 2007), which is undesirable when imaging proteins. For AFM imaging of protein fibrils, freshly cleaved mica appears to be the most convenient substrate.

2.2.4 Sample preparation

The preparation of a protein aggregate sample for AFM imaging is crucial for the quality, reproducibility, and reliability of the images that are produced and influences the apparent aggregate morphology (Nilsson, 2004).

A typical sample preparation procedure for the imaging of amyloid fibrils in air, used in this thesis, is to deposit several μl of protein sample solution on freshly cleaved mica, let it settle for several minutes in a humid environment (*e.g.* a closed petri dish containing a wet tissue), and then wash gently with several 100 μl of demineralized water to remove unbound material and buffer salts. Then, the sample can either be left overnight to dry in ambient air, or be blown dry with a gentle stream of N₂(g). For measuring in liquid, the sample can be washed with the buffer used for imaging, and the sample must be kept under a drop of liquid at all times. A typical imaging buffer would contain salt ions (for example 50 mM NaCl), and a pH buffer such as TRIS or HEPES.

2.3 Interpretation of AFM images of protein aggregates

In analyzing atomic force microscopy images, it is important to be aware of the properties and limitations of the physical imaging process, so as to avoid over-interpretation of features and maximize the yield of information from the images. This section first considers artifacts resulting from the imaging process, and then moves to the interpretation of height, amplitude, and phase images.

2.3.1 AFM imaging artifacts

Tip-sample convolution

The physical interaction of the tip with the sample may result in several artifacts in AFM images. A well-known phenomenon in AFM imaging is the apparent broadening of features on the surface due to the finite diameter of the probe tip. This effect is known as tip-sample convolution. A simple model for this effect approximates amyloid fibrils as having a cylindrical cross-section (fig. 2.4). This simple model allows calculation of the tip radius r_t from the triangle equation, given the measured sample radius r_s (half the fibril height) and the apparent width w :

$$(r_t + r_s)^2 = (r_t - r_s)^2 + (w/2)^2 \quad (2.1)$$

Solving for r_t yields

$$r_t = w^2/(16r_s) \quad (2.2)$$

Double-tip artifacts

Double-tip artifacts are the multiple appearance of topographical features on the same image, whereas the feature is there only once (see fig. 3.6 on page 60 for an example). The spacing between the double-tip artifact features is typically in the order of tens to hundreds of nm. Double-tip artifacts may be caused by two general mechanisms. The first mechanism is that the final end of the tip consists of several sub-tips, causing the feature to be scanned several times. The second cause of double-tip artifacts is contamination of the tip or the cantilever by material from the sample or the imaging medium. These contaminations may then act as a second tip. Double-tip artifacts are usually easily recognized since all objects in the image will have their artifact counterparts next to them at the same distance and in the same direction relative to the original object.

Figure 2.4. Tip-sample convolution affects reported object width

Simple one-dimensional models for tip-sample convolution for helical fibrils. Fig. 4.2A allows calculation of the tip radius assuming the tip (green) is spherical and the sample (red) has a spherical cross section. In fig. 4.2B, a family of curves is displayed indicating the approximate tip diameter given a certain apparent fibril width and fibril radius in an image, according to the model in figure A ($r_t = w^2/16r_s$, see the text).

r_t , tip radius; r_s , sample radius; w , apparent width

Inhomogeneous surface coverage

One artifact that may skew the results of an investigation of amyloid fibril properties by AFM is the inhomogeneous surface coverage resulting from the vast difference in length scale between the sample objects and the substrate surface. Figure 2.5 shows a sample that is distributed very inhomogeneously on the mica surface. This is more problematic in AFM than in optical microscopies because the field of view is a factor of 10^8 smaller (order of μm^2) than the size of the substrate (cm^2). Microscopists should therefore always attempt to obtain a representative picture of a sample by imaging at least several sites on a sample. Localization of features of interest may thus be very time-consuming. The use of combined AFM- and optical microscopes ameliorates this issue, provided that at least one dimension of the objects of interest is large enough to be visible in optical contrast.

2.3.2 Height images

Most work on the morphology of amyloid fibrils as measured by AFM uses height images. Height images are typically produced by the TM-AFM feedback system: as the tapping amplitude reduces when the tip-sample interaction changes due to the proximity of a topographical feature, the feedback system changes the voltage to the z -piezo to bring the tapping amplitude back to the setpoint value (see fig. 2.1). The voltage to the z -piezo is the signal that

Figure 2.5. Sample coverage on the substrate may be very inhomogeneous

(a)-(c): Tapping mode AFM height images in liquid of wildtype α -synuclein fibrils on different locations on the same mica substrate exemplify the risk of obtaining un-representative images if not enough sites are compared. Scale bars 1 μm .

is represented in the height image, after a conversion to length space by a calibration factor determined by imaging a calibration grid with known height differences.

The noise level in the z direction of height images ideally is limited only by the Brownian motion of the cantilever, and is typically in the order of 0.1 nm. Laterally, the resolution is limited by the size of the tip (section 2.3.1) and is on the order of 10 nm.

The accuracy of the height image of a biological sample (to what extent does it represent the ‘true’ height of the feature) depends also on the imaging medium, because dehydration plays a major role in the morphology of the structures (see chapter 3 for a detailed discussion).

2.3.3 Amplitude images

Amplitude or error images indicate to what extent the piezo element in the z direction was unable to track the topography of the surface. A large signal upon

Figure 2.6. AFM phase contrast shows high-resolution fibril features

TM-AFM height image of A30P α -synuclein fibrils (a) with the corresponding simultaneously recorded AFM phase contrast image (b). The phase image (b) shows that fibril ends (*circles*) and certain specific parts of the fibril bulk have material properties differing from that of the bulk fibril. Scale bar 500 nm. *Image size, resolution, scan speed: 2 μ m, 256 pix/line, 16 μ m/s.* A three-dimensional rendering of this image is shown on the cover of this thesis.

the tip entering or leaving a higher feature indicates scanning was done too fast, or at too low feedback gain, for the feedback loop to track the surface exactly. Error images thus tend to be first derivative images in the scanning direction. No height information can be inferred from amplitude images, but information on the lateral size of features may be readily quantifiable, and amplitude images provide a useful contrast mechanism for high-frequency spatial information.

2.3.4 Phase images

The contrast mechanism in tapping mode AFM phase contrast imaging is the lag between the oscillation of the cantilever and the driving oscillation (fig. 2.1). This lag is due to the interaction between the tip and the sample/substrate, which may depend on any number of material properties and electrical charge of tip and sample.

When good phase contrast is achieved, the information in the images can add much to regular height and amplitude images. Figure 2.6 demonstrates that the ends of the α -synuclein fibrils and certain specific points in the bulk of the fibrils have a decidedly different phase lag than the rest of the fibril. The resolution of these patches of alternate tip-sample interaction is higher than the resolution of the height image, presumably because only the very ‘tip of the tip’ participates in the interactions that cause the phase difference. Possibly for the same reason, tip-sample convolution does not reduce the phase resolution in the same way as it reduces the lateral resolution of height images.

The exact origin of the oscillation lag and thus of the phase contrast is unclear and probably involves the higher harmonic mode of the cantilever oscillation as the tapping cantilever touches the sample surface. Energy transfer between resonance modes of the cantilever prohibits the application of a simple model where all energy lost in the oscillation as measured by the decrease in amplitude is considered to be absorbed by the sample. It may well be that the ‘lost’ oscillation energy has transferred to higher order oscillation modes of the cantilever (Stopel, van Raaij *et al.*, unpublished data).

AFM phase contrast images may reveal information that is not present in height images. Phase images show that paired helical filaments (PHF) formed by tau protein, involved in Alzheimer’s disease, carry a ‘fuzzy coat’ with different tip-sample interaction properties than the fibril itself (Moreno-Herrero *et al.*, 2004). We observed a similar fuzzy coat around fibrils produced from wildtype α -synuclein (see fig. 2.7). The fuzzy coat may be attributed to the phenomenon that segments of the protein molecules are not incorporated in the fibril bulk and protrude from the fibril body. These external protein segments may have different hydrophobicity than the part of the protein in the fibril itself, which we hypothesize to cause the altered tip-sample interaction force.

The exact imaging conditions that produce strong phase contrast in amyloid fibrils are notoriously hard to reproduce. Whether or not a good phase contrast is achieved depends on the correct choice of tapping frequency, tapping amplitude, and setpoint ratio ($A_{\text{setpoint}}/A_{\text{free}}$), and on environmental variables such as air humidity, the thickness of a potential thin water layer on the dry sample, and surface charges on the tip and/or the sample.

Often, phase contrast images appear identical to the error image: in such situations the phase contrast is not due to some material property but an artifact due to the imaging process.

The examples given here indicate that phase contrast images hold great promise as a method of characterization of material properties of amyloid fibrils. As discussed, two factors hold back the application of phase contrast at this point: the first is the establishment of imaging parameters that will yield reproducible phase contrast images, and the second is a thorough understanding of the origin of the altered tip-sample interaction that produces the phase lag. The solution to these issues would constitute a substantial advancement in the field.

2.4 Insight in amyloid fibril formation through AFM

This section will review how the careful application of AFM has contributed to the scientific understanding of the biophysical processes underpinning amyloid fibril formation in roughly 2004 – 2008. Earlier papers are reviewed by Gosal *et al.* (2006).

Figure 2.7. Phase images show fibril material properties

TM-AFM in air phase contrast images show that Alzheimer's PHF and α -synuclein fibrils have a 'fuzzy coat' of material that has different tip-sample interaction than the fibril bulk. AFM height (A) and phase (B,C) images of Alzheimer's PHF (reprinted with permission from Moreno-Herrero *et al.*, 2004). The 'fuzzy coat' around the filaments cannot be observed in the height images. Height (D) and phase (E) images of wildtype α -synuclein fibrils display a similar feature. Note also that the phase image has much higher spatial resolution than the height image (the banded periodicity seen in (E) is ~ 12 nm), and that a double tip artifact is visible in (E). Scale bar in (D) and (E) 200 nm.

2.4.1 Polymorphism

The clearest example of where nanoscale imaging methods like AFM can contribute to the understanding of amyloid fibril formation is in the classification of the multitude of aggregate morphologies that occur in both *in vivo* and *in vitro* protein aggregation. Generally, amyloid fibrils are unbranched, linear fibrils with a diameter of around 10 nm and lengths that run into the micrometer range (see for example chapter 5 of this thesis). The presence of several types of filaments, differing in their diameter, and the observation of periodicity or even twist in the thicker fibrils, has led to the prominence of the hierarchical assembly model discussed in section 1.1 (Khurana *et al.*, 2003), although polymorphism observed in A β fibrils suggests other mechanisms also play a role (Goldsbury *et al.*, 2005). Fibrils formed by various proteins differ in the number of filaments in their mature fibrils. For example, β_2 -microglobulin (β_2 M) fibrils, involved in dialysis-related amyloidosis, form three types of mature fibrils: consisting of two or four intertwined filaments, or consisting of four filaments and forming a twisted-ribbon structure (Kad *et al.*, 2003). These authors also found fibrils exhibiting supercoiling: the super-helical twist period was twice the twist period, which gives the fibrils a zig-zag appearance. Whether the various species form sequentially or in parallel is as yet unclear.

Polymorphism does not only occur comparing different fibrils: fibrils formed by full length prion protein display polymorphism even within individual fibrils, differing in number of constituent filaments and twisting at different points along their length (Anderson *et al.*, 2006).

One assembly mechanism that may contribute to this behavior is ‘lateral seeding’: monomers and oligomers from solution may attach to an existing fibril surface and initiate a new filament alongside an existing one. This mechanism had been proposed by Jansen *et al.* (2005) for insulin fibrils. One argument against the generality of lateral seeding is that it implies that fibrils would grow in width indefinitely: it has been shown on fibrils formed by many different proteins and peptides that only a small range of fibril diameters is formed.

Another clear case of polymorphism by fibrils formed by one protein is the case of glucagon, a 29 amino acid hormone involved in maintaining blood glucose levels and commonly used in the pharmaceutical industry for treating hypoglycemia. Glucagon fibrils were characterized by TM AFM in liquid and found to have several distinct fibril types, distinguished by the number of filaments apparently wound together to form a fibril (De Jong *et al.*, 2006; Dong *et al.*, 2006). The mature fibril types had two, three and four filaments intertwined respectively. Glucagon forms more than just fibrils: combined quartz crystal microbalance and AFM measurements have shown that first, a multilayer of monomers forms, which grows thicker and more viscoelastic, before fibrils start appearing and a polymorphic network is formed (Hovgaard *et al.*, 2007). In AFM images of hen lysozyme, fibrils were found to be accompanied

by small spherical aggregates at low pH (Arnaudov and de Vries, 2005).

The structural properties of amyloid fibrils formed by several single point mutant versions of the same protein also display a certain amount of polymorphism. Disease-related mutants of α -synuclein formed fibrils differing in diameter and periodicity (this thesis, chapter 4).

One key issue in the field of amyloid fibril formation pertains to the early stage aggregates, or oligomers: whether oligomeric species are on-pathway or off-pathway in the formation of mature amyloid fibrils is as of yet unresolved (Kodali and Wetzel, 2007). In partial answer to this question, Podesta *et al.* (2006) analyzed the eccentricity of early stage insulin aggregates and found that there is already a shape preference present in oligomers before any fibrils are formed, indicating the oligomers may be on-pathway to forming fibrils.

2.4.2 Mechanical properties of amyloid fibrils

One step beyond characterizing morphological properties of the fibrils under various conditions by AFM imaging, is to deduce mechanical properties of the specimen under study from those images. We review efforts to deduce the Young's modulus of fibrils, the bending rigidity, the forces required to break fibrils, and the strength of bonds holding fibrils together.

From a materials science perspective, relevant properties of fibrillar materials include the bending rigidity C_B and the Young's modulus $Y = C_B/I$, where I is the inertia. By analyzing the shape of fibrils of α -lactalbumin, variants of insulin, β -lactoglobulin, and TTR_{105–115} as measured from TM AFM images in air, Knowles *et al.* found that the bending rigidity C_B varies over four orders of magnitude (from 10^{-24} to 10^{-28} Nm²). The elastic modulus Y is in the order of 2 – 14 GPa for the fibrils, comparable to that of spider dragline silk (Knowles *et al.*, 2007a). These values are larger than those of other filaments occurring in living cells, such as those of tubulin and actin, which depend on being reversible and highly regulated in order to perform their function in the cell. Collagen, on the other hand, has a modulus on the order of 5 – 11 GPa (Wenger *et al.*, 2007).

The mechanical strength of amyloid fibrils is also accessible to AFM force spectroscopy. The forces required to break amyloid fibrils by pulling on them with an AFM tip are in the order of 300 – 500 pN (Smith *et al.*, 2006, on insulin). These fibrils have a very high strength and mechanical stiffness. In experiments where TTR_{105–115} fibrils were ‘unzipped’ mechanically by pulling with an AFM tip, a sawtooth pattern in the extension curve indicates a sequential breaking of β -strands from the intermolecular β -sheet at a force of 20 pN (Fukuma *et al.*, 2006). This process of breaking of a material in steps, by removal of so-called ‘sacrificial bonds’ revealing ‘hidden length’, is a mechanism that applies to many materials with high macroscopic mechanical tensile

Figure 2.8. Force-extension curves show unzipping and elastic behavior Force-extension curves of $A\beta_{1-42}$ fibrils with stretch (*darker*) and release (*lighter*) traces yield structural information: force plateaus indicate unzipping of sheets from the fibril (a) while elastic behavior is also observed (b), and sometimes even combinations of both (c). ΔF indicates the height of the force plateau. Reproduced with permission from Karsai *et al.* (2006).

strength (Fukuma *et al.*, 2006). These authors even speculate that the self-assembling nature of amyloid fibrils allows them to self-heal under appropriate conditions, which if true would make amyloid fibrils even more interesting as a nanomaterial. Amyloid fibrils appearing in other settings, such as in a natural adhesive secreted by an alga, also show force plateaus indicative of mechanical unzipping of fibrils, and large number of unfolding events indicating the fibrils consist of cross-linked proteins (Mostaert *et al.*, 2006). Molecular dynamics simulations have shown that $A\beta$ dissociation is highly anisotropic: $A\beta$ fibrils are strong but brittle when pulled perpendicular to the fibril axis, and less strong but soft when pulled along the fibril length (Raman *et al.*, 2007).

It is also possible to extract single monomers from fibrils. Single molecule force spectroscopy experiments have shown that cysteine-mutant prion protein molecules, attaching specifically to a gold-coated AFM tip, can be pulled out of an amyloid fibril with a force on the order of 100 pN, at a loading rate around 10 nN/s (Ganchev *et al.*, 2008).

The force holding monomers together may be measured by immobilizing monomeric protein molecules to an AFM tip and covalently binding them to an amino-functionalized mica surface via glutaraldehyde cross-linking and then pulling them apart using AFM force spectroscopy. The resulting dimer dissociation force increases strongly for α -synuclein, $A\beta$, and lysozyme monomers when they are pulled off each other in low pH solution (McAllister *et al.*, 2005). This indicates a difference in structure of the protein: CD spectroscopy confirmed that in the lower pH solutions, more β -sheet was present. In this investigation, the pull-off force for an α -synuclein-dimer ranged from 50 – 800 pN depending on the pH, being strongest at around pH 4.

The thorough analysis of force-extension curves may reveal much about

the strength of bonds holding fibrils together. Karsai *et al.* (2006) performed force spectroscopy on $A\beta_{1-42}$ fibrils and found that some fibrils show plateaus of constant force while being extended, indicating the steady ‘unzipping’ of a filament from a fibril, while others display reversible non-linear elastic stretching without any hysteresis (fig. 2.8). Both of these phenomena are completely reversible: even the segment that is unzipped in fig. 2.8a re-attaches and can be unzipped again many times (Karsai *et al.*, 2006). The unzipping force attributed to one $A\beta_{1-42}$ filament is 23 pN, and the distance between two $A\beta$ molecules is 4.7 Å (Serpell, 2000) so that the associated bond free energy is $\Delta G = F\Delta x = 23 \text{ pN} \cdot 0.47 \text{ nm} = 1 \cdot 10^{-20} \text{ J}$, corresponding to $\sim 6 \text{ kJ/mol}$, which is similar to the free energy of one hydrogen bond of $\sim 4 - 8 \text{ kJ/mol}$ (see table 5.1 on page 84).

Chapter 5 of this thesis describes how a statistical-mechanical model verified by measuring fibril length as a function of initial concentration, yields similar results for mature α -synuclein fibrils. The free energy of the bond between filaments as determined using this method is on the order of 10 kJ/mol, whereas the bond between monomers in a filament is much stronger, at around 16 kJ/mol (van Raaij *et al.*, 2008). In an analysis of the surprisingly strong structural order (Knowles *et al.*, 2006) of twisted insulin, SH3, A-chain and TTR fibrils, the energetic ‘cost’ of a defect in the twist angle of the fibrils was found to also be in the order of 16 – 20 kJ/mol (Knowles *et al.*, 2007b). Taken together, these data all point toward the prevalence of hydrogen bonding as a main factor in fibril self-assembly (Dobson, 2003).

2.4.3 Kinetic properties of amyloid fibril assembly

A third type of information that illuminates the time course of amyloid fibril assembly is the kinetics of the amyloid fibril assembly process. The kinetics of aggregation are often followed by biochemical methods, such as the fluorescence of a dye that binds to β -sheet folded protein (Ban *et al.*, 2003). The benzothiazole dye Thioflavin T is often used for following the kinetics of amyloid fibril formation, although it has been shown that ThioT binding to amyloid fibrils is rather unspecific, and various explanations have been offered for its binding to amyloid fibrils (Khurana *et al.*, 2005; Krebs *et al.*, 2005) and alternatives are available (Lindgren *et al.*, 2005; Volkova *et al.*, 2007, 2008).

Time-lapse AFM imaging (Stolz *et al.*, 2000) takes images either at different incubation times from a vial (Dong *et al.*, 2006) or *in situ* on surface-adsorbed growing fibrils (Goldsbury *et al.*, 1999). These authors have shown that amylin fibrils grow at rates of around 1 nm per minute and grow bidirectionally.

α -Synuclein fibrils exhibit growth rates between 10 – 50 nm/minute on mica (Hoyer *et al.*, 2004). Interestingly, this growth appears to happen in steps: there are periods of stagnation and periods of rapid growth. This finding resonates with the work of Kellermayer *et al.*, who performed scanning

Figure 2.9. Controlled dimer dissociation yields kinetic parameters

By rupturing α -synuclein dimers with an AFM tip (a) at a range of loading rates (b), Yu and Lyubchenko (2008) determined the off-rates, and thus transition times for 2 states in dimers as 4.0 s for the first and 0.08 s for the second transition (*fitted lines in (b)*). Reproduced with permission from Yu and Lyubchenko (2008).

force kymography: continuous scanning along the length of one $A\beta_{1-42}$ fibril on graphite shows that fibrils grow in 7 nm ‘bursts’, where the growth rate sometimes exceeds 100 nm/s during a short amount of time (Kellermayer *et al.*, 2008). These AFM kymography experiments, with high spatial and time resolution, promise to be very useful for understanding the mechanisms of protein fibril formation.

AFM force spectroscopy can also yield insights into the kinetics of amyloid fibril formation. An important kinetic parameter is the lifetime of the various intermediate aggregate species. By plotting the rupture force against the loading rate in a pulling experiment on α -synuclein dimers (fig. 2.9), the lifetime of the dimers was determined to be 4.0 s for the first transition (Yu and Lyubchenko, 2008). This number is in stark contrast to the dynamics of α -synuclein monomer conformation, which takes place at ns to μ s time scales. α -Synuclein dimers may thus serve as nuclei for further aggregation.

The force spectral fingerprints of molecules pulled from a surface using AFM spectroscopy have also revealed three classes of conformations of α -synuclein monomers: weak, ‘ β -like’, and random coil (Sandal *et al.*, 2008), although the authors have not been able to corroborate the existence of these populations using optical spectroscopy techniques.

2.5 Conclusion

This chapter reviewed the application of atomic force microscopy to understand the biophysical properties of amyloid fibril formation. Tapping mode imaging,

both in air and in liquid, has contributed to the understanding of polymorphism and mechanical properties of amyloid fibrils. The trend towards quantification of morphological parameters will help put insights into structure of amyloid fibrils on an even more solid footing. AFM force spectroscopy contributes to the understanding of the structure of amyloid fibrils by quantifying the forces that hold dimers, filaments and fibrils together, and even the lifetimes of the early aggregates.

The interpretation of height and amplitude images typically present no problems. The origin of tapping mode AFM phase contrast, on the other hand, and its translation to material properties of the sample, is as yet unresolved. Understanding phase contrast, and pinning down the imaging parameters yielding reproducible phase contrast, remains an important challenge and its resolution will add a valuable tool to the biophysicists' scanning force microscopy toolbox.

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The influence of AFM imaging conditions on the apparent morphology of protein aggregates

Atomic force microscopy (AFM) is a scanning probe microscopy technique that is well suited to characterize protein aggregate species at the nanoscale. One aspect that is often overlooked in the interpretation of AFM images is that it is crucial to consider the effect of the AFM imaging conditions on the reported aggregate morphology. The most important factor is whether imaging is performed under liquid, usually an aqueous buffered solution with salts, or in ambient air.

In this study we quantify the effect of the imaging medium on the apparent morphology of protein aggregates formed by α -synuclein, a protein implicated in Parkinson's disease. We describe how the choice of imaging environment and AFM probe influence the interpretation of AFM height images in studies of protein aggregate morphology. We find that α -synuclein fibrils appear $\sim 25\%$ less high in air than in liquid, and that sharper tips do not yield extra structural information on α -synuclein morphology. We also discuss the advantages and disadvantages of imaging in air *versus* in liquid.

The data presented in this chapter was obtained in large part by master's degree student Kim Sweers (figures labeled with *KS*).

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3.1 Introduction

Atomic force microscopy (AFM), introduced in chapter 2, is well suited to study the mechanical and morphological properties of biological materials at the nanometer scale. One field where such studies have made an important contribution to the understanding of a biological problem, is protein aggregation (reviewed in Gosal *et al.*, 2006, and in chapter 2 of this thesis).

The aggregation of proteins into a diversity of species, such as oligomers, fibrils and amorphous aggregates, is a process relevant to many human diseases. Parkinson’s disease, to name but one example, is associated with large intracytoplasmic neuronal inclusions consisting of the protein α -synuclein. Somewhere along the process of misfolding and aggregation, irreversible neuronal damage is done that results in the debilitating symptoms of the disorder. *In vitro* (and sometimes *ex vivo*) studies of the protein aggregates in various states of aggregation using AFM have dramatically advanced the knowledge of the fundamental biophysics of these processes (Gosal *et al.*, 2006).

One aspect of the imaging of protein aggregates using AFM that is not often analyzed thoroughly is the influence of the AFM imaging system, and particularly the measurement environment, on the reported morphology of the protein aggregates. Reports that do consider the effect of the measurement environment draw conflicting conclusions: the apparent diameter of amyloid fibrils has been reported both to decrease (Moreno-Herrero *et al.*, 2004) and increase (Chamberlain *et al.*, 2000) when changing from a liquid to an ambient air environment.

Our goal in this chapter is to quantify the effect of the choice of measuring in air or in liquid on the apparent morphology of protein aggregates formed by α -synuclein. Since contact mode AFM damages fragile protein aggregates under all imaging environments (section 2.2.2), we will only consider tapping mode AFM.

We will first discuss the AFM measurement environments that are amenable to the study of protein aggregate morphology. Then we discuss the influence of the environment on the forces between AFM tip and sample, and on the dynamic behavior of the AFM cantilever.

In order to quantify the effect of the imaging environment on apparent protein aggregate morphology, we measure the height of aggregates in various states of aggregation imaged in air and in liquid. We also test the effect of using ultrasharp (1 nm) tips on the apparent morphology of protein fibrils. Finally, the salt concentration in the imaging buffer may change during a measurement due to liquid droplet evaporation. This changes the electrostatic double-layer force and the hydration forces (Butt *et al.*, 2005, sec. 6.2), which influences tip-sample interaction, but may also affect the adsorption of aggregates to the mica substrate. To assess this effect, we quantify the desorption of small protein aggregates as a function of salt concentration.

3.1.1 AFM imaging environments

The measurement environments most commonly used in TM-AFM are air, liquid, and vacuum. In air, one measures either in ambient air or under an N_2 atmosphere; measurements in liquid are usually done with high purity water, with or without pH buffers, and with or without various salts that influence the conformation or folding state of the biomolecules and their adhesion to the substrate. Vacuum AFM can yield very high resolution images of hard materials but is not often used for biological samples.

In studies of biomolecules, imaging in air is usually easier to perform than imaging in liquid, but it necessarily involves dehydration of the biomolecules and thus constitutes a very un-physiological environment. Many studies therefore opt to image under aqueous buffers with appropriate salts, so that the proteins stay hydrated and in a more or less natural conformation. It is conceivable that the thin water layer that is always present on the hydrophilic mica surface in air due to air humidity will keep the fibrils hydrated, but this is not substantiated (Gosal *et al.*, 2006). Some authors also perform AFM imaging of amyloid fibrils under isopropanol (Stine Jr. *et al.*, 1996, on $A\beta$ fibrils) to reduce the pull-out (adhesion) forces that are stronger in water than in alcohols (Weisenhorn *et al.*, 1992), and observe fibrils similar to those measured under a water based buffer. If the objective of the study is to probe biomolecular interactions such as protein-protein or protein-DNA interactions in realtime, imaging in buffer is the only option.

3.1.2 Tip-sample interaction forces

The forces exerted by an AFM tip on a sample surface in air and in liquid have been discussed by Weisenhorn *et al.* (1989). In air, there are two transitions when a tip retracts from a surface: one when the tip lifts off the surface, and one when the tip breaks free of the thin water layer adsorbed to the surface. In water, there is just the transition from lifting off the surface itself. Atomic resolution by AFM is typically obtained only on stiff, solid samples in air or vacuum (and not in liquid): repulsive forces acting at $\text{\AA}$ distances are able to sense point-to-point differences on the surface on the atomic scale, whereas the attractive forces that govern imaging in liquid are able to resolve only structures with sizes of the order of the tip radius r_t (Goodman and Garcia, 1991).

The main forces acting on an AFM probe in tapping mode are the oscillating driving force F_{drive} , the interaction between the tip and the sample $F_{\text{tip-sample}}$, and in liquid a drag force F_{drag} on the cantilever, directed opposite of $F_{\text{tip-sample}}$. Several forces contribute to the tip-sample interaction: short-range forces, such as Born repulsion and hydration repulsion; long range Van der Waals (vdW) forces; capillary forces; and electrostatic interactions. Their origin, direction, range and magnitude as a function of imaging medium are

summarized in table 3.1.

It is clear from table 3.1 that the presence, range and strength of the forces between an AFM tip and a sample depend strongly on the imaging medium. The most important differences are the absence of capillary forces and the ten-fold reduction of vdW forces in liquid compared to in air. TM AFM induces elastic and non-elastic deformations of the sample, making it difficult to establish the magnitude of interaction forces at very short range.

In AFM on biological samples, the short-range hydration repulsion that is due to the removal of the last molecular water layers trapped between a tip and a sample is often neglected (Butt *et al.*, 2005, sec. 6.2), even though AFM imaging of hydration layers on lipid bilayers has shown that these layers are stable enough to present multiple energy barriers to nanoscale objects attempting to approach the bilayers (Fukuma *et al.*, 2007). The potential of the hydration force is $U_A = A \exp -r/\lambda_H$ (Butt *et al.*, 2005), with typical amplitude $A = 10^{-3} - 10 \text{ J/m}^2$ and typical decay lengths $\lambda_H = 0.2 - 1.4 \text{ nm}$. If we assume $A = 1 \text{ J/m}^2$, $\lambda_H = 1 \text{ nm}$, then at a distance $r = 1 \text{ nm}$, the magnitude of the hydration force F_H would be

$$F_H = -\frac{dU_A}{dx} = \frac{A}{\lambda_H} \exp -r/\lambda_H \sim 0.4 \text{ nN} \quad (3.1)$$

The strongest contribution to the tip-sample interaction when imaging in air is from the capillary force (table 3.1). VdW forces can also be long-ranged since it falls only as $1/D$ in a sphere-surface geometry: the vdW potential is $w(D) = -AR/6D$, where D is the distance between sphere and surface, A is the Hamaker constant, and R is the radius of the sphere (Israelachvili, 1991, chapter 11).

The electrostatic interaction depends strongly on charges on tip and sample. Mica is hydrophilic and negatively charged at all pH values. Glass also carries a net negative charge, but both glass and mica can be coated with for example poly-L-lysine (PLL) to obtain a positive surface charge at neutral pH. The SiO_2 layer on a Si tip is negatively charged above pH 3 (Butt *et al.*, 2005, sec. 6.1); a Si tip is much more likely to bear a substantial surface charge than an Si_3N_4 tip. In aqueous medium, the electrostatic force is the electrostatic double-layer repulsion, which becomes more and more screened at higher salt concentrations, but hydration forces become stronger with increasing salt concentration (Butt *et al.*, 2005, sec. 6.2).

In amplitude modulated tapping mode AFM, the combination of the forces described above and their non-linear dependence on tip-sample separation leads to the coexistence of two stable states of oscillation of the cantilever (Garcia and Perez, 2002, sec. 2.4.2), as discussed in section 2.2.2.

contributing force	origin	direction	range		magnitude	
			in air	in liquid	in air	in liquid
$F_{\text{short range}}$ (Born repulsion)	charge overlap	repulsive	< few Å ^a			
$F_{\text{short range}}$ (hydration repulsion)	removal of water layer	repulsive	0.2 – 1.4 nm ^b		0.4 nN ^b	
$F_{\text{long range}}$ (Van der Waals) ^c	dipole interactions	attractive	up to 10's of nm ^a		6 nN ^d	reduced 10-fold or more ^a ; 0.9 nN ^d
$F_{\text{electrostatic}}$	electrostatic interactions	depends on sign of charges	up to μm ^e	10's of nm ^f	10 – 100 nN ^e	~ 10 nN ^g
$F_{\text{capillary}}$ ^h	capillary effect	attractive	100's of μm	not present	100 – 300 nN ⁱ	not present

Table 3.1. Comparison of forces contributing to tip-sample interaction in tapping mode AFM in air and in liquid.

Forces are listed in order of increasing range. Values for magnitude are rough guides to the magnitude of the force at the range specified below.

- a. Goodman and Garcia (1991)
- b. Butt *et al.* (2005, sec. 6.2)
- c. The term ‘Van der Waals force’ is sometimes used as a container term including many types of non-covalent interactions. According to Atkins and De Paula (2006, 7th ed., p. 696), “Van der Waals forces are the interactions between closed-shell molecules” including “the repulsive interactions that prevents matter from collapsing completely”, which are considered Born repulsion here.
- d. Weisenhorn *et al.* (1989, for a tip with $r_t = 20$ nm)
- e. Butt *et al.* (2005, fig.15), between 1-100 nm
- f. The decay length of the electrostatic interaction in a liquid (Debye length $1/\kappa$) scales with the (monovalent) ion concentration c as $\lambda = 0.305 \text{ nm}/\sqrt{c}$ (Israelachvili, 1991, section 12.15). So at a typical $c(\text{NaCl}) = 50 \text{ μM}$, $\lambda = 43$ nm.
- g. Butt *et al.* (2005, fig. 20), between 1-10 nm.
- h. Also called meniscus force
- i. Farshchi-Tabrizi *et al.* (2008, depending on relative humidity)

Figure 3.1. Many cantilever resonances occur in a liquid cell

A Bode plot of the frequency response of cantilever MSCT-F in the Bioscope I/Nanscope IV system with fluid cell shows many resonances. There are no definite rules for picking the best one for imaging. The spectra also depend on the distance to the surface (*solid line*, far from surface (> 1 mm); and *dashed line*, close to the surface ($2\ \mu\text{m}$)).

3.1.3 Dynamic behavior of AFM cantilevers

Imaging in a liquid environment also changes the resonance behavior of the cantilever from its ‘normal’ behavior in air. The resonance frequency of the cantilever is about 25 % of its value in air due to hydrodynamic damping and the extra mass due to the displaced liquid. In liquid there are many more due to the sample chamber and the liquid droplet (fig. 3.1). The frequencies of those resonances depend on the dimensions of the sample chamber and the liquid droplet, so finding the best resonance mode may require some trial and error. In practise, using peak with the highest amplitude does not always yield the best images. Figure 3.1 also shows that at lower frequencies, the frequency response depends on the tip-sample distance, but at the ideal tapping frequencies around 32 kHz there is hardly any effect. The ‘jumps’ in the phase plot are due to the ‘wrapping’ of the signal at ± 180 degrees: every resonance peak produces a 180 degree phase shift.

The choice of imaging in air or in liquid also influences AFM phase contrast (section 2.3.4). A ‘clean’ second order oscillatory system exhibits a 180 degree phase shift between the driving sine wave and the detected deflection signal at its resonance frequency. The extent of phase contrast then depends on the degree of damping present in the system, represented by the quality factor Q

(Marion and Thornton, 1995, chapter 3):

$$Q \equiv \frac{\omega_R}{2\beta} \quad (3.2)$$

where $\omega_R = \sqrt{k/m - 2\beta^2}$ is the resonance frequency of a driven and damped system and β is the damping coefficient. A higher Q (less damping, higher resonance peak, slower response to height features) means a sharper phase transition and thus more phase contrast. In air, there is much less damping than in liquid, so in air, AFM cantilevers have a higher Q factor (typically $\sim 100-300$), and in liquid a lower Q factor (typically $\sim 1-3$) and correspondingly low phase contrast. An advantage of measuring in liquid is that the quality factor is inversely related to the response time of the system to height features: a lower Q means a faster response and thus a better lateral resolution (Gosal *et al.*, 2006).

The sensitivity to changes in phase depends on the distance from the tip to the surface. From the phase plot in air (fig. 3.2a), it can be seen that phase sensitivity become smaller when close to the sample. The amplitude of the resonance peak itself is also damped substantially (when driven at the same driving voltage). This is likely due to the long-range capillary and Van der Waals forces acting on the cantilever in air. A similar size frequency window around a resonance peak in liquid (fig. 3.2b) shows that several peaks are spaced closely together. Comparing the dashed and solid lines in both plots, we see that in liquid, the effect of the distance of the cantilever to the surface is negligible.

It is possible to use the same cantilevers for imaging in air and in water-based buffer, ideally with a force constant in the order of 0.5 N/m (Putman *et al.*, 1994). The most important difference between measuring in air *versus* in liquid in terms of the motion of the cantilever is that in air, the cantilever deflection decreases symmetrically with decreasing tip-sample distance, whereas in liquid, only the bottom envelope of the oscillation changes (Putman *et al.*, 1994). This is due to the much lower Q in liquid caused by the damping of the oscillation by the liquid. The oscillation amplitudes can be 10 times lower in liquid than in air because in liquid, the tip does not have to break free of the thin water layer that is always present on the hydrophilic mica surface in air, and consequently much less damage is done to the sample.

3.2 Methods

Protein expression, purification and aggregation

Human α -synuclein with Parkinson's disease-related point mutation E46K was expressed in a bacterial system, purified and induced to aggregate as described

Figure 3.2. Cantilever resonance is complex in AFM in liquid

Bode plots of resonance of cantilever MSCT-F in air (a) and liquid (b) on the Bioscope I/Nanoscope IV system. The plot in air shows only one resonance at the cantilever’s advertised resonance frequency, but in the liquid cell there are several resonances close together. In liquid, the frequency response does not depend on distance nearly as much as it does in air (*solid line*, far from surface ($\sim 250\ \mu\text{m}$); and *dashed line*, close to the surface ($1\ \mu\text{m}$)). The phase sensitivity is the slope of the phase *vs.* frequency curve.

in chapter 4.

AFM sample preparation

All samples were deposited in $4\ \mu\text{l}$ aliquots on freshly cleaved mica. Samples were allowed to settle for 2 minutes in a humid environment. Samples for measurement in air were rinsed with $400\ \mu\text{l}$ Milli-Q water to remove any unbound aggregates and salts, and were then blown dry with a gentle stream of nitrogen. Samples for measurement in liquid were rinsed with $400\ \mu\text{l}$ of imaging buffer (10 mM HEPES, 50 mM NaCl, pH 7.4) and were kept wet at all times to avoid artifacts due to protein dehydration and salt crystallization.

AFM probe selection

We used MSCT-AU, tip F (Veeco, Plainview, NY, USA) Si_3N_4 triangular cantilever probes; nominal tip radius $r_t = 10\ \text{nm}$, nominal cantilever spring constant $k = 0.5\ \text{N/m}$ and typical resonance frequency $f_{\text{res}} = 132\ \text{kHz}$. This

probe was selected because of its reliable performance in our lab, and because the same tip can be successfully applied to both imaging in air and in liquid. For the probe comparison test, we also used Veeco ORC8-F and NP-s!0-A, Nanoworld NLCR-10, MikroMasch NSC36-B and HI'RES-DP18 and DP19 probes (Sweers, 2008). The data shown in fig. 3.5 is from MSCT-AU-F and HI'RES DP19 ($k = 3.5$ N/m, $f_{res} = 75$ kHz, and $r_{tip} = 1$ nm).

X, Y and Z axes were calibrated using a test grid.

AFM imaging in air

Images were made using a lab-built AFM (van der Werf *et al.*, 1993) in tapping mode. Unless otherwise noted, image parameters were: scan area $2 \times 2 \mu\text{m}^2$, 256×256 pixels, scan speed $16 \mu\text{m/s}$. Tapping amplitude in air was always less than 100 nm: it was set just above the minimum amplitude needed to break free from tip-sample adhesion due to capillary forces. Feedback gains were set to just below the point of cantilever oscillation.

All images shown in this chapter are topography (height) images. Raw topography data was corrected for scanner bow (3rd order least-mean-squares average profile) and scanner jumps (0th order line-wise leveling) while excluding any objects in the image from the calculation, using SPIP image analysis software (Image Metrology, Hørsholm, Denmark), as detailed in appendix 7.4.

AFM imaging in liquid

Imaging in liquid was accomplished using the same setup, settings and probe as for imaging in air, except that the tapping amplitude was set to < 10 nm, and the resonance frequency was typically ~ 32 kHz. The imaging buffer contained 10 mM HEPES, 50 mM NaCl, and the imaging droplet size was about $250 \mu\text{l}$.

Fibril height measurement

The average height was determined using the SPIP analysis package, by making a line profile along the long axis of the fibrils and placing cursors at the fibril ends. The average height along the profile can then be read. Any non-zero background heights were averaged and subtracted from the measured height. Only fibrils longer than ~ 120 nm (2 periods of helical twist, see chapter 4) were taken into account.

Effect of NaCl concentration on aggregate adsorption

A mica disk with α -synuclein oligomers was imaged as described above in a $250 \mu\text{l}$ droplet of buffer (10 mM HEPES, 50 mM NaCl). The number of oligomers present in 12 images of $2 \times 2 \mu\text{m}^2$ was counted. The concentration of NaCl was increased in 10 mM steps by the addition of appropriate volumes

Figure 3.3. Fibrils tend to cluster upon drying

AFM height images of E46K α -synuclein fibrils as a function of incubation time and imaging environment. Apart from a tendency to cluster when dried, and a reduced apparent height in air, the gross morphology of E46K α -synuclein fibrils as measured by AFM in air *versus* in liquid is similar. *Images KS. Image size: 2 μ m.*

of concentrated NaCl in buffer. After a 12 minute incubation period for each concentration step, a new set of images was made on the same area of the sample and the number of oligomers was counted again.

3.3 Results

3.3.1 Influence of sample preparation in air and in liquid

The drying of a sample that is necessary for AFM imaging in air results in fibrils clustering together on the mica (fig. 3.3). This agglomeration hinders the individual characterization of the fibrils, and may lead to misinterpretation of the degree of aggregation in a sample. As incubation time progresses, more fibrils are found to agglomerate when measuring in air (fig. 3.3a-d). In liquid, we did not observe substantial clustering of fibrils (fig. 3.3e-g). We did observe more double-tip artifacts in liquid, such as in fig. 3.3g, presumably caused by protein sticking to the tip and resulting in a larger apparent width of the fibrils.

	<i>height (nm) t = 48 h</i>	<i>height (nm) t = 72 h</i>	<i>height (nm) t = 96 h</i>
<i>oligomers:</i>			
ambient air	4.38 ± 0.9 (N = 107)		
liquid	4.32 ± 1.18 (N = 108)		
<i>fibrils:</i>			
ambient air	5.22 ± 0.45 (N = 18)	5.7 ± 1.08 (N = 62)	6.25 ± 1.26 (N = 48)
liquid	7.44 ± 1.06 (N = 17)	6.75 ± 1.26 (N = 72)	8.51 ± 1.75 (N = 52)
		5.62 ± 0.47 (N = 35)†	6.81 ± 0.82 (N = 24)
		7.82 ± 0.73 (N = 37)	9.96 ± 0.68 (N = 28)
reduction*	29.8 %	17.5 %	26.5 %

Table 3.2. Apparent height of E46K α -synuclein fibrils and oligomers as a function of aggregation time and imaging medium

* The reduction in apparent fibril height in air relative to that in liquid.

† The height distribution in liquid at $t = 72$ h and $t = 96$ h appears to have two modes, see fig. 3.4. *Data KS/MvR.*

3.3.2 Fibril morphology as a function of imaging environment

The apparent diameter of the fibrils increases during the aggregation process, both as measured in air and as measured in liquid (fig. 3.4). The apparent height is larger when measured in liquid than when measured in air. In liquid, we observe two populations of fibrils. If we average the heights of the lower mode, cutting it off at 6.5 nm ($t = 72$ h) and 8.5 nm ($t = 96$ h) respectively, we observe that the average fibril height of the lower mode is close to the average fibril height of the fibrils measured in air (table 3.2). It may then be that in liquid, we see two distinct populations of fibrils, filaments and mature fibrils, whereas in air we only observe one of them (likely the dehydrated mature fibrils).

If we consider the entire distribution of fibrils in liquid to be one species of fibril, the average apparent fibril height decreases by a percentage that depends on the state of the aggregation but does not increase or decrease linearly with aggregation time (table 3.2).

3.3.3 Influence of probe properties on apparent morphology

The sharpness of the AFM tip influences the apparent morphology of protein aggregate samples under study. We tested a several probes as listed under methods, differing in resonance frequency, spring constant and tip sharpness (Sweers, 2008). We found that the image resolution increased only marginally upon use of tips that were specified to be much sharper (fig. 3.5). We found the Si₃N₄ Veeco MSCT-F tip, with specified $k = 0.5$ N/m, $f_{res} = 120$ kHz, and $r_{tip} = 10$ nm, to be easy to handle, have reliable probe-to-probe properties,

Figure 3.4. Fibril height appears larger in liquid than in air

Apparent fibril height histograms based on AFM images of α -synuclein fibrils on mica in increasing stage of aggregation, both in buffer (blue, right hatched bars) and in ambient air (red, left hatched bars). The histograms for $t = 72$ and 96 h suggest that in liquid, two fibril populations are present. *Data KS.*

and produce reasonable resolution images (fig. 3.5a). We compare this tip to a very sharp silicon MikroMasch DP18-HI'RES probe, specified as $k = 3.5$ N/m, $f_{res} = 75$ kHz, and $r_{tip} = 1$ nm using the same sample (fig.3.5b). The observed fibril morphology is similar: both fibrils profiled have the same average height and similar periodicity (45.5 nm *versus* 42,0 nm) (fig. 3.5c and d). The 25-line averaged perpendicular profiles also shown indicate the DP18 tip gives the fibril an apparent width of 44 nm, whereas the MSCT-F tip makes the fibril appear to be 60 nm wide. The simple tip-sample convolution model in eqn. 2.2 yields estimated tip radii of 45 and 24 nm respectively, in stark contrast to the 10 and 1 nm specified by the manufacturer. A disadvantage of the super sharp probes is that they are much more fragile and were harder to handle than the MSCT probe.

Figure 3.5. Sharper tips give slightly better resolution

Height images of E46K α -synuclein fibrils made in tapping mode AFM in air, with profiles showing the differences between a MSCT-F probe (a) and a DP-18 (super sharp) probe (b). Scale bars 250 nm. The *segmented lines* on the fibril mark the locations of the height profiles shown in (C) and (D), and the *dotted boxes* mark the locations of the 25-averaged cross-section profiles. Horizontal scale bar in (C) and (D) 250 nm, vertical scale bar 2 nm. Data KS/MvR. Image size, resolution, scan speed: (A) and (B): 1 μm , 256 pix/line, 8 $\mu\text{m/s}$

One disadvantage of imaging in liquid is that aggregates are more prone to tip-induced damage when imaged in liquid (fig. 3.6). Consecutive scanning leads to the tip damaging the sample: the tip carries material away in the direction of the scanning motion. Also note that the tip produced a double tip artefact: the fibril appears twice. This is likely caused by some protein being stuck to the tip.

Tapping mode AFM in liquid can provide very accurate nanoscale topography of protein aggregates. However, even the gentlest tapping mode AFM procedure is a mechanical process: in our experiments, the tip touches the surface some 32,000 times per second. We have observed repeated instances of fibril breakage due to the AFM tip, where the disassembly clearly occurred by the subsequent removal of segments of the fibril (fig. 3.7).

Figure 3.6. AFM in liquid is prone to induce damage to biological samples

Four consecutive height images of an α -synuclein fibril on mica in liquid show that even under gentle tapping conditions ($A_{\text{tap}} \sim 3$ nm) it is possible to damage a fibril. Scan direction is top-bottom. Note also that the tip was already contaminated with protein material (double-tip artifact). Scale bar 200 nm.

Two striking observations can be made based on fig. 3.7: first, the inner segments are damaged and removed by the scanning first, not the outermost ones; and second, the fibril parts that remain, in for example the third frame, have about half the fibril height. The first observation points to a segmented association model being a more appropriate model of α -synuclein assembly than the hierarchical assembly model. The second observation however, suggests that in any given segment, the fibril consists of at least two filaments.

3.3.4 Influence of salt concentration on aggregate adsorption

During long imaging sessions under liquid, the liquid droplet will partially evaporate. To assess the influence of the resulting increased salt concentration on the adsorption of protein aggregates, we quantified the oligomer density on a mica surface. We chose oligomers because due to their small size there can be many more per μm^2 of mica than of larger aggregates, increasing efficiency of the measurement.

The adhesion of the α -synuclein oligomeric aggregates is unaffected by a moderate increase in the concentration of monovalent sodium and chloride ions (from 50 to 130 mM) that may occur when the imaging water droplet partially evaporates during the course of a 2-hour AFM imaging session (fig. 3.8). A linear fit to the number of oligomers adsorbed to the mica per unit area *versus* NaCl concentration indicates that the number of oligomers still attached to the surface remains constant to within less than 0.1 % per mM, which is well within the error margins of the measurement.

Figure 3.7. Fibril disassembly by scanning with an AFM tip

In this series of time-lapse tapping mode AFM height images in buffer, the fibril dissociates in a segmented fashion. Note that the inner segments are affected first, and not the outermost ones. The line profiles indicate that the segment height reduces to approximately half the mature fibril height before the segments disappear. Finally, it is interesting that the disassembly does not take place in the scan direction (which is horizontal), but perpendicular to it. Scale bar on AFM image 100 nm, horizontal scale bar on profiles 50 nm; vertical scale bar 2 nm. These are 5 images of a series of 30.

3.4 Discussion

Apparent protein aggregate morphology

Imaging under air *versus* under liquid leads to differences in the observed sample morphology. We observe that α -synuclein fibrils imaged in air appear between 17 and 30 % lower than the same fibrils imaged under liquid. These findings confirm that morphological properties of protein aggregates as measured in air should be corrected for the dehydration associated with measuring in air. Our findings are consistent with measurement on other amyloid-forming

Figure 3.8. Aggregate adsorption to mica does not depend on [NaCl]

Adsorption of oligomeric α -synuclein aggregates to mica as a function of NaCl concentration. Adsorption at [NaCl] = 50 mM is taken to be 100 %. Red dashed line is a least-mean-squares linear fit to the data. The number of oligomers counted at [NaCl] = 50 mM was 156. *Data KS.*

proteins, but conflict with others. Moreno-Herrero *et al.* (2004) compare the morphology of Alzheimers' paired helical filaments (PHF) consisting of tau protein when imaged under air and liquid, and find that in air the filaments appear 20 % less high and that peak-valley ratios are different, presumably due to dehydration and differences in capillary-, electrostatic- and Van der Waals forces. A reduction in apparent height when imaged under air relative to when imaged under liquid is also found in other types of biological samples, such as *E. coli* cells (> 50% reduction, Beckmann *et al.*, 2006)), and DNA (30 – 50% reduction, Liu *et al.*, 2005). However, there are also studies that report an *increase* in apparent height in protein aggregates under air: amyloid fibril height in TTR_{10–19} fibrils was 1 – 2 nm higher in air than under liquid (Chamberlain *et al.*, 2000). These authors attribute the lower apparent height in liquid to the flexibility of the hydrated fibrils in response to the force exerted by the AFM tip: the deformation by the tapping.

The observed morphological variations between fibrils imaged in air *versus* under liquid may then be due to differences in the state of the sample (mainly its hydration state, which may in turn influence mechanical properties such as its Young's modulus) and due to differences in the forces governing tip-sample interactions. The absence in liquid of the capillary force, which is the longest-ranging and strongest tip-sample interaction force in air, makes it possible to use much lower tapping amplitudes in TM-AFM in liquid, and thus deposit much less energy in the sample with every 'tap'. Still, we found that α -synuclein fibrils can be disassembled by an AFM tip in tapping mode in liquid. Khurana *et al.* cite the excellent agreement between AFM measurements in air and EM measurement of fibril morphology as a reason to opt for conducting AFM experiments in air (Khurana *et al.*, 2003), but this agreement may be

due to that in both experiments, fibrils are dehydrated and do not show their physiologically relevant hydrated morphology.

Influence of the probe and tip-induced damage

The main tip property determining the resolution of AFM images is the tip diameter. The use of very sharp (1 nm) tips yielded slightly better images than ‘regular’ (10 nm) tips but, in the dozens of images we acquired, no extra information on the structure of the α -synuclein fibrils. The sharper tips did prove to be much more fragile. Even following the manufacturers’ recommendations of using low tapping amplitudes and high setpoint ratios, the tips broke quickly and making large numbers of images was not possible. The other high resolution tips tested in (Sweers, 2008) faced similar problems. Because of this combination of low structural information gain and strong increase in operational difficulty imparted by the ‘super-sharp’ tips, we decided to use the MSCT-F tip in the remainder of this work.

We observed two types of tip-induced damage. The primary mechanism of tip-induced damage is that small fragments of the fibril may be carried along by the tip in the scanning direction. An unexpected second mechanism, is that repeated scanning on an α -synuclein fibril in buffer sometimes results in its falling apart in segments. The segments may yet consist of several filaments, since the height of the segments reduces to about half the original fibril height before the segments disappear. The observation that the first and last segments of the fibril are the ‘last ones standing’, may be the result of a different protein conformation at the ends of the fibril. We speculate that these extra hydrophobic conformations make for good binding partners both for additional monomeric or oligomeric protein units, and the mica surface. The interpretation of the fibril ends having different conformation is consistent with our finding that in tapping mode phase contrast AFM in air, the ends often display a strong phase contrast as compared to the rest of the fibril (fig. 2.6 on page 33). Based on these observations, we may also tentatively suggest that the new fibril ‘ends’ that are formed by the removal of segments from the bulk of a fibril do not change conformation: they do not seem to bind more strongly to the mica than they did before the neighboring segments were removed.

Our finding of fibrils being more fragile in liquid than in air agrees with the findings of Mesquida *et al.* (2007). These authors studied the synthetic peptide TTR_{105–115} corresponding to the G β -strand of the human plasma protein transthyretin, which self-assembles into fibrils with similar size and morphology as α -synuclein fibrils. The TTR_{105–115} fibrils were not damaged by the tip in tapping mode in air, but they fell apart after scanning in liquid. Mesquida *et al.* determined that their fibrils were damaged by indenting in contact mode in air by applying forces in the 40 – 100 nN range. They estimate the fibrils in liquid were damaged by tapping forces as low as 1 nN and speculate

that acoustic waves in the water, set up by the vibrating cantilever, may be the cause of this effect.

Measuring the morphology of amyloid fibrils by TM-AFM in air does not measure true heights, but does allow for identification of aggregate species (Kad *et al.*, 2003). Measurements of fibril length would not be affected so much by dehydration, so those experiments would benefit from the simpler sample preparation and sample storage possibility inherent to imaging in air. The comparison of properties of fibrils formed by mutants of a protein, or of fibrils formed by different proteins, should be done in liquid to more accurately represent their true morphology and avoid errors due to possible variable degrees of dehydration. Also the mechanical properties will likely be affected by dehydration, so those should be measured in liquid as well.

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Quantitative analysis of *in vitro* α -synuclein fibril morphology

High resolution atomic force microscopy is a powerful tool to characterize nanoscale morphological features of protein amyloid fibrils. Comparison of fibril morphological properties between studies has been hampered by differences in analysis procedures and measurement error determination used by various authors. We describe a fibril morphology analysis method that allows for quantitative comparison of features of amyloid fibrils of any amyloidogenic protein measured by atomic force microscopy. We have used tapping mode atomic force microscopy in liquid to measure the morphology of fibrillar aggregates of human wildtype α -synuclein and the disease-related mutants A30P, E46K and A53T. Analysis of the images shows that fibrillar aggregates formed by E46K α -synuclein have a smaller diameter (9.0 ± 0.8 nm) and periodicity (mode at 55 nm) than fibrils of wildtype α -synuclein (height 10.0 ± 1.1 nm), periodicity has a mode at 65 nm). Fibrils of A30P have smaller diameter still (8.1 ± 1.2 nm) and show a variety of periodicities. This quantitative analysis procedure enables comparison of the results with existing models for assembly of amyloid fibrils. Both hierarchical assembly and segmented association models can be made plausible by experimental observations.

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4.1 Introduction

The misfolding of proteins is at the heart of many human diseases (Uversky and Fink, 2004; Dobson, 2003; Soto, 2003; Forman *et al.*, 2004). In Parkinsons disease, the misfolding and subsequent aggregation of the natively unfolded protein α -synuclein is an essential factor (Lundvig *et al.*, 2005). In familial forms of Parkinsons disease, three point mutations in the α -synuclein gene have been identified that result in single amino acid substitutions in the α -synuclein protein: A30P, E46K, and A53T (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998; Zarranz *et al.*, 2004). Differences in the aggregation kinetics and/or aggregate morphology among the mutant proteins may yield new insights into the general process of protein misfolding and aggregation. The nanoscale morphology of amyloid fibrils can be visualized with atomic force microscopy (AFM) or electron microscopy (see for example Hoyer *et al.*, 2004; Choi *et al.*, 2004).

The aggregation of α -synuclein into mature fibrils has been described by models based on AFM images (Khurana *et al.*, 2003) and is supposed to proceed through various stages, each with characteristic morphological features such as typical fibril heights and periodicities. As noted by Khurana *et al.* (2003), there are many studies on the morphology of fibrillar aggregates of various amyloidogenic proteins, but there has been little uniformity in the analysis and description of fibril characteristics.

We describe a quantitative analysis procedure that is applicable to atomic force micrographs of amyloid fibrils originating from any amyloidogenic protein. The procedure takes into account the limitations of the physical process of imaging a biological sample with atomic force microscopy, such as tip-sample convolution. We applied the quantitative image analysis procedure to AFM images (made in tapping mode and in liquid) of fibrils of wild-type and disease-related mutant α -synuclein formed after 72 h of aggregation.

The analysis reveals grossly similar morphology but varying heights and periodicities among the disease-related mutants. Fibrils from A30P and E46K α -synuclein appear smaller in height and correspondingly lower in peak height, trough height, and modulation depth than wild-type fibrils. Height measurements of wildtype α -synuclein fibrils in this study are consistent with those measured by others. We measure 10.0 ± 1.1 nm average height for the fibrils formed by wild-type α -synuclein, as compared to 11 ± 2 nm (Hoyer *et al.*, 2002) and 9.8 ± 1.2 nm (Khurana *et al.*, 2003).

4.2 Methods

Expression and purification of recombinant human α -synuclein

Expression of human wild-type and the A30P, E46K and A53T disease mutants of α -synuclein was performed in *E. coli* B121(DE3) in a pT7-7 based expression system. After IPTG induction bacterial cell pellets were harvested by centrifugation and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM Pe-fabloc protease inhibitor cocktail. Cell lysis was carried out by sonication and followed by centrifugation at $10,000 \times g$ for 30 min. at 4 °C. Streptomycin sulfate precipitated DNA was removed by centrifugation at $13,500 \times g$ for 30 min. at 4 °C. An ammonium sulfate precipitation step was performed to selectively precipitate the α -synuclein protein. After centrifugation at $13,500 \times g$ for 30 min. at 4 °C the pellet was dissolved in 10 mM Tris-HCl, pH 7.4, 1 mM Pe-fabloc and 0.2 μ M filtered. The resulting solution was loaded onto a Resource Q anion exchange column on an Äkta Basic chromatography system (GE Healthcare Biosciences, Little Chalfont, UK). Fractions collected during elution with a salt gradient were assayed for the presence of α -synuclein protein by SDS-PAGE followed by Coomassie staining. Fractions containing α -synuclein were pooled, dialyzed against 10 mM HEPES, 50 mM NaCl, pH 7.4 and concentrated to 250 μ M. Protein concentrations were determined by measuring the absorbance at 275 nm using an extinction coefficient of $5,600 \text{ M}^{-1}\text{cm}^{-1}$. Purified protein was stored at -75°C in 0.5 ml aliquots.

Induction of aggregation

The monomeric protein solutions of 100 μ M wildtype and mutant α -synuclein were incubated at 37°C in 10 mM HEPES, 50 mM NaCl, pH 7.4 under constant stirring at 300 rpm in glass vials. The volume per vial was 500 μ l. All aggregations were performed in triplicate. Aliquots of 20 μ l were withdrawn at 24 h intervals to accommodate for both ThioT and AFM measurements. While ThioT measurements were made immediately, samples for AFM imaging were stored at 4 °C in 16 μ l aliquots before measurement. No change in aggregate morphology was observed between fresh samples and samples stored at 4 °C.

Kinetics of aggregation measured by Thioflavin T fluorescence

The fluorescence increase of Thioflavin T (ThioT) upon binding to a β -sheet secondary structure was used as an indicator of protein aggregation state. At time points chosen 24 h apart, 4 μ l aliquots of aggregate mixture were added to 2 ml of a 5 μ M ThioT solution in 50 mM sodium glycinate, pH 8.2. Fluorescence was excited at 457 nm in a fluorescence spectrophotometer (Cary Eclipse, Varian Inc, Palo Alto, CA, USA), and emission spectra were recorded from 477 – 600 nm. The entrance and exit slit widths were set at 10 nm.

ThioT reference spectra were subtracted from every measurement. The intensity quoted in fig. 4.4 is the peak intensity of a skewed Gaussian function fitted to the ThioT spectrum.

Atomic force microscopy of protein aggregates

Atomic force microscopy images were made on a custom built instrument (van der Werf *et al.*, 1993) using Si_3N_4 tips (Veeco MSCT-AU tip F) with a spring constant of $k = 0.5 \text{ N/m}$ and a nominal tip radius of 10 nm. The measurements were made in tapping mode in liquid. The tapping amplitude was less than 4 nm and tip-sample contact time was minimal, such as to minimize the force exerted on the sample and therefore the influence of the scan on the aggregate morphology. All AFM images have 512×512 pixels and a lateral resolution of 11 nm/pixel or better. Images were made at a typical scan speed of $40 \mu\text{m/s}$. Buffers were based on Milli-Q water (resistivity $> 18 \text{ M}\Omega\text{cm}$) and $0.22 \mu\text{m}$ filtered to remove any impurities.

Samples for AFM were prepared as follows: $4 \mu\text{l}$ of protein sample was placed on unmodified freshly cleaved mica and allowed to adsorb in a humid environment for 2 minutes. Unbound protein was gently washed off with $100 \mu\text{l}$ of buffer (10 mM HEPES, 50 mM NaCl, pH 7.4). The sample was then mounted on the AFM stage and $100 \mu\text{l}$ of fresh buffer was applied. The samples remained in buffer throughout the experiment to maintain as much of a physiologically relevant environment as possible and to avoid drying artifacts in the fibril morphology and deposition of buffer salts on the mica. Fibril characterization was done on unprocessed AFM height images.

In images used for presentation purposes, height discontinuities between subsequent scan lines were removed by 0th order line-wise leveling, and piezo drift was compensated by a 3rd order least-mean-squares average profile using SPIP software (Image Metrology A/S, Hørsholm, Denmark).

Quantitative analysis of fibril morphology

In order to make quantitative statements about the morphology of fibrils formed by wildtype and mutant α -synuclein variants, we developed a quantitative morphological analysis procedure (fig. 4.1). The length ab is considered the fibril length (a and b are placed just inside the ramp-up and ramp-down, which are ignored because of tip-sample convolution). The reported fibril height is the average height between a and b . The periodicity of the fibril is calculated as the distance cd divided by, in this case, 15 periods. The ‘typical’ peak and trough heights are indicated by e and f . Finally, the height difference ef is the measured modulation depth. There is a certain subjectivity in the choice of which peaks to consider ‘typical’, due to the limited spatial resolution of the

Figure 4.1. Amyloid fibril characterization procedure applied to an E46K α -synuclein fibril.

The top panel shows an AFM topography image of an α -synuclein fibril (left panel) with a manually drawn segmented line profile (right panel) on the curved fibril. The bottom panel shows the height profile of the fibril.

image (in this case, the lateral resolution is 11 nm/pixel) and due to possible peak height variations in the fibril itself.

All fragments were profiled and characterized individually. Those fibril fragments not classified as periodic were irregular in height. Height values are averaged over all N fibrils; the other parameters are averaged over the periodic fibrils. The standard deviation is used as the measurement error.

Calculation of tip radius and expected trough height

To assess the reliability of the measured parameters, we use a simple one-dimensional model for tip-sample convolution (fig. 4.2a), which allows calculation of the tip radius assuming the tip (*green*) is spherical and the sample (*red*) has a spherical cross section.

The legend to fig. 4.2 defines the symbols the coordinates (x, y) used below. The tip center c has coordinates (x_c, y_c) ; the point of contact between tip and sample 0 has coordinates (x_0, y_0) ; the trough location t has coordinates (x_t, y_t) . The tip radius r_t is computed from the triangle equation, given the measured sample radius r_s and apparent width w (fig. 4.2):

$$(r_t + r_s)^2 = (r_t - r_s)^2 + (w/2)^2 \quad (4.1)$$

Figure 4.2. Tip-sample convolution affects reported object width, not height and periodicity.

Simple one-dimensional models quantify tip-sample convolution for the width (a) and apparent trough height (b) of helical fibrils. r_t , tip radius; r_s , sample radius; w , apparent width; o , contact point; c , tip center; t , apparent trough position; and p , periodicity.

Solving for r_t gives

$$r_t = w^2 / (16 \cdot r_s) \quad (4.2)$$

The finite diameter of the AFM tip also influences the apparent trough height (fig. 4.2b). To compute the expected trough height, we define the tip as a circle, with

$$r_t^2 = (x - x_c)^2 + (y - y_c)^2 \quad (4.3)$$

where the tip x position x_c is at $p/2$, as can be seen from Fig.4.2b. The bottom half of the circle is then described by rearranging equation 4.3 into $y_{tip} = y_c - \sqrt{r_t^2 - (x - p/2)^2}$.

The top contour of the sample, which in the twisted filament model is a double helical structure, is given by

$$y_{sample} = \frac{3}{2}r_s + \frac{1}{2}r_s \cos(\pi x/p) \quad (4.4)$$

where $x = 0$ to $p/2$. At the contact point, their derivatives with respect to x are equal:

$$\begin{aligned} \frac{dy_{sample}}{dx} &= \frac{dy_{tip}}{dx} \\ -\frac{1}{2}r_s \sin(\pi x/p)\pi/p &= (x - p/2)/\sqrt{r_t^2 - (x - p/2)^2} \end{aligned} \quad (4.5)$$

This equation can be solved numerically for x to yield the contact point x position x_0 . Substitution of x_0 into the equation for the sample then yields the y contact coordinate y_0 , from which tip center height y_c and trough height $y_t = y_c - r_t$ follow.

In the case of our α -synuclein fibrils, the trough height is overestimated in the images since the tip radius is large relative to the periodicity. This complicates attributing the observed periodicity to a supposed helical structure of the fibrils. We consider the measurements of the periodicity to be reliable since they were found to be equivalent for fibrils in any orientation with respect to the scan direction, and because the tip radius does not affect the periodicity observed but only the modulation depth, as shown in fig. 4.2b.

4.3 Results

4.3.1 Morphology as a function of aggregation state

The morphology of α -synuclein aggregates depends on the state of the aggregation. Fig. 4.3 displays a series of AFM images taken from aliquots of aggregated α -synuclein taken out of the incubator at times indicated in the accompanying Thioflavin T-intensity curve. Initially, there is hardly any fibrillar material to be seen, but at the same time the ThioT signal goes up, indicating the formation of β -sheet folded protein, more and longer fibrils begin to appear. After the initial peak value in the ThioT curve, the intensity typically drops somewhat and in the AFM images, we observe large amorphous agglomerates of fibrils and not many ‘single’ fibrils any more. The drop in ThioT signal may be due to some fraction of the protein being ‘inside’ a large amorphous aggregate and not being accessible to the intercalating dye anymore.

4.3.2 Aggregation kinetics of disease-related α -synuclein mutants

The kinetics of aggregation of disease-related α -synuclein mutants are not all identical (fig. 4.4). E46K α -synuclein has a somewhat faster halftime of aggregation than the other three. The final intensity of the ThioT signal varies strongly among the mutants, but sometimes also within a triplicate set (fig. 4.4a). We found no apparent correlation between final ThioT intensity and fibril density as deposited on the mica. The A53T aggregation did not produce fibrils whose morphology was quantifiable with AFM but instead only amorphous aggregates.

Figure 4.3. Morphology of α -synuclein aggregates as a function of aggregation state.

Time-lapse AFM images show the formation of fibrillar α -synuclein aggregates as a function of incubation time. At $t = 0$ h, there is no fibrillar material, consistent with a near-zero Thioflavin T fluorescence intensity shown in the *inset*. Fibrils form slowly and after $t = 96$ h, many mature fibrils have formed and ThioT signal is at its maximum. AFM images of the aggregates at later time points indicate that large, amorphous aggregates have formed.

Figure 4.4. Mutant α -synuclein fibrillation kinetics and morphology differ from wildtype.

Kinetics of (A) wildtype, (B) A30P, (C) E46K, and (D) A53T human α -synuclein aggregation, measured by Thioflavin T fluorescence emission. Each experiment was performed in triplicate (squares, circles, and triangles in each of the graphs) and all experiments were performed using the same environmental parameters. The insets show parts of atomic force micrographs of the aggregates at $t = 72$ h (as indicated by the highlighted data point in each graph). Scale bars 100 nm, except A53T scale bar 500 nm.

α -synuclein variant	N fibrils	Fibril height (nm)	Typical trough height (nm)	Typical peak height (nm)	Modulation depth (nm)	Periodicity (nm)
wt	35 (22)	10.0 ± 1.1	9.3 ± 1.3	11.4 ± 1.2	2.1 ± 0.6	81 ± 24 (mode 65 nm) [†]
A30P	26 (21)	8.1 ± 1.2	7.4 ± 1.4	8.9 ± 1.4	1.7 ± 0.6	103 ± 20
E46K	26 (17)	9.0 ± 0.8	7.7 ± 1.0	9.8 ± 1.1	2.0 ± 0.5	76 ± 34 (mode 55 nm)
A53T	0*					

Table 4.1. Quantitative comparison of α -synuclein mutant fibril morphology in liquid.

The number of fibrils classified as periodic is given in brackets under N fibrils.

* In our experiment, A53T α -synuclein did not aggregate into fibrils but into large amorphous aggregates ($> 100\mu\text{m}$ in diameter) that could not be characterized.

[†] Fig. 4.5 contains a more accurate representation of the range of periodicities.

4.3.3 Nanoscale morphology of mutant α -synuclein fibrils

The morphological analysis procedure as presented in fig. 4.1 reveals grossly similar morphology but varying heights and periodicities among the disease-related mutants (table 4.1). Fibrils from A30P and E46K α -synuclein appear smaller in height and correspondingly lower in peak height, trough height, and modulation depth than wild-type fibrils. Height measurements of wildtype α -synuclein fibrils in this study are consistent with those measured by others. We measure 10.0 ± 1.1 nm average height for the fibrils formed by wild-type α -synuclein as compared to 11 ± 2 nm (Hoyer *et al.*, 2002) and 9.8 ± 1.2 nm (Khurana *et al.*, 2003). Most E46K fibrils display a periodicity of around 55 nm (as in fig. 4.6 fibril A), but much larger periodicities (fibril B) or unperiodic fibrils (fibril C) are also observed. The periodicities of E46K and wild-type α -synuclein fibrils have modes at 55 and 65 nm, respectively, and fibrils formed by A30P α -synuclein display a wider variety of periodicities (fig. 4.5). Quantitative discussion of morphological features of objects studied by AFM requires consideration of the influence of scanning system properties on the resulting image. One of the main limiting factors in deriving fibril properties from AFM images is tip-sample convolution, as evidenced by the large difference between the apparent width and the height of fibrils that are expected to be approximately round in cross section.

Figure 4.5. Distribution of periodicities in fibrils of disease-related α -synuclein mutants.

Wild-type and E46K α -synuclein fibrils have modes at 65 and 55 nm, respectively. A30P shows a wider variety of periodicities. Bin size is 10 nm.

Figure 4.6. Tapping mode atomic force micrograph of E46K α -synuclein in liquid after 72h of aggregation.

The color scale represents 17.6 nm in height. The fibril fragments marked A, B, and C are profiled along their length in the bottom panel indicating the range of morphologies observed in E46K fibrils. Vertical scale bar 2 nm; horizontal scale bar 200 nm.

Fibril	r_s (nm)	w (nm)	p (nm)	r_t^* (nm)	y_t^* (nm)	$y_t^\dagger$ (nm)	$y_t^\ddagger$ (nm)
A	4.5	90.7	56.2	114.3	7.5	6.8	7.0
B	4.75	91.2	186.4	109.4	7.2	7.1	7.0

Table 4.2. Comparison of measured *versus* modeled trough heights.

Fibrils A and B refer to the fibrils highlighted in fig. 4.6. Sample radius r_s is half the peak height; w was measured from the image as the average width of 17 line profiles perpendicular to the fibril; p , periodicity; r_t , tip radius; y_t , trough height.

* from the convolution model; † from the hierarchical assembly model; ‡ as measured.

4.4 Discussion

In the hierarchical assembly model of α -synuclein fibril formation, as proposed by Khurana *et al.*, the trough height would be $y_t = (3/2)d$, where d is the protofilament diameter (equal to the radius of the mature fibril r_s). We can now compare the trough height values as modeled with the tip-sample convolution model, as modeled with the hierarchical assembly model, and as actually measured, for two fibrils with a different periodicity (table 4.2). Based on this comparison, we conclude that the morphological features we observe are those one would expect of fibrils formed by twisted protofilaments, but we note that the lateral resolution of our images is not high enough to prove unambiguously that this model is indeed correct.

This study introduces a quantitative morphological analysis procedure of fibrillar aggregates of amyloidogenic proteins. We show that high-resolution atomic force microscopy under sample-favorable imaging conditions (imaging under buffer, low tapping amplitude (~ 4 nm), and low interaction force), combined with detailed image analysis, allows nanoscale comparison of fibrils formed by human wild-type α -synuclein and disease-related α -synuclein mutants. The fibrils formed by the disease-related mutants have a similar overall morphology, but a smaller diameter and a different periodicity than fibrils formed by wild-type α -synuclein.

The observed fibril morphology is compatible with the hierarchical assembly model of α -synuclein fibrillization described in (Khurana *et al.*, 2003). We emphasize that great care should be taken in deriving structural models from AFM data. We believe that the careful quantitative approach to determining fibril morphological characteristics reported here should be used more widely, and will be of increasing importance considering the growing number of studies that use advanced scanning probe microscopies for nanometer scale characterization of protein fibrils.

4.5 References

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Statistical-mechanical modeling of α -synuclein fibrillization

The initial concentration of monomeric amyloidogenic proteins is a crucial factor in the *in vitro* formation of amyloid fibrils. We use quantitative atomic force microscopy to study the effect of the initial concentration of human α -synuclein on the mean length of mature α -synuclein fibrils, associated with Parkinson's disease. We determine that the critical initial concentration, below which low-molecular-weight species dominate and above which fibrils are the dominant species, lies at approximately 15 μ M, in good agreement with earlier measurements using biochemical methods. In the concentration regime where fibrils dominate, we find that their mean length increases with initial concentration. These results correspond well to the qualitative predictions of a recent statistical-mechanical model of amyloid fibril formation. In addition, good quantitative agreement of the statistical-mechanical model with the measured mean fibril length as a function of initial protein concentration, as well as with the fibril length distributions for several protein concentrations, is found for reasonable values of the relevant model parameters. The comparison between theory and experiment yields for the first time an estimate of the magnitude of the free energies associated with the intermolecular interactions that govern α -synuclein fibril formation.

The work described in this chapter is the result of a collaboration with dr. Jeroen van Gestel and prof. Simon de Leeuw of the Physical Chemistry and Molecular Thermodynamics group, TU Delft, Delft, The Netherlands.

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5.1 Introduction

The aggregation of proteins into amyloid or amyloid-like fibrils is a process of crucial importance in many neurological disorders (Chiti and Dobson, 2006; Rochet and Lansbury, 2000; Tycko, 2004). In the case of Parkinson's disease (PD) the hallmark pathological features are Lewy bodies: intracellular neuronal inclusions consisting mainly of misfolded and aggregated α -synuclein (Goedert, 2001; Shults, 2006). Nanoscale knowledge of the morphology of the protein aggregates in these inclusions may help increase understanding of the etiology of the disease.

Earlier biophysical research has shown that aggregates of various amyloidogenic proteins typically take the form of thread-like fibrils. These fibrils may assemble hierarchically, that is, by protofilaments winding together (Khurana *et al.*, 2003), but also by lateral association without formation of a helical structure (Jansen *et al.*, 2005; Segers-Nolten *et al.*, 2007). Similar fibrillar structures have been observed in vitro formed by various disease-related and non-disease-related proteins, such as α -synuclein (Khurana *et al.*, 2003; Segers-Nolten *et al.*, 2007; Hoyer *et al.*, 2004; van Raaij *et al.*, 2006), glucagon (De Jong *et al.*, 2006; Dong *et al.*, 2006), insulin (Khurana *et al.*, 2003; Jansen *et al.*, 2005), A β peptide 1-40 (Kodali and Wetzel, 2007), prion protein (Anderson *et al.*, 2006), and others. The morphology of the resulting aggregates depends on such diverse factors as solution conditions (Hoyer *et al.*, 2002), the shape of any preformed aggregates which may serve as nuclei (Jansen *et al.*, 2005), and mutations in the amino acid sequence of the protein (van Raaij *et al.*, 2006, and chapter 4 of this thesis).

Based on these observations, structural models describing the assembly of amyloid fibrils have been proposed. The dominant structural model for α -synuclein fibrillization (Khurana *et al.*, 2003) proposes that two protofilaments (linear chains of β -sheet folded monomers) wind together to form an intermediate fibril, and two of these intermediate fibrils in turn wind together to form a so-called 'mature fibril'. (Note that various authors use different terminologies to describe the various species that arise during the aggregation process. We follow the definitions of Kodali and Wetzel (2007)).

We will quantify the free energies of the interactions involved in α -synuclein fibril formation by testing a statistical-mechanical model of amyloid fibril formation by van Gestel and de Leeuw (2006). We will first introduce kinetic models of α -synuclein fibril formation, the forces and energies involved in protein self-assembly, and describe the main features of the statistical-mechanical model.

5.1.1 Kinetic models of protein self-assembly

The aggregation of proteins into amyloid fibrils is considered to be a nucleation-polymerization process (Wood *et al.*, 1999). As such, the initial concentration of α -synuclein is expected to have a profound effect on the fibril length. The concentration of α -synuclein present in neural cells is a factor relevant to the etiology of PD, since triplication of the α -synuclein gene (and subsequent overexpression of the protein) is associated with familial PD (Singleton *et al.*, 2003). Another clue to the significance of α -synuclein concentration for the etiology of the disease is that in pathological conditions, α -synuclein aggregates are also found in glial cells, where in vitro overexpression of α -synuclein leads to cell death (Stefanova *et al.*, 2001). Apart from overexpression, the ‘effective concentration’ of α -synuclein can also change from its normal value due to reduced degradation or unspecific molecular crowding. These effects have been found to significantly reduce the aggregation lag time in in vitro experiments (Shtilerman *et al.*, 2002; Munishkina *et al.*, 2004).

5.1.2 Forces and energies involved in protein self-assembly

The main force driving self assembly of protein structures is the hydrophobic interaction (Nelson *et al.*, 2004, chapter 8): the tendency of hydrophobic parts of macromolecules to stay away from polar molecules such as water. Hydrophobicity is not the only factor driving protein self-assembly though: the balance between hydrogen bonding and electrostatic, hydrophilic and hydrophobic interactions determines the (mis-)folding and amyloid formation of proteins (van der Linden and Venema, 2007). Entropic forces may also play a role: as large molecules in a solution aggregate together, the many water molecules surrounding them gain more freedom to move. As such, aggregation of macromolecules increases entropy.

The net stability of a protein’s secondary structure is usually on the order of about $20k_B T_r$ (Lodish *et al.*, 2000), which compares to the free energy of a few hydrogen bonds. The bond energies (the amount of energy required to break a bond) of the bonds holding the supramolecular aggregates together are often also in the order a few hydrogen bonds (table 5.1).

5.1.3 Statistical-mechanical model of amyloid fibril formation

In order to capture the essential factors that allow many different proteins to form similar fibrils, we use a general statistical-mechanical model of amyloid fibril formation that was recently outlined by van Gestel and de Leeuw (2006). This model couples a theory describing self-assembly and conformational transition to a description of the association of linear chains. The model focuses on the formation of linear, unbranched amyloid fibrils commonly observed in

		E (kcal/mol) [†]	E (kJ/mol)	E ($k_B T$)
covalent bond	single	51 – 110	213 – 460	87 – 188
	double	120 – 170	502 – 711	205 – 290
	triple (C≡C)	195	816	333
hydrogen bond	in water	~ 5	~ 21	~ 8.6
	in protein/ nucleic acid	~ 1 – 2	~ 4 – 8	~ 1.6 – 3.2
van der Waals interaction		~ 1	~ 4	~ 1.6
average thermal energy of a molecule at temperature T is $E = k_B T$ [‡]		0.59	2.45	1

Table 5.1. Typical bond energies

[†] Values in kcal/mol were taken from Lodish *et al.* (2000, p. 17), values in kJ/mol and $k_B T$ calculated from that, with $T = T_r = 22^\circ\text{C} = 295\text{ K}$. 1 kcal/mol = 4.184 kJ/mol.

[‡] At body temperature $T_b = 37^\circ\text{C} = 310\text{ K}$ bond energies in kcal/mol and kJ/mol are about 5 % higher.

studies of protein aggregation, and does not address amorphous aggregation. The model predicts the effect of protein concentration on the properties of a dilute solution of fibrillogenic protein molecules, given the free energies associated with various intermolecular interactions. For reasonable values of these free-energy parameters, the model predicts that there exists a critical concentration, below which most protein molecules are present as free monomers. It also predicts fibril formation above this concentration, with the fibril length increasing with protein concentration. The existence of a critical concentration is consistent with the notion of amyloid fibril formation being a nucleation-polymerization process (Chiti and Dobson, 2006; Wood *et al.*, 1999).

In this investigation we test the predictions of the statistical-mechanical model introduced in (van Gestel and de Leeuw, 2006), both qualitatively and quantitatively, using atomic force microscopy (AFM) to image mature α -synuclein fibrils formed in vitro at various initial protein concentrations. Our experimental results demonstrate that α -synuclein fibril formation is well described by the model. We determine the critical concentration for α -synuclein fibrillization to be 15 μM . From the measured dependence of the average fibril length on the protein concentration, we extract values for the free energies of interactions in the fibrils: the free energy of interaction between adjacent β -folded monomers is found to lie between -16.2 and -15.4 kJ/mol and the lateral interaction between protofilaments in the fibril has a bond energy between -11.0 and -7.4 kJ/mol.

5.2 Methods

Expression, purification and aggregation of recombinant human α -synuclein

Wildtype α -synuclein was expressed and purified as described in chapter 4. Protein solutions taken from stock at -80°C were defrosted and centrifuged for 1 h at $21,000 \times g$ to remove any pre-formed aggregates or contaminating particles. Native gradient polyacrylamide gel electrophoresis confirmed the presence of only monomeric α -synuclein. The initial protein concentration was determined by measuring the absorbance at 275 nm with a NanoDrop ND-1000 absorption spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA) and using an extinction coefficient $\epsilon(275\text{nm}) = 5600 \text{ M}^{-1}\text{cm}^{-1}$ ($1400 \text{ M}^{-1}\text{cm}^{-1}$ per tyrosine residue). Monomeric α -synuclein was diluted to the desired initial protein concentrations in the $5 - 250 \mu\text{M}$ range in a buffer containing 10 mM HEPES and 50 mM NaCl at pH 7.4. We estimate the concentration uncertainty to be 6 % for all initial concentrations based on analysis of absorption measurements and pipetting accuracy.

Aggregation was performed in a temperature-controlled shaking incubator (ThermoMixer, Eppendorf AG, Hamburg, Germany) at 37°C while shaking at 500 rpm. This shaking frequency speeds up the aggregation process to a manageable timescale but is considered ‘gentle’. Any shear-force induced fibril breakage would occur to the same extent for all concentration conditions in this experiment since incubation conditions were equal. The aggregation of amyloidogenic proteins is not an artifact of agitation: aggregation occurs in undisturbed solutions, only much slower (Ramirez-Alvarado *et al.*, 2000). Each vial contained $400 \mu\text{l}$ of protein solution and all aggregations were performed in duplicate. Before samples were taken out of the aggregation vessels, they were rotated at an angle and aspirated in order to maximize homogeneity of the sample without disrupting any aggregates.

Samples were taken for detailed AFM analysis after 20 to 28 days, when the aggregation reactions had reached their final equilibrium state as verified from fibril morphology measurements. The presence of monomeric protein in equilibrium with fibrils was not only confirmed by measuring the 275 nm absorbance of the supernatant after pelleting the fibrils by centrifugation, but also from native gradient polyacrylamide gel electrophoresis.

Acquisition of AFM images

Aggregated protein solutions were deposited in $4 \mu\text{l}$ aliquots on freshly cleaved mica and incubated for 2 minutes in a humid environment to avoid drying of the droplet and salt crystal formation. The samples were then gently rinsed with $200 \mu\text{l}$ of MilliQ water (resistivity $> 18 \text{ M}\Omega\text{cm}^{-1}$) and blown dry with a

gentle flow of $N_2(g)$. Aggregates of all sizes were found to adhere equally well to freshly cleaved mica without further surface modification (see for example Hoyer *et al.* (2004)).

Atomic force microscopy images were acquired on a custom built standalone AFM instrument (van der Werf *et al.*, 1993) and on a Multimode AFM with a Nanoscope IV controller (Veeco, Santa Barbara, CA, USA) in tapping mode under ambient conditions. The drying of protein aggregate samples for imaging in air influences their morphology (especially height and periodicity) but does not affect the observed fibril length. We used Veeco Probes MSCT-AU tip F (Si_3N_4), nominal tip radius 10 nm, spring constant $k = 0.5$ N/m; and MikroMasch NSC36/Cr-Au tip B (Si), nominal tip radius < 10 nm, spring constant $k = 1.75$ N/m. Tapping amplitude was between 50 – 100 nm, depending on tip-sample adhesion assessed on a measurement-by-measurement basis. For the aggregates formed by 5 and 10 μM α -synuclein solutions, images were taken at a pixel resolution of 4 nm/pixel (image size 4 μm), for the 20 – 30 μM aggregates at 20 nm/pixel (image size 20 μm) and for the 50 – 250 μM aggregates at 40 nm/pixel (image size 20 μm).

Measurement of fibril length distributions

Raw AFM height images were processed using Scanning Probe Image Processor (Image Metrology, Hørsholm, Denmark) to remove sample tilt and scanner bow. Sample tilt was removed using manual tilt correction while monitoring x and y cross sections until both cross-sections were horizontal. Then, any scanner bow artifacts were corrected using a second or third order average profile fit. To minimize distortion of apparent morphology of the objects in the image, the fit was calculated excluding these objects by setting limits on the z color scale. Finally, any line-to-line scanner jumps were corrected by a zeroth order linewise fit. Lengths of individual fibrils were measured using segmented line profiles in ImageJ (Rasband, 1997-2006). To minimize observer bias, all fibrils that fit the following criteria were included in the analysis:

1. the fibril lies completely within the image;
2. the fibril can be unambiguously distinguished from any overlapping fibrils;
3. the fibril appears in the image as larger than 4 pixels.

We estimate the accuracy of the individual fibril length measurements to be 40 nm (20 and 30 μM concentrations) and 80 nm (50 – 250 μM concentrations), mainly limited by tip-sample convolution and pixel resolution. The analysis procedure is demonstrated in fig. 5.1. The aggregates formed by initial concentrations of 5 and 10 μM were amorphous with reported sizes on the order of 20 nm, which corresponds to the ‘tip-sample-convolution resolution’.

Figure 5.1. AFM images illustrating the length measurement procedure.

Using plane-corrected height images (A), lengths were measured using manually placed segmented line profiles for all fibrils that could be resolved individually, did not fall off the edge of the image and were larger than 4 pixels (B). Scale bar 2 μm .

The size of these aggregates was characterized by their height instead of their length.

Statistical-mechanical modeling of α -synuclein fibril length as a function of initial concentration

We have adapted a statistical-mechanical model of protein aggregation in dilute solution that was recently outlined (van Gestel and de Leeuw, 2006) to the specific case of α -synuclein fibrillization (fig. 5.2). The model assumes that only two conformational states of the protein molecules are sufficiently populated to have an effect on the aggregation behavior: proteins can either be in a β -strand conformation, or they can be in a less ordered conformation. The model then describes the properties of mature fibrils in an equilibrium situation.

One key prediction is the distribution of the lengths of mature fibrils as a function of initial concentration. The statistical-mechanical model does not attempt to model the early stages of aggregation. It would in principle be possible to extend the model to include parameters that represent monomer conformation. However, for every conformation taken into account, we need an extra free energy parameter. A model with an infinite number of adjustable parameters may be complete but will not be very informative. It will also be next to impossible to independently determine the appropriate values for these parameters from experimental data.

It would also in principle be possible to include an activation step into the model. However, the concentration of ‘activated’ monomers (that are in a conformation capable of adding to a fibril) will be extremely small since they would be incorporated into the fibrils immediately. The equilibrium model then

Figure 5.2. Definition of parameters of the statistical-mechanical model

(a) Schematic and simplified representation of relevant species in α -synuclein fibrillization. Intrinsically disordered monomers (left) misfold and aggregate to form protofilaments (middle). Mature fibrils (right) can consist of up to four laterally interacting protofilaments. In this cartoon, molecules represented by discs possess the β -strand conformation that characterizes amyloid fibrils, while those represented by blobs do not.

(b) All interactions between protein molecules in the fibril have a free energy associated with them, as described in the text.

simplifies to one without the activation step. The kinetics of the process would be influenced by an activation step, but since our interest lies in the morphology of the resulting species, that does not pose a problem. Any conformational changes in the monomers will likely involve such small free energy changes that it would not influence the predictions of our model if we took them into account.

The model accounts for three species that participate in the aggregation process: monomers, protofilaments and fibrils (fig. 5.2). Monomers are defined as single protein molecules that possess a non- β conformation. Protofilaments are linear chains of interacting monomers, each of which can be in a non- β state or in a β -strand state. Mature fibrils are defined as rod-like aggregates, which in the case of α -synuclein contain up to four protofilaments. In this paper we assume that the fibril ends can be in either a β or a non- β conformation. The experimentally observed helical twist in the mature fibril is not represented in the model, and all processes are assumed to be reversible.

Because proteins that possess different conformations interact differently, we introduce two free-energy parameters: one that accounts for the interaction between two proteins that are both in a β -strand conformation (labeled P in fig. 5.2), and one for the interaction of two proteins that are not both in this conformation (E). Furthermore, we introduce an interaction free energy for lateral protein-protein contacts (F), and a free-energy penalty that is applied

whenever an ordered region and a disordered one meet $(R)^1$.

According to the current structural model of α -synuclein fibrillization, mature α -synuclein fibrils consist of two intertwined intermediate fibrils, which in turn consist of two intertwined protofilaments (Khurana *et al.*, 2003). This is reflected in the theory by only taking into account fibrils that consist of four or less protofilaments. Each fibril contains $(p - 1) \cdot m$ lateral protein-protein contacts, with p the number of protofilaments making up the fibril, and m the length of each protofilament expressed in the number of protein molecules. Combining the model with self-assembly theory allows us to obtain values for the mean aggregate size, the distribution of fibril lengths, and the mean fibril length.

The temperature at which aggregation is performed (or modeled) affects the kinetics of amyloidogenesis, but not so much the fibril morphology or the equilibrium concentrations. In the model, as in the experiment, temperature was kept constant at the physiologically relevant value of 37 °C.

In a statistical-mechanical description of a polydisperse system of dissolved protein aggregates, two characteristics are of vital importance. The first is the number density ρ , which gives the total number of particles (aggregates and monomers) that are present in solution, and the second is the volume fraction ϕ of protein molecules, which in effect counts the total number of protein molecules present. In van Gestel and de Leeuw (2006), it was determined that ρ and ϕ equal

$$\rho = z + z^2 k + \frac{xz^3 k^2 \lambda_1}{1 - zk\lambda_1} + \frac{yz^3 k^2 \lambda_2}{1 - zk\lambda_2} + \sum_{p=2}^4 \rho_{\text{fibrils}}(p) \quad (5.1)$$

and

$$\phi = z + 2z^2 k + \frac{xz^3 k^2 \lambda_1 (3 - 2zk\lambda_1)}{(1 - zk\lambda_1)^2} + \frac{yz^3 k^2 \lambda_2 (3 - 2zk\lambda_2)}{(1 - zk\lambda_2)^2} + \sum_{p=2}^4 \phi_{\text{fibrils}}(p) \quad (5.2)$$

respectively. In the above equations, x , y , λ_1 and λ_2 are prefactors depending on the description of the protofilament ends. These prefactors depend only on Boltzmann factors σ and s (defined below). The equations thus contain five variables: the fugacity $z = \exp(\mu)$, with μ the chemical potential of protein molecules given in units of the thermal energy ($k_B T$ with k_B Boltzmann's constant and T the absolute temperature), and the Boltzmann factors

¹The symbols for some of the free energies are different than those in van Gestel and de Leeuw (2006). E replaces M , to avoid confusion with the molar mass, and P replaces P^* , because in the current context it is not necessary to distinguish between the β -bond free energy and the excess β -bond free energy.

$f = \exp(-F)$, $k = \exp(-E)$, $s = \exp(-P + E)$, and $\sigma = \exp(-2R)$. The free energies F , E , P and R are introduced above (fig. 5.2), and are also given in terms of the thermal energy.

In eqs. 5.1 and 5.2 the number density and volume fraction have each been split into five terms that can be used separately if required. The first term in each equation gives the number density or volume fraction of monomers, the second term that of dimers, the (combined) third and fourth terms of protofilaments of all lengths, and the final term for fibrils of all lengths, containing p protofilaments. These latter terms equal

$$\rho_{\text{fibrils}}(p) = f^{-2}(f^2 z^2 k s)^p \left[1 - \frac{k s f z)^p}{f} \right]^{-1} (1 + \sigma^{1/2} k z)^{2p} \quad (5.3)$$

and

$$\phi_{\text{fibrils}}(p) = \frac{p(k s f^2 z^2)^p}{f^2 - f(k s f z)^p} (1 + \sigma^{1/2} k z)^{2p} \left[\frac{2 - (k s f z)^p / f}{1 - (k s f z)^p / f} + \frac{2\sigma^{1/2} k z}{(1 + \sigma^{1/2} k z)} \right] \quad (5.4)$$

If ϕ and ρ are known, the mean number of protein molecules per particle can be calculated as

$$\langle N \rangle = \frac{\phi}{\rho} \quad (5.5)$$

By taking the last term from eq. 5.1 and 5.2, we can calculate the mean aggregation number for fibrils only in a similar way

$$\langle N \rangle_{\text{fibrils}} = \frac{\phi_{\text{fibrils}}}{\rho_{\text{fibrils}}} \quad (5.6)$$

or alternatively, for all fibrils containing $p = 4$ protofilaments

$$\langle N \rangle_{\text{fibrils}, p=4} = 4 \left[\frac{2 - (k s f z)^4 / f}{1 - (k s f z)^4 / f} + \frac{2\sigma^{1/2} k z}{(1 + \sigma^{1/2} k z)} \right] \quad (5.7)$$

To calculate the mean length (expressed in number of monomers) of such fibrils, one then only needs to divide the mean aggregation number by the number of protofilaments, p .

$$\langle L \rangle_{\text{fibrils}, p=4} = \frac{\langle N \rangle_{\text{fibrils}, p=4}}{4} \quad (5.8)$$

In order to compare theory and experiment we need to convert experimental units to those reflected in the theoretical model. The volume fraction of (initially monomeric) protein ϕ is calculated as $\phi \equiv V_{\text{protein}}/V_{\text{solution}} = Mc/\rho_{\text{protein}}$, since $V_{\text{protein}} = m_{\text{protein}}/\rho_{\text{protein}} = c \cdot M \cdot V_{\text{solution}}/\rho_{\text{protein}}$, where V_{protein} is the volume occupied by the protein molecules, V_{solution} is the total volume, m_{protein} is the mass of the dissolved protein, c is the protein molar concentration, M is the protein molar mass, and ρ_{protein} is the protein mass density.

The mass density of α -synuclein was estimated according to Fischer *et al.* (2004, eq. 2), inserting $M = 14.460$ kDa for the molar mass, giving $\rho = 1.46 \cdot 10^3$ mg/ml. The mass density is assumed to be constant upon folding and aggregation of the protein. This assumption may be an oversimplification, but pending detailed structural information about the fibril architecture we consider this estimate to be the best justified estimate available (see section 5.2.1 below).

A second conversion is that between the units in which length is measured. In the theory, the length of a fibril is given as the degree of polymerization divided by the number of protofilaments per fibril, i.e., in terms of a number of molecules, rather than in nanometers. The ‘length of one protein molecule’ along the fibril long axis equals one inter- β -strand distance of 0.47 nm (Serpell *et al.*, 2000). A fibril that contains four protofilaments and has a length of 1 μm would thus contain approximately $8.5 \cdot 10^3$ monomers.

Finally, the theory requires that the conformation of the end monomers of the fibrils be specified. This can be done in three ways: we can force all fibril ends to be in a non- β conformation, we can fix them in a β conformation, or we can allow them to attain either of these conformations (van Gestel and de Leeuw, 2006). The first of these boundary conditions causes the model to predict the formation of unrealistically long fibrils for reasonable values of the free-energy parameters. The other two descriptions of fibril end conformation yield realistic, and equivalent, results. We chose to allow both conformations since the actual conformation of fibril ends is not known.

5.2.1 Estimates of physical properties of α -synuclein fibrils

Linear number density of monomers in α -synuclein fibrils

It will be useful to estimate some basic physical properties of an α -synuclein fibril. To approximate the linear number density of monomers in a mature fibril (the number of monomers per μm) we make two assumptions: (i) that there are $n_f = 4$ filaments per mature fibril, as in the hierarchical assembly model (Khurana *et al.*, 2003) and (ii) that the inter-monomer distance in the fibril length direction is equal to one inter- β -sheet distance of $d_m = 0.47$ nm. This

second assumption is justified by X-ray diffraction experiments that demonstrated that α -synuclein fibrils show the same cross- β structure as is known for A β fibrils (Serpell *et al.*, 2000). We then estimate that the linear number density N of α -synuclein monomers in a mature fibril is

$$N = n_f/d_m \cdot 1000 \text{ nm} = 8.5 \cdot 10^3 \text{ monomers per } \mu\text{m of fibril.} \quad (5.9)$$

This is a very densely packed structure compared to for example actin filaments, which has ~ 360 monomers per μm of fibril in a 2-stranded fibril with an inter-monomer spacing of 5.5 nm (Howard, 2001, chapter 7).

Mass density of α -synuclein fibrils

Another important basic physical parameter is the mass density ρ of the protein molecules, both in its intrinsically disordered monomeric form and incorporated in a fibril as a β -sheet-folded molecule. As a starting point, Lodish *et al.* (2000) states that proteins in general have a mass density of $\rho = 1.35 \text{ g/ml}$. This rough estimate was refined by Fischer *et al.* (2004) who found that protein density is a molecular weight-dependent function, which increases substantially with decreasing molecular weight below 20 kDa. At a molecular weight of $M = 14.46 \text{ kDa}$, α -synuclein would have a mass density of $\rho = 1.46 \text{ g/ml}$ (Fischer *et al.*, 2004, equation 2). They do not specify which fold or packing the proteins they analyzed are assumed to have. In our lab, in gel electrophoresis experiments the intrinsically disordered α -synuclein runs at 19 kDa, and in gel filtration chromatography experiments it has an apparent molecular weight of 50 kDa. The fact that the apparent molecular weight is larger than the actual molecular weight implies that α -synuclein, when monomeric in solution, has a reasonably extended conformation.

When α -synuclein is incorporated in a fibril, we can estimate the mass density as follows. We approximate the fibril as a cylinder, with a radius $r = 5 \text{ nm}$, length L , and a volume $V = \pi r^2 \cdot L$. With the linear number density $N = 8.5 \cdot 10^3 \text{ monomers per } \mu\text{m}$ of fibril estimated above, and with $m = (N/N_A) \cdot M$, with N_A Avogadro's number, we obtain

$$\rho_{\text{fibril}} = \frac{m}{V} = \frac{NM}{N_A \pi r^2 L} = 2.6 \text{ g/ml} \quad (5.10)$$

The mass density of a fibril thus likely falls in the range of 1.35–2.6 g/ml. In this chapter, we use the Fischer estimate of 1.46 g/ml for α -synuclein, whether monomeric or fibrillar, and note that based on the above the actual mass density may be higher.

Estimation of concentration of α -synuclein in a neural cell

In order to relate the critical concentration for α -synuclein fibrillization found in our theory and experiments to the in vivo situation we need to approximate the concentration of α -synuclein in a neural cell. It is estimated that α -synuclein comprises a fraction of $f_a \cong 0.5 - 1.0$ % of brain cytosolic protein (Shults, 2006). If we assume that proteins make up for $f_p = 20$ % of a cell's weight, and the average mass density of a cell is $\rho_c = 1.03$ g/ml (both estimates from Lodish *et al.* (2000)), we can approximate the concentration of α -synuclein in a neuron as

$$c_a = \frac{n_a}{V_c} = \frac{m_a}{MV_c} = \frac{f_a f_p \rho_c}{M} = 70 - 140 \mu\text{M} \quad (5.11)$$

using $n_a = m_a/M_a$, $m_a = f_a f_p m_c$ and $m_c = \rho_c V_c$, where c_a is the molar concentration of α -synuclein in the cell, n_a is the number of moles of α -synuclein in the cell, m_a is the total mass of the α -synuclein, and V_c and m_c are the cell volume and mass respectively, which cancel in the equation.

5.3 Results

5.3.1 Influence of initial protein concentration on fibril formation

Under the conditions employed in this investigation, α -synuclein forms no fibrillar aggregates when the initial concentration is 5 or 10 μM (see representative images A and B in fig. 5.3). The apparent lateral dimensions of these aggregates are exaggerated by tip-sample convolution (about 20 nm). Their height is in the order of a few nanometers (see also fig. 5.4a,b). The aggregation with an initial concentration of 20 μM produced many small aggregates similar to the ones observed for 5 and 10 μM , and a small amount of short fibrils, most of them under 1 μm in length (fig. 5.3c). For higher concentrations (30 μM up to 250 μM) progressively more and longer fibrils were observed (fig. 5.3d-f).

5.3.2 Critical α -synuclein concentration for fibril formation

The fibril length measurements for each initial concentration condition are summarized in the length distribution histograms shown in fig. 5.4. The distribution of mature α -synuclein fibril lengths depends on the initial protein concentration: the distributions display a tail towards longer fibril lengths that becomes more pronounced at higher concentrations (fig. 5.4c-g). The shortest fibrils (less than 100 nm in length) at each aggregation condition are under-represented in the histograms because the tip-sample convolution and pixel resolution require

Figure 5.3. Fibril length depends on initial concentration.

AFM height images of wildtype α -synuclein aggregates formed at initial protein concentrations increasing from 5 to 250 μM . All scale bars 1 μm .

a minimum length for identification as a fibril in the AFM images, in this case 40 – 80 nm.

To quantify the effect of increasing protein concentration, the numerical average of the fibril lengths was calculated from each length distribution. Under our experimental conditions, there is very little fibrillization below a threshold initial concentration of approximately 15 μM ; above this concentration progressively longer fibrils form (fig. 5.5). See the next section for a discussion of the error on the critical concentration estimate. The vertical error bars at the 5 and 10 μM data points in fig. 5.5 are set to 20 nm because that is the ‘tip-sample convolution resolution’: the lateral size (‘length’) of the aggregates appears as 20 nm due to the finite AFM tip size, but the real length is smaller. The resolution of the length measurements is taken as the uncertainty on the means of the other concentrations, and is 40 nm for 20, 30 and 50 μM , and 80 nm for the higher concentrations.

Figure 5.4. Fibril length distributions for wildtype α -synuclein fibrils formed in vitro from a range of initial protein concentrations (5, 10, 20, 30, 50, 100, 250 μ M)

For 5 and 10 μ M samples the aggregate height instead of length was measured as indicator of aggregate size (a-b), since no measurable fibrillation occurred. The solid lines in the distributions for 20 – 250 μ M (c-g) are theoretical predictions using the same free-energy parameters as in fig. 5.5. The reported n is the number of fibrils measured at each concentration. Bin sizes are 0.4 nm (for 5 and 10 μ M concentrations) and 100 nm (20 – 250 μ M).

Figure 5.5. Concentration dependence of mean α -synuclein fibril length. The data points are the mean lengths from the fibril length distributions (fig. 5.4), the solid line is the concentration dependence predicted by the statistical-mechanical model (lateral binding free energy $F = -3.8k_B T$, binding free energy between β -folded monomers $P = -6.2k_B T$).

5.3.3 Free energy of interactions in α -synuclein fibrils

We use the experimentally determined mean fibril lengths to establish the free energies of the interactions in α -synuclein fibrils as modeled by the statistical-mechanical model. While the original model contains four free-energy parameters, only two of these parameters influence the predicted concentration dependence of the mean fibril length. These are the free energy of an interaction between β -folded monomers P , and the lateral-interaction free energy, F (see fig. 5.2). The other two parameters, that describe the interaction between disordered protein molecules and the transition between a disordered and an ordered regime along the fibril axis, turn out to have a negligible effect. This observation indicates that the mature fibrils that dominate the system at high enough protein concentrations contain very few disordered protein molecules. A similar result was found by Nyrkova *et al.* and co-workers in their analysis of protein fibril formation by a synthetic peptide 2000.

To quantitatively compare the experimental results to the theory, we fixed the values of all four free-energy parameters, and calculated the unknown fugacity z from the protein concentration using eq. 5.2. Subsequently, eq. 5.8 was used to calculate the mean fibril length for the fixed values of the free-energy

parameters and at the specified concentration. By repeating this process for different values of ϕ , we obtain the dependence between the protein concentration and the mean fibril length (fig. 5.5). By systematically varying the free-energy parameters P and F we conclude that the mean fibril length at high concentrations only depends on the β -bond free energy P and not on F , whereas the predicted critical concentration depends on both free-energy parameters. Observing the experimental results (fig. 5.5), we set the limits between which the critical concentration must fall at 10 and 20 μM , and use this as a criterion to determine which values of the free energy parameters yield good agreement between theory and experiment. We then specify the error margin of the critical concentration as $c_{crit} = 15 \pm 5 \mu\text{M}$. The constant slope of the curve (fig. 5.5) at high concentrations is virtually independent of the value of either parameter.

Good agreement between theory and experiment is found when the β interaction free energy P lies between -6.3 and -6.0 times $k_B T$, and the lateral-association free energy F is between -4.3 and -2.9 times $k_B T$, with k_B Boltzmann's constant and T absolute temperature. The solid line in fig. 5.5 shows the predicted concentration dependence of the mean fibril length for $P = -6.2 k_B T$ and $F = -3.8 k_B T$. These values correspond to $P = -15.9 \text{ kJ/mol}$ and $F = -9.8 \text{ kJ/mol}$ at the fibrillization temperature of 37°C .

Using the same values for the free-energy parameters, we compared theoretically calculated length distributions to those measured by AFM. The statistical-mechanical model reproduced the experimental results semi-quantitatively for concentrations above 10 μM (fig. 5.4). We plotted the number density (proportional to the number of fibrils per unit volume) of fibrils with a length that falls within each specified interval (expressed as a number of monomers m), divided by the total fibril number density. The total fibril number density was calculated by summation of the fibril number density over all fibril lengths. This is summarized in eq. 5.12. Only fibrils consisting of four protofilaments were taken into account in the calculation, because fibrils that contain fewer than the maximum allowed number of protofilaments tend to stay very short (van Gestel and de Leeuw, 2006).

$$\frac{\rho(p=4, m)}{\rho(p=4)} = \frac{\rho(p=4, m)}{\sum_{N=1}^{\infty} \rho(p=4, m)} = (ksfz)^{4m-8} f^{2-m} \left[1 - \frac{(ksfz)^4}{f} \right] \quad (5.12)$$

The theory predicts an exponential decay of the number of fibrils of a given degree of polymerization with their length (solid lines in fig. 5.4c-g). The theory provides an excellent prediction for the fractions of long fibrils, but the agreement is less obvious for short fibrils. In particular, fewer short fibrils (with lengths below a few hundred nm) are found experimentally than would be expected from the model prediction. This may be due to the finite resolution of

the AFM imaging and length measurement procedure. Both the theory and the AFM measurements indicate that virtually no fibrils are formed in aggregations with initial protein concentrations of 5 and 10 μM .

5.4 Discussion

The critical concentration for α -synuclein fibrillization of $15 \pm 5 \mu\text{M}$ is in the same order of magnitude as an earlier reported critical concentration for α -synuclein of 28 μM , determined by quantitative amino acid analysis (Wood *et al.*, 1999). The critical concentration is well below the estimated in vivo concentration of α -synuclein in neural cells (70 – 140 μM). This indicates the possibility of amyloid fibril formation at normal physiological conditions, not necessarily involving overexpression of α -synuclein. The fibril lengths we find are also realistic: fibrils up to several μm do not have to fold upon themselves to fit in a Lewy body with an approximate diameter of 10 μm .

We determined the free energies of two types of bond within an α -synuclein amyloid fibril: the free-energy difference between bound and unbound states of the monomers, reflected by the parameter P which we determined to lie between -16.2 and -15.4 kJ/mol, and the free energy of the lateral bond between two protofilaments in a fibril, reflected by the parameter F , which is between -11.0 and -7.4 kJ/mol. These values imply that the bonds that make up the fibrils are about two to four times as strong as a hydrogen bond in a protein ($\sim 4.2 - 8.4$ kJ/mol), four times as strong as a typical Van der Waals interaction (~ 4.2 kJ/mol) and roughly 20 times weaker than a single covalent C-C bond (~ 347 kJ/mol).

The bond between the monomers in a protofilament is about 1.5 times as strong as the bond between protofilaments. Considering nanodeformation experiments using AFM tips, one would expect ‘unzipping’ of protofilaments to occur rather than breakage of fibrils perpendicular to the long axis. This prediction is consistent with results on A β reported by Kellermayer *et al.*, who used an AFM tip as a nanomanipulation tool (2005). Our value for the lateral association free energy F is very similar to their free energy of lateral binding of ~ 9.6 kJ/mol determined using mechanical unzipping of β -sheets from A β fibrils.

The statistical-mechanical model we employ holds under two conditions. The first is that the protein solution is dilute enough that the effects of inter-aggregate interactions (such as those of the excluded-volume type) may be neglected. This condition is likely satisfied, as the α -synuclein concentrations used in this investigation are in the micromolar range (100 μM is equivalent to 1 molecule per 16,000 nm³), and the concentration of fibrils is much lower still. The second condition is that the processes described by the model are

reversible, where the observed species can be reasonably assumed to be in thermodynamic equilibrium with the surroundings. Fibrillization of α -synuclein as of Alzheimer's A β protein (Wetzel, 2006), can be considered to be reversible: fibrils were shown to dissociate under high hydrostatic pressure (Foguel *et al.*, 2003).

The application of the statistical-mechanical theory to actual morphological data allows us to look in a new way at the interactions involved in α -synuclein fibrillization and to quantify the strength of the bonds involved. The model is equally applicable to other amyloid-forming proteins, provided that the maximum number of protofilaments per fibril and the intermolecular distance along the fibril long axis are known. Its predictions of the mean fibril length, the critical concentration and the fractions of fibrils with a given length will be valuable in establishing quantitative insights into the biophysics of fibril formation in other proteins. Detailed analysis of the kinetics and energetics of the aggregation process are essential to map the energy landscape for fibrillization, and to fill in the gaps in suitable theoretical models consistent with the physics of these complex biopolymer systems.

The existence of a critical concentration for aggregation is particularly interesting because critical monomer/nucleus concentration fluctuations may trigger the onset of nucleation (Podesta *et al.*, 2006), a process analogous to protein crystallization (ten Wolde and Frenkel, 1997). Since there is a growing consensus that early aggregate species are likely responsible for disease etiology, detailed morphological studies of the intermediate species around the critical concentration for fibrillization will yield key insights into potentially cytotoxic intermediates on the pathway to fibrillization, and to the development of intervention strategies for inhibition of aggregation or for fibril dissolution.

5.5 References

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Morphology and protein secondary structure content of Lewy pathology in nerve tissue sections of a Parkinson's disease patient

In vitro research on the aggregation and fibrillation of α -synuclein has contributed greatly to the molecular level understanding of Parkinson's disease and related neurodegenerative disorders. However, there is no *a priori* reason to expect the morphology and structural content of aggregates formed *in vitro* to be identical to the properties of *in vivo* inclusions.

To address this issue, we have made tapping mode atomic force microscopy and Raman spectroscopy images of fixed and dried nerve tissue sections from the autonomic ganglia (sympathetic trunk) of a Parkinson's disease patient. We compare the morphology and spectroscopic characteristics of Lewy pathology in tissue to the properties of *in vitro* produced and aggregated α -synuclein.

The surface morphology of deparaffinated and dried Lewy inclusions shows height differences of 20–100 nm on a length scale of ~ 200 nm, but we do not observe the fibrillar aggregates found *in vitro*. Analysis of the amide-I band of Raman spectra of Lewy pathology reveals the presence of mainly β -sheet folded protein comparable to *in vitro* solutions of α -synuclein fibrils, but not monomers. Finally, we discuss the effects of various aspects of sample preparation (formaldehyde fixation, deparaffination, and drying procedures) on the observed tissue properties.

The work described in this chapter is the result of a collaboration with dr. Rob de Vos and coworkers at the Laboratorium Pathologie Oost Nederland, Enschede, The Netherlands. Parts of the work described in this chapter will be published as:

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6.1 Introduction

The neuropathology of α -synucleinopathies such as Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) is characterized by the presence of proteinaceous inclusions within cellular dendrites and cell bodies or somata of neural cells. These inclusions appear as Lewy neurites (LN) in dendrites and axons and as Lewy bodies (LB) in the somata of affected nerve cells (Braak *et al.*, 2003, and see section 1.3 of this thesis). For the purposes of this investigation, LN and LB will collectively be called 'Lewy pathology' or 'Lewy inclusions'. Lewy pathology consists mainly of misfolded and aggregated α -synuclein (Shults, 2006; Braak *et al.*, 2006). The morphology of various species of α -synuclein aggregates have been studied in a laboratory context by many groups, using purified protein expressed in a bacterial system and aggregated *in vitro* (see for example Khurana *et al.*, 2003; Hoyer *et al.*, 2004, and this thesis). These fibrillar aggregates have been found to appear as long, unbranched threads consisting of multiple filaments. The diameter of the mature fibrils is well-defined and typically in the order of 10 nm (chapter 4) and their lengths are in the μm range (chapter 5). The question arises whether these aggregates are structurally the same as the aggregates found in Lewy inclusions in the brain tissue of a PD patient (Chamberlain *et al.*, 2000).

6.1.1 Ultrastructure of Lewy pathology

In vitro experiments have shown that fibrils have a tendency to cluster together after prolonged incubation. The fibrils then form a large, amorphous mass of aggregated protein. In a PD patient, the 'incubation time' is in the order of decades, so the presence of fibrillar structures in Lewy pathology is not obvious.

Investigation of the ultrastructure of Lewy bodies in the stellate ganglion using transmission electron microscopy (TEM) has shown that several types of Lewy bodies may be defined: filamentous Lewy bodies, which have a central core and radiating filaments; granular LBs; and 'LB related swellings' (Forno and Norville, 1976). Clear filaments are also present in TEM images of Lewy bodies by Duffy and Menefee (1965). Oligodendroglial inclusions, a pathological hallmark of multiple system atrophy, contain α -synuclein fibrils coated with amorphous material in the central core of the inclusions (Gai *et al.*, 2003).

One of the main techniques used to characterize the morphology of protein aggregates *in vitro* is atomic force microscopy (AFM) (for reviews, see Gosal *et al.* (2006) and chapter 2 of this thesis). Compared to electron microscopy, AFM has the decided advantage that samples need little sample preparation. The information in (transmission) EM and AFM images is largely complementary: in the case of protein aggregates, EM gives accurate lateral dimensions, where AFM measures the topography of the sample. AFM can also be applied to fixed tissue sections: Matsko (2007) show that AFM phase contrast

Figure 6.1. Comparison of fluorescence and Raman spectroscopy

Energy diagrams of fluorescence and non-resonant Raman scattering. Raman spectroscopy probes the molecular vibrational energy levels of the ground state of a molecule by monitoring the intensity and frequency distributions of inelastically scattered photons that are produced when the molecule is irradiated with a monochromatic light source. Compared to fluorescence microscopy, Raman scattering has a short excited state lifetime (fs/ps), a narrow emission bandwidth (0.5 nm), low emission intensity, and requires no label.

images of *C. elegans* give complementary information to TEM images. To our knowledge, AFM has not yet been used to study nerve tissue sections.

6.1.2 Structural characterization by Raman spectroscopy

AFM and EM reveal the morphology of a sample, but not the chemical composition. Vibrational spectroscopies such as Raman spectroscopy do yield insight into the chemical composition of a sample. Raman spectroscopy measures the intensity and spectral distribution of light emitted by a sample after it has been excited by an intense monochromatic light source such as a laser. The light that is emitted by the sample has a spectral intensity distribution that is characteristic of the vibrational modes in the molecules of the sample (fig. 6.1). Since different chemical groups in molecules have characteristic Raman signatures due to their specific stretching and bending modes, Raman spectroscopy can be used to determine the chemical ‘fingerprint’ of a sample.

In a biological context, Raman spectroscopy can be used to characterize molecular species such as proteins (Tuma, 2005). When using a Raman imaging system (also known as Raman microspectroscopy system), the distribution of DNA, lipids, and proteins in cells is also accessible (Baena and Lendl, 2004; van Manen *et al.*, 2005). Raman spectroscopy has also been applied to tissues, both for *in vivo* diagnostics, either confocal (Caspers *et al.*, 2003) or through a fiber-optical probe (Pappas *et al.*, 2000), and *ex vivo* on sections of a wide

variety of tissues (reviewed in Manoharan *et al.*, 1996; Krafft and Sergo, 2006), including human brain tumors (Mizuno *et al.*, 1994) and Parkinson’s disease affected monkey brain tissue (Ong *et al.*, 1999). A key issue these investigators faced was that histologically stained samples were necessary to identify features of interest on the tissue sections, but the staining interfered with the Raman measurements through a strong fluorescent background. In this work, we resolve this issue by using alternately stained and unstained consecutive sections to identify features of interest for AFM and Raman spectroscopic imaging on unstained sections. An important advantage of Raman spectroscopy relative to fourier transform infrared spectroscopy in the study of cells and tissues is that water gives less background signal in Raman than in IR spectra. The intrinsic fluorescence from tissue materials that swamps Raman signal when exciting the sample at visible wavelengths can be avoided by choosing an excitation wavelength that is well in the red (this work) or near infra-red (Mizuno *et al.*, 1994).

6.1.3 Quantification of protein secondary structure content

One useful tool in the Raman spectrum analysis of protein secondary and tertiary structure are the amide-I and -III bands. These are due to C=O and C-N stretch vibrations and N-H bend vibrations in the peptide backbone. These vibrations are very sensitive to the local environment of the atoms in the backbone and will give specific peaks when substantial fractions of the protein molecules in the sample have the same secondary and tertiary structure. The amide-I and -III bands are usually found at $1640 - 1690\text{ cm}^{-1}$ and $1230 - 1300\text{ cm}^{-1}$ respectively (Maiti *et al.*, 2004). We use the amide-I band to quantitatively measure the relative amounts of different types of secondary structure (specifically, α -helix, β -sheet, random coil) that occur in the protein sample. Band fits of the amide-I band of monomeric, oligomeric and fibrillar forms of α -synuclein in various buffers have shown that *in vitro* monomeric α -synuclein possesses both α -helical and β -sheet secondary structure. As fibrils are formed, the majority of the peptide bonds change conformation to β -sheet (Maiti *et al.*, 2004; Apetri *et al.*, 2006). In this work, we extend the understanding of the structure of amyloid fibrils by comparing the spectra of *in vitro* monomeric and fibrillar α -synuclein to Raman spectra measured on *ex vivo* human Lewy pathology and the surrounding tissue for the first time.

We will characterize the morphological and protein secondary structure content of Lewy pathology, and compare to monomeric and *in vitro* aggregates forms of α -synuclein. To this end we perform atomic force microscopy and

Raman spectroscopic imaging of nerve tissue sections obtained from the autonomic ganglia of the sympathetic trunk¹ of a Parkinson's disease patient.

We find that the surface morphology of Lewy inclusions has height differences in the order of 20 – 50 nm on a 200 nm length scale. We observed no fibrillar structures similar to those found by using TEM on sections (Forno and Norville, 1976; Duffy and Menefee, 1965). The surface morphology of the surrounding tissue shows deeper pits and has a less dense structure.

The protein secondary structure content of Lewy pathology as reflected in the Raman spectra on the other hand does very clearly indicate the presence of large quantities of β -sheet folded protein, comparable to *in vitro* formed α -synuclein fibrils.

The preparation of tissue sections for analysis, whether by EM, AFM or Raman spectroscopy, requires fixation of the tissue (usually with formaldehyde) and subsequent embedding in a supportive medium (typically paraffin or an epoxy resin) to allow microtome sectioning. The tissues used in this study were paraffin embedded. We used xylene deparaffination and two drying methods, ethanol gradient air drying, and critical point drying, to prepare the samples for AFM and Raman imaging. The potential influence of these sample preparation techniques on the observed morphology and spectral data will also be discussed.

6.2 Methods

Human nerve tissue sections

Nerve tissue was obtained from the autonomic ganglia (sympathetic trunk) from a patient who had been diagnosed with PD. The case fulfilled published criteria for macroscopic and microscopic diagnosis of PD (Ward and Gibb, 1990; van de Berg *et al.*, 2007). The tissue was embedded in paraffin and cut by a microtome in slices with a thickness of 2 μm . A series of ten consecutive sections were alternately stained and left unstained. The 5 sections to be used for localization of Lewy pathology were stained with hematoxylin-eosin (HE) for nuclei (purple) and cytoplasm (blue), and with anti- α -synuclein-DAB chromogen (brown) to identify the Lewy pathology (fig. 6.2a).

The 5 remaining (unstained) sections were deparaffinated using either of two procedures: (1) by immersing the slide three times in xylene (5 minutes, 5 minutes, and 1 minute) and then passing the slide through an ethanol gradient (99.8% (2x), 95% (2x), 70% (2x), milliQ water (3x)) and leaving it to dry in ambient air; or (2) by immersing three times in xylene, then immersing in

¹The autonomic ganglia are clusters of neurons forming the junction between nerves originating in the central nervous system and those innervating the tissues of the body. The sympathetic chain or trunk is located alongside the spinal cord and is part of the nervous system responsible for functions requiring fast responses (Paxinos and Mai, 2004)

Figure 6.2. Consecutive sections were stained and left unstained to locate Lewy pathology

Microphotographic maps of consecutive stained (left) and unstained (right) sections of nerve tissue of a Parkinson's disease patient and allow for identification of Lewy inclusions (*arrows* in inset). The sections have a diameter of ~ 2 mm and a thickness of $2\ \mu\text{m}$.

pure ethanol, and critical point drying using liquid CO_2 as the intermediate medium. The influence of the drying procedures on the observed morphology will be discussed in section 6.3.7.

Full microphotographic maps consisting of 17 – 20 stitched photos per section were made using a Zeiss Axiovert 200 inverted microscope with a 10x objective and an Axiocam MRc5 color camera. We aligned the microphotographic maps of subsequent sections to locate cellular and subcellular features in the unstained sections by comparing them to the adjacent stained section, where cells, nuclei and Lewy pathology are readily visible (fig. 6.2, and see the results section).

Atomic force microscopy of nerve tissue sections

We used tapping mode atomic force microscopy (TM-AFM) in air to characterize the surface of the deparaffinated tissue sections. We used a Veeco Bioscope I microscope with a MSCT-Au tip F cantilever ($f_{\text{res}} \sim 130$ kHz, $k = 0.5$ N/m) and free tapping amplitudes in the order of 100 nm. The setpoint for the tapping feedback was $A_{\text{set}}/A_{\text{free}} \sim 0.3$, resulting in relatively hard tapping. In our experience, more gentle tapping ($A_{\text{set}}/A_{\text{free}} \sim 0.9$) did not allow the feedback system to adequately track the surface topography of the tissue sections, with

maximum height variations on the order of 1 μm on a 100 nm length scale, which occurred at gaps in the tissue sections due to microtome sectioning.

Images were acquired at resolutions between 4 – 40 nm/pixel (1024×1024 pixels for images $> 10 \mu\text{m}$ and 512×512 pixels for images with a field of view smaller than $10 \mu\text{m}$). Scan velocity was at or below 40 $\mu\text{m/s}$ to avoid sample damage, leading to acquisition times of up to 34 minutes per frame for the images with a 40 μm field of view. There was no damage to the surface of the sections due to the imaging, as observed by repeated AFM imaging.

Confocal Raman imaging of nerve tissue sections

Confocal Raman imaging was performed using a lab-built laser-scanning confocal Raman spectroscopy instrument (Uzunbajakava *et al.*, 2003). The sample was excited with $\lambda_0 = 647.1 \text{ nm}$ Kr-ion laser light with an intensity at the sample of around $P_{\text{sample}} = 40 \text{ mW}$ through a $40\times$ objective. This intensity does not induce photodamage in the tissue as indicated by unchanged white light microscopy images and the stability of the Raman signals themselves. A custom-written LabVIEW program coordinated the movements of the scanning mirror with the spectral measurements.

The Raman spectrum was detected using a thermo-electrically cooled CCD camera containing a back-illuminated chip with 1340×100 pixels (Pixis 100, Princeton Instruments, NJ, USA) mounted on a Jobin-Yvon HR460 spectrograph with a 600 lines/mm grating. Images were collected at 32×32 pixels, with 1 second integration time per pixel. The spectral axis was calibrated using a toluene sample with known Raman peak locations.

Analysis of Raman images

A dark-current spectrum, originating from the camera readout electronics, was subtracted from the spectrum measured in each pixel of an image. Then, singular value decomposition (SVD) was performed to reduce noise on the individual spectra (Uzunbajakava *et al.*, 2003). The spectra were then clustered using principal component analysis (PCA) and hierarchical cluster analysis (HCA) (see for example van Manen *et al.*, 2005). This procedure identifies the major components present in the spectra of each of the image pixels and groups pixels with a similar spectrum into clusters. The minimum number of clusters needed for adequate discrimination of Lewy pathology and cellular background was determined by systematically increasing the number of clusters until two of the cluster-average spectra were virtually identical.

The spectra of Lewy pathology shown in the results section were obtained by subtracting the cluster average spectrum of the cellular background from the cluster average spectrum of the Lewy pathology. The spectra were normalized

to the CH₂ bending band at 1447 cm⁻¹, according to

$$I_{norm} = \frac{I - I(1492 \text{ cm}^{-1})}{I(1447 \text{ cm}^{-1}) - I(1492 \text{ cm}^{-1})} \quad (6.1)$$

$I(1492 \text{ cm}^{-1})$ was the nearest minimum to the band maximum at 1447 cm⁻¹. This was used as a background estimator throughout this work.

Raman spectroscopy on monomeric and fibrillar α -synuclein

Monomeric α -synuclein was expressed in a bacterial system and purified as described in chapter 4. A 4 μ l sample of 485 μ M α -synuclein (7.0 mg/ml) in a 25 mM TRIS-HCl buffer, pH 7.5, was introduced in a 0.3 x 0.3 mm square ID glass cuvette (Vitrecon Inc). Raman spectra were recorded using the instrument described above. Integration time per spectrum was 5 $\times$ 120 s and the laser power at the sample was 100 mW. Recording 5 spectra of 120 s instead of one of 600 s facilitates cosmic ray event removal. Excitation powers below $\sim$ 160 mW did not damage protein samples in buffer as indicated by unchanging Raman spectra, presumably because heat can dissipate freely through the liquid. The buffer spectrum was recorded using the same parameters and subtracted from the protein spectrum. For presentation purposes, the resulting spectrum smoothed using 5 point adjacent-averaging.

Fibrillar α -synuclein aggregates were obtained by shaking a 100 μ M wild-type α -synuclein solution in 50 mM TRIS, 100 mM KCl at 37 °C as described in chapter 4. The presence of fibrils was confirmed by AFM. Fibrils were deposited on a CaF₂ slide and 50 s spectra were recorded on the Raman microscope at $P_{sample} = 160$ mW. The spectrum was recorded and analyzed as described above.

6.3 Results

6.3.1 Surface morphology of Lewy pathology

The procedure of alternately staining and not staining consecutive sections allows for localization of specific cells and subcellular features such as Lewy pathology on unstained sections (fig. 6.3a,b). The Veeco Bioscope I system allows the operator to position the AFM cantilever over an area of interest with accuracy of several micrometers, which is accurate enough for locating micrometer-sized features of interest (fig. 6.3c).

Height images produced by tapping mode AFM in air of nerve tissue sections yield surface topography information (fig. 6.3d-f), even though the sections have been formaldehyde fixed, embedded in paraffin, sectioned by a microtome, deparaffinated in xylene, and critical point dried. Under our imaging conditions,

error (amplitude) and phase images of tissues and Lewy pathology did not yield extra information: there was no appreciable tapping mode AFM phase contrast (data not shown).

The surface morphology of Lewy pathology as measured by AFM displays a roughness on several length scales (fig. 6.3d-f). Line profiles of the Lewy pathology quantify the typical roughness: on a $5\text{ }\mu\text{m}$ length scale, pits are visible with a depth of $300 - 500\text{ nm}$ (fig. 6.3g), and on a length scale of $\sim 200\text{ nm}$ the surface shows height differences of $20 - 50\text{ nm}$ (fig. 6.3h). We did not observe any fibrillar structures similar to those found in *in vitro* aggregations of α -synuclein (typical diameter 10 nm , typical length in the micrometer range) on the Lewy pathology surface, irrespective of the relative orientation of sectioning surface and fibril axis.

6.3.2 Surface topography of surrounding tissue

The surface morphology of the tissue surrounding the Lewy pathology appears different from that of the Lewy pathology itself. We used the feature localization procedure comparing stained and unstained consecutive section to locate a large cell (fig. 6.4a, b). In the corresponding AFM height image, nucleus and cytoplasm can be readily identified (fig. 6.4d). It is also apparent that the tissue surrounding the large cell has a different structure.

Higher magnification AFM height images on the cell (fig. 6.4d, e) indicate that the cell has a less dense network of structures than the Lewy pathology (compare fig. 6.3). We observe height differences on the order of 500 nm on a length scale of $2\text{ }\mu\text{m}$ (fig. 6.4f), and height differences on the order of $50 - 100\text{ nm}$ on a length scale of $400 - 600\text{ nm}$ (fig. 6.4g). We speculate that these height differences represent inclusions and organelles present in the cytoplasm, such as the endoplasmatic reticulum, mitochondria, and lysosomes, and possibly also parts of the cytoskeletal network. The Lewy pathology (figs. 6.3 and 6.11) thus has a denser packing than the surrounding tissue on the $100 - 200\text{ nm}$ length scale.

6.3.3 Chemical composition of Lewy pathology

The Raman spectrum of Lewy pathology differs considerably from that of the surrounding tissue (fig. 6.5). The amide-I and -III bands, are much stronger in the Lewy pathology than in the surrounding tissue (fig. 6.5c and e). This indicates that the overall signal intensity in the Lewy inclusion is greater than in the tissue around it, presumably due to a very dense packing of protein. To get a proper quantification of the intensity of a Raman band it is important to subtract the local background. We approximated the background in the amide I region as a linear background (fig. 6.5d), and found that the intensity of the local background-subtracted β -sheet band at $1660 - 1680\text{ cm}^{-1}$ on this

Figure 6.3. Nanoscale surface topography of Lewy pathology (critical-point dried)

The Lewy inclusion indicated by the white arrow in (A) was identified on an unstained deparaffinated section (B) and imaged with TM AFM in air (the black triangle in (C) is the AFM cantilever). Figures (D), (E), and (F) show progressively higher magnification AFM height images of the Lewy inclusion. To quantify the surface roughness, line profiles (G) and (H) were measured on the low- and high-magnification images (D) and (F), respectively. *Image size, z-range, resolution, scan speed:* (D): 40 μm , 4 μm , 1024 pix/line, 40 $\mu\text{m/s}$. (E): 13.2 μm , 1.5 μm , 1024 pix/line, 26 $\mu\text{m/s}$. (F): 2 μm , 0.4 μm , 512 pix/line, 26 $\mu\text{m/s}$.

Figure 6.4. Nanoscale surface topography of a cell in a deparaffinated nerve tissue section (air dried)

The same procedure as in fig. 6.3 applied to the surrounding tissue. *cyt*, cytoplasm, *nucl*, nucleus. The Lewy inclusion marked * in (C) was too high for this color scale. Image size, z-range, resolution, scan speed: (C): 80 μm , 3.5 μm , 1024 *pix/line*, 80 $\mu\text{m/s}$. (D): 20 μm , 1.5 μm , 1024 *pix/line*, 20 $\mu\text{m/s}$. (E): 4.5 μm , 0.6 μm , 1024 *pix/line*, 4.5 $\mu\text{m/s}$.

Figure 6.5. Raman imaging spectroscopy of a Lewy inclusion

The same Lewy inclusion (A) that was investigated by AFM in fig. 6.3 was imaged using Raman imaging spectroscopy. (B) shows the total intensity in the Amide I β -sheet band of the Raman spectra, while (C) shows the the same band with the local background removed as indicated in (D). Typical spectra (E) correspond to the bulk of the inclusion (x), the edge of the inclusion (*), and the surrounding tissue (+). Scale bar in (A) 25 μm , in (B) and (C) 10 μm .

particular Lewy inclusion is stronger at the edges and at the center of the inclusion than in the bulk (fig. 6.5c).

The difference spectrum between the spectrum of a Lewy inclusion and a spectrum the surrounding tissue gives the spectral signatures of materials present only in the inclusion and not in the surrounding tissue (fig. 6.6). To exclude pixel-to-pixel variations in the Lewy pathology and tissue spectra, we use cluster-averaged spectra to calculate the difference spectrum. The difference spectrum of the Lewy pathology features a strong amide-I band, indicating high protein content, and a narrow peak due to phenylalanine at 1003 cm^{-1} .

Comparison of the cluster-averaged background-subtracted Lewy pathology spectra of several lesions on different sections indicates the Raman spectra of various Lewy inclusions are comparable but not identical (fig. 6.7). The amide-III feature is not as prominent in the inclusion labeled ‘LB3’, although the amide-I feature is present. All three inclusions had a strong peak at 1003 cm^{-1} . This peak is typically attributed to a ring breathing mode of the benzene ring in

Figure 6.6. Hierarchical cluster analysis allows for subtracting the average tissue Raman spectrum from the average Lewy pathology spectrum (A) a Lewy inclusion is found on a stained section. The *dotted line* indicates the original edge of the tissue section, as compared to the white light image (B) of the unstained section. The *dotted box* in (A) and (B) indicates the approximate site of the Raman spectral image. (C) A 3-cluster hierarchical cluster analysis Raman image of the Lewy pathology. The spectra grouped together on the inclusion are colored orange, the surrounding tissue is blue. Image size is 15 μm . (D) shows the cluster average spectra of the Lewy pathology (orange), the surrounding tissue (blue) and the difference spectrum (green).

the amino acid phenylalanine (Movasaghi *et al.*, 2007). It is interesting to note that the lesion labeled ‘LB3’ has an elongated shape and a spectral signature differing from the other two, but its morphology is comparable to other Lewy lesions (compare fig. 6.3, which is ‘LB2’, with fig. 6.11, which is ‘LB3’).

Figure 6.7. Comparison of Raman spectra of Lewy inclusions

Different Lewy inclusions on different sections have similar but not identical Raman spectra. (A) shows three Raman cluster images of different inclusions. (B) shows the corresponding Lewy-only Raman spectra, as well as the spectrum of pure solid paraffin (intensity axis scaled down relative to the other spectra, see the text). The most intense paraffin peaks (at 1062, 1132, and 1295 cm^{-1}) appear in one of the inclusions but not in the others (arrowheads). Image size: 15 $\times$ 15 μm .

Figure 6.8. Raman spectra of monomeric and fibrillar α -synuclein and Lewy pathology.

The fingerprint region shows clear differences in the amide-I and -III bands (*highlighted*) that indicate protein secondary structure. Respective backgrounds of buffer and tissue have been subtracted; the spectra have been normalized to the 1447 cm^{-1} CH_2 bending mode and shifted vertically for clarity of presentation.

6.3.4 Protein secondary structure content of Lewy pathology

We compared the Raman spectrum of Lewy pathology to the spectra of monomeric and *in vitro* fibrillized α -synuclein (fig. 6.8). The spectra have been normalized to the 1447 cm^{-1} band (see the methods section). All three spectra show the typical protein backbone amide-I and -III bands. The Lewy pathology spectrum also shows a strong peak at 1003 cm^{-1} due to the amino acid phenylalanine. All three spectra show a large number of minor peaks throughout the fingerprint region, which are attributed to vibrational modes in the constituent amino acids and will not be analyzed further in this work. In this analysis, we focus on the amide-I band.

The amide-I band is due to the $\text{C}=\text{O}$ stretching vibration in the polypeptide backbone, and it is very sensitive to the local hydrogen bonding network. For this reason, this band gives much information on the secondary structure of the protein present in the sample. A three band curve fit of the $1550 - 1700\text{ cm}^{-1}$ spectral region with Lorentzian peaks (*cf.* Maiti *et al.*,

Figure 6.9. Amide I bandfits quantify relative contribution of protein conformations

Lewy inclusions consist of protein with β -sheet folded secondary structure, similar to α -synuclein fibrils. Multiple Lorentzians (green solid lines) were fit to the 1550–1700 cm^{-1} regions of the Raman spectra shown in fig. 6.8 (blue solid lines). The red dotted lines are the sum of the Lorentzians. Lewy pathology clearly contain much β -sheet folded protein (peak at $\sim 1670 \text{ cm}^{-1}$). The intensity axes are scaled independently for clarity of presentation.

2004) shows that monomeric α -synuclein possesses bands at ~ 1650 , 1670 and 1685 cm^{-1} (fig. 6.9a), whereas in *in vitro* grown fibrils, the amide I peak has narrowed into one band at $\sim 1670 \text{ cm}^{-1}$ (fig. 6.9b). In Lewy inclusions the $\sim 1670 \text{ cm}^{-1}$ peak is much more intense than the other two, but the spectrum has contributions from the 1650 and 1685 cm^{-1} peaks as well (fig. 6.9c).

The peak at $\sim 1650 \text{ cm}^{-1}$ is assigned to α -helical structure, the peak at $\sim 1670 \text{ cm}^{-1}$ to β -sheet conformation, and the peak at $\sim 1685 \text{ cm}^{-1}$ may be due to short β -strand fragments, random and turn conformations, and polyproline II structure (Maiti *et al.*, 2004). The peaks at 1579 and $1610 - 20 \text{ cm}^{-1}$ in the spectra are due to ring modes of aromatic residues (Movasaghi *et al.*, 2007), and are included in the fit only because they could not be baseline separated from the amide I feature.

The relative contributions of the three amide I peaks have been quantified in table 6.1. The β -sheet peak in the fibrillar species is narrower than in monomeric α -synuclein and in the Lewy pathology, indicating the β -sheet packing in the fibrils is very regular. The α -helix peak is wider in the Lewy pathology than in α -synuclein monomers, indicating a larger degree of conformational freedom for the proteins present in the Lewy inclusion. It should be noted that in Lewy bodies and neurites around 50 species of protein have been identified, not just α -synuclein (Shults, 2006). The spectra shown in fig. 6.9 prove however that β -sheet folded protein is by far the major component of Lewy pathology.

peak assign- ment	α -synuclein monomers			α -synuclein fibrils			Lewy pathology		
	center cm^{-1}	width cm^{-1}	area %	center cm^{-1}	width cm^{-1}	area %	center cm^{-1}	width cm^{-1}	area %
α -helix	1647	26	31				1651	34	31
β -sheet	1668	26	45	1666	14	100	1668	23	62
other	1686	14	24				1687	20	7

Table 6.1. Quantitative analysis of secondary structure content

Peak positions, widths, and areas of the three ~ 1650 , ~ 1670 and $\sim 1685 \text{ cm}^{-1}$ Lorentzian peaks fitted to the amide-I band of the Raman spectra of monomeric synuclein, synuclein fibrils and Lewy pathology in fig. 6.9. These numbers are intended to give an indication of the relative contributions of the secondary structures and should be regarded as such.

6.3.5 Influence of formaldehyde fixation on fibril morphology

Tissues that are prepared for pathological examination need to be fixed in order to prevent degradation. The fixation of tissue samples with formaldehyde introduces cross-links between proteins in the tissue. First, a fast addition reaction takes place within minutes, where the formaldehyde molecule adds to the amine group. Then, a slow condensation reaction takes place (hours-days, weeks for whole tissues), that cross-links the proteins. The conformation of proteins remains unchanged by formaldehyde fixation (Mason and O’Leary, 1991). We fixed a solution of *in vitro* formed α -synuclein fibrils in 4 % paraformaldehyde for 3,5 h. AFM height images of the fibrils indicate that the qualitative morphology of α -synuclein fibrils did not change after fixation (fig. 6.10).

6.3.6 Influence of paraffin embedding on tissue Raman spectra

Histological tissue sections are typically embedded in paraffin to provide mechanical support for sectioning. In order to perform AFM and Raman spectroscopy measurements, the paraffin needs to be removed, since otherwise it may be the paraffin surface that is measured instead of the tissue surface. The strongest peaks of solid paraffin (fig. 6.7) are almost an order of magnitude more intense than the Raman signal from the tissue at equal excitation power (the paraffin spectrum shown in fig. 6.7 has been scaled down for clarity of presentation), but are not present in the tissue spectra. In the spectrum of one of the imaged Lewy inclusions however, minor peaks at 1062, 1132, and 1295 cm^{-1} can be observed (fig. 6.7), and peaks at 1440 and 1463 cm^{-1} may contribute to the CH_2 bending band at 1447 cm^{-1} . This indicates that in most tissue sections, all traces of paraffin have been removed by the deparaffination procedure with xylene, but this particular inclusion appears to contain some residual paraffin.

Figure 6.10. Formaldehyde fixation does not change gross morphology of α -synuclein fibrils

AFM height images of fibrils grown *in vitro* without shaking. (A) control sample, (B) fibrils fixed in 4 % formaldehyde for 3,5 h.

6.3.7 Influence of drying procedure on tissue morphology

To assess the influence of the tissue section drying process on the apparent surface morphology, we dried the deparaffinated tissue sections either in air or by critical point drying (CPD). During critical point drying, liquid ethanol surrounding and pervading the sample is replaced by liquid CO_2 , which is then heated to its critical temperature (40 °C). The CO_2 then transitions to a vapor state, after which it is slowly released from the pressure chamber. The advantage of this procedure is that the sample is never exposed to the surface tension forces associated with a retreating water-air interface, which may damage the cellular structures. Especially in electron microscopy, it has been shown that CPD results in fewer drying artifacts than evaporative drying. However, we observe similar surface morphology of Lewy pathology on tissue sections dried using either procedure (compare height differences at 200-300 nm length scale in fig. 6.3h to fig. 6.11f). We thus conclude that the surface morphology of Lewy pathology in deparaffinated tissue sections is not adversely affected by evaporative drying.

6.4 Discussion

This work takes a first step in translating *in vitro* determined properties of α -synuclein fibrils to the surface morphology and protein content of Lewy lesions found in Parkinson's disease patients. The method of alternately staining and leaving unstained consecutive sections to locate lesions of interest for further

Figure 6.11. Nanoscale surface topography of a Lewy inclusion (air-dried)

The inclusion indicated by the white rectangle in (A) was identified on an unstained deparaffinated section (B) and imaged with TM AFM in air. Figures (C) and (D) show progressively higher magnification surface topography images of the inclusion. To quantify the surface roughness, line profiles (E) and (F) were measured. *Image size, resolution, scan speed:* (C): 20 μm , 1024 pix/line, 40 $\mu\text{m/s}$. (D): 5 μm , 1024 pix/line, 20 $\mu\text{m/s}$.

microscopic study has worked well. The problem of histological staining interfering with the Raman signal from the tissue sections as described by (Ong *et al.*, 1999) is solved with this method. By making full microphotographic maps of the entire sections it has been possible to locate specific cells and sub-cellular features on a sample 40,000 times the area of those features, on several different microscope systems.

The efficiency of the method could be further improved by selecting tissue sections with a large number of Lewy lesions near the edge of the section, where it is relatively easier to locate them than in the middle of a section, and by having combined instruments, eliminating the need for physically moving the sample between measurements using different modalities.

Morphology

We have characterized for the first time the surface morphology of Lewy lesions using atomic force microscopy. The surface morphology of Lewy pathology as measured by our AFM experiments shows height differences in the order of 20-50 nm on a length scale of several 100 nm. These findings are not consistent with the prevailing picture of Lewy bodies consisting of fibrils radiating from a dense core, as has been observed by transmission electron microscopy. Rather, we see a surface consisting of scale-like features. These are similar to those found in AFM phase contrast images of oligodendroglial inclusions in brain tissue by Pountney *et al.* (2004), and are attributed by these authors to be the end faces of the fibrils. The structures we see are too big to be construed as such, even when considering tip-sample convolution effects. Instead, we speculate the scale-like morphology is due to amorphous α -synuclein aggregates, or fibrillar aggregates coated with amorphous material, as was seen in TEM images of α -synuclein fibrils in oligodendroglial inclusions (Gai *et al.*, 2003).

The greater average height (around 1,5 μm) of the Lewy inclusions relative to the surrounding tissue (around 1 μm) suggests that the inclusions are more resistant to microtome sectioning than the tissue at large: we can speculate that the inclusions are compressed when the knife cuts the section and ‘rebound’ afterwards. This would also explain why the Raman signal intensity on the Lewy lesions is greater than in the surrounding tissue: there is more material in the focal volume.

The absence of AFM phase contrast in our measurements once again underlines the necessity to develop a reliable method to obtain reproducible phase contrast, as discussed in chapter 2. AFM phase contrast should yield useful material property information on tissue sections, as demonstrated for sections of *C. Elegans* in (Efimov *et al.*, 2007; Matsko, 2007).

A possible extension of these results could lie in making adhesion images using functionalized AFM tips: certain receptors are still functional even after formaldehyde fixation and the localization of those receptors on the various

substructures in the tissue could be imaged.

Protein secondary structure content

The protein structural content of Lewy pathology had not been directly compared to the secondary structure content of its main component, the protein α -synuclein. The Raman spectra of Lewy pathology show they contain mainly β -sheet folded protein, with structural content similar to that of mature α -synuclein fibrils. In light of the fact that Lewy pathology consists mainly of α -synuclein (Shults, 2006), we interpret the prominence of β -sheet folded protein in the inclusions to be due to fibrillized, or at least β -sheet-folded, α -synuclein. The situation is analogous to that of fibrillar A β *versus* isolated and purified senile plaques from the brain of Alzheimer's disease patients (Dong *et al.*, 2003). These authors also found a strong β -sheet peak in the amide-I region for both fibrils and plaque. The Raman spectra of solutions of monomeric and fibrillized α -synuclein conform to the spectra measured by Maiti *et al.* (2004). The amide-I bandfitting has also been used in characterization of various silk fibres made by spiders, which also show a strong β -sheet component at around 1669 cm^{-1} (Lefevre *et al.*, 2007). These authors attribute the high extensibility of spider silk to the fact that the protein chains are not completely extended and have a high content of extensible structures.

Sample preparation methods

We have also considered the influence of the measurement conditions, and specifically the sample preparation methods, on the observed morphological and spectroscopic properties of the system. In the case of tissue sections, four main processes may influence the resulting sample: (i) formaldehyde fixation, (ii) paraffin embedding, (iii) microtome sectioning, (iv) deparaffination.

Our finding that the structure of the protein fibrils does not appear to change after formaldehyde fixation, is consistent with literature reports that the secondary structure of proteins does not change upon formaldehyde fixation, as measured by FTIR spectroscopy (Mason and O'Leary, 1991; Ikeda *et al.*, 1998). The structure of the protein aggregates appears to be locked in by the cross-linking effected by the formaldehyde. Formaldehyde fixation was also found to be the best fixative for cells to be imaged by AFM (Moloney *et al.*, 2004). We found that the microtome blade did not leave marks on the $2\text{ }\mu\text{m}$ thick sections that hindered AFM imaging. Presumably, any scratches made by the knife blade would be on a length scale far greater than the tens of micrometers typically covered by an AFM image.

As for the paraffin embedding and subsequent deparaffination, we found that xylene deparaffination procedures typically used in pathological laboratories suffice to remove nearly all traces of paraffin. There was only one section

that had trace amounts of paraffin left in the Lewy lesions, as evidenced by Raman spectroscopy. Alternative embedding methods include use of epoxy resins (Matsko and Mueller, 2004). The quality of the resin determines in large part the contrast that can be achieved in height and phase images of tapping mode AFM. The quality of AFM imaging may be further improved by applying chemical etching to resin-embedded sections (Tiribilli *et al.*, 2005).

6.5 References

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Conclusions and future directions

The objective of the research described in this thesis has been to contribute to the understanding of the etiology of Parkinson's disease by investigating the nanoscale morphology and structure of aggregates formed by α -synuclein.

We have developed a method to quantify the morphology of amyloid fibrils formed *in vitro* based on atomic force microscopy images, quantified the differences between amyloid fibrils formed by wild-type and Parkinson's disease-related mutants of α -synuclein, determined free-energy parameters of interactions in α -synuclein fibrils using a statistical-mechanical model of α -synuclein aggregation, and analyzed Lewy pathology in nerve tissues affected by Parkinson's disease.

In this last chapter, we draw conclusions from the results and interpret them in the context of the etiology of Parkinson's disease. We discuss how these results hint at potential future directions for research in this field.

7.1 Morphology and structure of fibrillar α -synuclein aggregates

The successful treatment and potential prevention of Parkinson's disease and the other synucleinopathies can only come closer if we understand what causes the damage that cripples the neurons that are affected by the disease. Since pathologists find lesions consisting of aggregated α -synuclein in the brain tissue of deceased Parkinson's disease patients, the properties of these aggregates may hold a clue to the mechanisms of the disease.

This work has attempted to contribute to the basic biophysical understanding of the formation, structural properties, and morphology of fibrillar α -synuclein aggregates. We asked how to quantify the fibril morphology as measured by atomic force microscopy, what the influence of disease-related point mutations on the fibril morphology might be, how the process of α -synuclein aggregation can be modeled biophysically, and how the aggregates found *in vitro* compare to those in the brain tissue of Parkinson's disease patients.

The following sections will draw conclusions from the results presented in the chapters of this thesis, and offer interpretations of these conclusions in the context of the etiology of the synucleinopathies. We will end by suggesting future directions for research attempting to bring the treatment of neurodegenerative disorders one step closer.

7.1.1 Quantitative AFM of protein fibrils

Atomic force microscopy can be used for qualitative imaging, but the usefulness of AFM as a technique for the nanoscale characterization of biological systems increases substantially when the morphological and nanomechanical properties of the system under study can be made quantitative.

Quantification of properties of amyloid fibril systems as measured by AFM is complicated by several factors, such as (i) tip-sample convolution effects disguising the true morphology of the sample, (ii) incomplete knowledge of the contrast mechanisms rendering image interpretation difficult, and (iii) distortion of sample morphology by the imaging environment.

The main effect of tip-sample convolution is to increase the apparent width and trough height of twisted fibrillar aggregates, but the periodicity of twisted fibrillar structures is not affected (section 4.2). It also imposes a minimum length in at least one lateral direction to make useful quantifications of fibril length (sec. 5.2). Using a sharper tip increases the lateral resolution in the image but does not yield additional insight into the structure of α -synuclein fibrils (sec. 3.3.3).

The interpretation of height and amplitude images is straightforward, but phase imaging has not been successfully interpreted in terms of a material

property of the soft, biological sample (sec. 2.3.4). This challenge is further discussed in section 7.3.3.

The imaging environment (air or an aqueous pH-buffered solution) has a pronounced effect on the observed morphology, particularly on the apparent height of the fibrils. Tapping mode AFM in air yields 17–30 % lower apparent protein fibril height than in liquid (sec. 3.3.2), presumably due to dehydration of the protein. Measuring in liquid is more physiological and thus probably yields an apparent morphology closer to the ‘true’ morphology, but it also increases tip-sample damage (sec. 3.3.3).

Morphological properties of amyloid fibrils such as length, average height, modulation depth, and periodicity can be measured quantitatively and accurately by AFM imaging in liquid and subsequent segmented line profile analysis. This procedure yields quantitative data for characterization and comparison of protein fibril morphological properties (chapter 4).

7.1.2 Fibrils from disease-related α -synuclein mutants

Fibrils formed by disease-related single-point mutants A30P and E46K of α -synuclein are morphologically similar but not identical to wild-type fibrils. The average fibril height is marginally lower: 10.0 ± 1.1 nm for wildtype, 8.1 ± 1.2 and 9.0 ± 0.8 nm for A30P and E46K, respectively (sec. 4.3.3). These mutants form fibrils displaying a degree of polymorphism: the fibril diameters are narrowly distributed, but the periodicities vary (between roughly 60 and 140 nm for A30P), and fibrils with no discernible periodicity also occur.

The effect of single point mutations on many disease-related proteins is to accelerate aggregation (Chiti *et al.*, 2003), suggesting the mutations are pathogenic precisely because of the increased aggregation rate. The morphology of the A30P and E46K mutant α -synuclein fibrils characterized in chapter 4 is not different in such a way that it would imply an alternative pathological mechanism to that of sporadic Parkinson’s disease, and as such supports the idea that the point mutations are pathogenic due to increased aggregation relative to wildtype (Conway *et al.*, 2000; Choi *et al.*, 2004). Inasmuch as the pure morphology does not appear to yield clues to the specific mechanisms of the disease-related mutants, the solution may be in the differences between wildtype and the mutant proteins in their interactions with partners such as enzymes involved in protein folding (Gerard *et al.*, 2008, FK506) or crosslinking (Segers-Nolten *et al.*, 2008, tissue transglutaminase).

7.1.3 Free energies of interactions in α -synuclein fibrils

The process of self-assembly of α -synuclein fibrils can be described with the statistical-mechanical model based on self-assembly theory and a description of conformational transitions described in van Gestel and de Leeuw (2006). The

experimental distribution of fibril length as a function of initial monomeric protein concentration corresponds well to the predictions of the model (sec. 5.3.2), and yields free energy parameters for the interactions between monomers in a filament and between filaments present in a fibril.

The interaction between two monomers in a filament (around 16 kJ/mol) is somewhat stronger than that between filaments (around 10 kJ/mol, sec. 5.3.3). Both are only as strong as a few hydrogen bonds in protein ($\sim 4 - 8$ kJ/mol), and many times weaker than a single covalent bond. These findings resonate with the idea that hydrogen bonding is the main factor in the stability of protein fibrillar aggregates (Dobson, 2003).

7.1.4 α -Synuclein aggregates *in vitro* and in nerve tissue

The surface morphology of Lewy pathology in nerve tissue sections from a Parkinson's disease patient can be characterized with TM AFM height imaging in air (sec. 6.3.1). The Lewy inclusions have scale-like features on the order of 20 – 50 nm on the 200 nm length scale. These height differences do not resemble fibrillar structures observed in TEM images (Forno, 1996), whether the microtome sectioning was parallel or perpendicular to the fibril long axis. We hypothesize that this difference in morphology is caused by a coat of amorphous material surrounding the fibrils. This phenomenon has been observed for α -synuclein fibrils in oligodendroglial (GCI) inclusions associated with multiple system atrophy (Gai *et al.*, 2003). The fibrils in GCI inclusions were only visible in TEM images after the removal of a substantial amount of amorphous material by extensive washing (Gai *et al.*, 2003). This may explain why no fibrillar aggregates are visible on the surface of sectioned Lewy inclusions. Gai *et al.* state that the amorphous coating is a difference between GCIs and Lewy pathology, since TEM images show that LBs consist of a denser core with fibrils radiating outward (Forno, 1996), suggesting different assembly mechanisms may be at work. Our AFM data suggests however that GCI and Lewy pathology may be quite similar after all, if we interpret the scale-like morphology to represent amorphous material. The secondary structure content of Lewy pathology does indicate the inclusions consist of β -sheet folded protein, so presumably there are fibrils present in the Lewy pathology.

The protein secondary structure content of Lewy inclusions is similar to that of mature α -synuclein fibrils (sec. 6.3.3) in that the Lewy inclusions, like mature α -synuclein fibrils, have β -sheet folded protein as their main component. Analysis of the amide-I band of the Raman spectra shows that in contrast to fibrils and Lewy pathology, monomeric α -synuclein has a substantial content of α -helix and other secondary structures (sec. 6.3.4), likely being an ensemble of dynamically changing conformations (Maiti *et al.*, 2004).

Figure 7.1. Single-stranded filaments stay short, multi-stranded filaments grow long

Redrawn after Howard (2001). In a single-stranded filament (a), breakage is equally likely everywhere, but in multi-stranded fibrils (b), dissociation only occurs at the ends.

7.2 Structural models of α -synuclein assembly

The hierarchical assembly model (HAM) of amyloid fibril formation (Khurana *et al.*, 2003) holds that fibrils are formed by successive intertwining of filaments. Our data indicates that this is only one of several possible aggregation mechanisms. Lateral association of segments, as described for insulin by Jansen *et al.* (2005), also explains certain features of α -synuclein fibrils, such as the segmented disassembly we observed (sec. 3.3.3).

There are also models that state that elongation of amyloid fibrils proceeds by linear aggregation of preformed nucleation units, similar to the aggregation of colloidal particles, and resulting in a beads-on-a-string appearance of fibrils (Xu *et al.*, 2001).

From a polymer physics perspective, the hierarchical assembly model, where single-stranded filaments are formed first and only then intertwine to form mature fibrils, sounds quite improbable: single-stranded filaments will always tend to stay short (no more than several ‘subunits’ long) because the energy required to break them at an arbitrary point in the chain is low and the same throughout the filament (Howard, 2001, chapter 9). In a multi-stranded filament, in contrast, the subunits are bound by several interactions, and dissociation of the end units is more likely to occur than fibril breakage (fig. 7.1).

From a structural point of view, however, the HAM does describe many morphological features of amyloid fibrils, such as their twisted morphology and the presence of thinner untwisted filaments. We thus consider the HAM to be a useful model for describing the end-state morphology of a subset of the fibrils. Competing mechanisms occurring simultaneously are lateral assembly of segments and amorphous aggregation, as outlined in fig. 1.3 on page 7.

7.3 Future directions for research in α -synuclein aggregation

7.3.1 Structure of Lewy pathology

Unraveling how Lewy bodies and neurites are formed may hold a clue to nerve cell death and its prevention (Forno, 1996). The Raman spectroscopic work presented in chapter 6 proves that it is possible to extract information on protein secondary structure content from Lewy inclusions *in situ*, namely embedded in the tissue they were formed in, but many questions remain. For example, are there protein secondary structural variations within individual Lewy inclusions? Are there significant structural and protein content variations between Lewy bodies and neurites? These questions can be addressed by making Raman images of a larger number of Lewy inclusions in different patient samples, ideally also from patients with familial Parkinson's disease to study the influence of the point mutations.

Atomic force microscopy may address the question of the coating of the α -synuclein fibrils with amorphous material. AFM tips can be used to indent the sample and to remove material by scratching the surface at a known force. If the presumed amorphous aggregates coating the fibrils can be removed mechanically in this manner, the underlying fibril structure should become visible.

Although the method of alternatively staining and not staining consecutive sections, as employed in this work, allows for localization of Lewy pathology on unstained tissue sections *in situ*, it is perhaps more efficient to attempt to separate the Lewy pathology from the tissue by laser microdissection (Leverenz *et al.*, 2007). The disadvantage of such a procedure is the loss of cellular context, but the procedure would allow for a more rapid characterization of larger number of Lewy inclusions than with our method.

7.3.2 Homologous and heterologous seeding

Since the brains of the elderly often are affected by more than one neurodegenerative disorder (Braak *et al.*, 2003), the interplay between the molecules involved in those diseases (for example between α -synuclein and A β in Alzheimer's disease) may yield new insights in the disease mechanisms. One important question is if and how fibrillation and aggregation of one protein species influence the aggregation of the other (Westermarck, 2005).

Several aspects of homologous and heterologous seeding of α -synuclein aggregations have already been investigated. The primary structure of the protein used as the seed is important in aggregation propensity: the α -synuclein homologs β -synuclein and γ -synuclein do not normally aggregate, and cannot be seeded by α -synuclein (Biere *et al.*, 2000). β - and γ -synuclein even inhibit the aggregation of α -synuclein (Uversky *et al.*, 2002). Heterologous seeding

efficiency depends strongly on sequence similarity between the seed and the aggregating protein, but this influence is less strong when the sequence differences are in regions of the polypeptide chain which do not end up in the fibril core (Krebs *et al.* (2000, 2004), on hen lysozyme). In fact, from seeding experiments with mutants of Alzheimer-related protein A β , it appears that the seeding efficiency is influenced more by the shape of the fibril (particularly the hydrogen bonding network) than by the primary sequence (O’Nuallain *et al.*, 2004). This finding resonates with the result that when human α -synuclein is seeded by fibrils of such diverse proteins as GroES (from *E. coli*), hen lysozyme, or bovine insulin, the resulting fibrils adopt the diameter of the seed fibril (Yagi *et al.*, 2005). It has even been found that seeds made of non-pathogenic materials such as silk fibrils can enhance amyloid formation in mice (Lundmark *et al.*, 2005).

In vitro, the solution conditions are also important for seeding efficiency: homologous seeding of α -synuclein almost completely abolishes the lag phase when the seeds are formed under the same solution conditions, but the reduction of lag phase is much less pronounced when the seed aggregates are formed at different conditions than the seeded incubation, such as a lower pH (Hoyer *et al.*, 2002).

One possible extension of this work that would address the interplay between α -synuclein and A β is to perform *in vitro* seeding of solutions of α -synuclein with pre-formed A β aggregates, and vice versa, and quantitatively characterize the morphology of the resulting aggregates using the procedures described in this thesis. Such data would indicate whether the fibrils take on the secondary structure of the seeds, as is the case for strains of the prion protein (Jones and Surewicz, 2005). Seeds can be produced readily from mature fibrils by sonication: 30 s sonication of α -synuclein fibrils yield fibril fragments retaining morphological properties such as diameter and periodicity (fig. 7.2).

7.3.3 Interpretation of AFM phase contrast

The phase contrast that is sometimes apparent in tapping mode AFM has been used as a contrast mechanism, even in the study of tissue sections (Matsko, 2007), where it proved to yield more contrast than height images. However, the imaging conditions that will produce reliable and reproducible phase contrast remain elusive.

Another issue is that it has not been possible to couple the time lag between the tapping driving signal and the measured cantilever deflection to a material property of the sample under study (Burnham *et al.*, 1997). The difficulty is compounded by the transfer of energy from the fundamental resonance mode into higher order resonance modes of the cantilever at every single contact between tip and sample. The resulting cantilever deflection is a linear combination of all these modes, which makes attributing the phase lag to a sample

Figure 7.2. Sonicated mature fibrils retain morphological properties

(a) TM-AFM height image of mature E46K α -synuclein fibrils in air. Scale bar 5 μ m.

(b) AFM height image of the above fibrils after sonication at the same magnification. Scale bar 5 μ m.

(c) Zoom-in on (b). Scale bar 250 nm. *Image size, resolution, scan speed: (a) and (b) 20 μ m, 512 pix/line, 40 μ m/s; (c) 1 μ m, 256 pix/line, 2 μ m/s.*

material property very difficult.

It is clear from figs. 2.6 and 2.7 on pages 33 and 35, however, that phase imaging does yield information not otherwise accessible by AFM. The unequivocal attribution of the degree of phase lag to a material property of the sample would constitute a major advance in the application of atomic force microscopy to the study of amyloid fibrils.

7.3.4 Aggregation intermediates and interactions

This work has focused on the morphological and structural properties of the end products of α -synuclein aggregation: mature α -synuclein fibrils *in vitro*, and Lewy lesions *ex vivo*. These investigations lead to understanding of the mechanisms of α -synuclein self-assembly, but have not given insight in the mechanisms by which damage is inflicted on neurons in the living tissue of a Parkinson's disease patient.

Intermediate species along the aggregation pathway, such as early-stage oligomers, have been implicated as pathogenic agents. The study of their interactions with various components of the cell (such as cell membranes, other proteins, enzymes involved in protein folding (Gerard *et al.*, 2006), the ubiquitin-proteasome system (Ciechanover and Brundin, 2003), etc.) may lead to identification of the mechanisms of cell damage.

The methods developed for mature fibrils in this work may expedite the characterization of α -synuclein oligomers and their interactions. Sufficiently

Figure 7.3. Cells expressing α -synuclein may provide a more life-like environment for α -synuclein aggregation

(a) White light image of formaldehyde fixed and dried SHSY5Y neuroblastoma cells on a glass slide during AFM imaging (*triangular shadow* is from AFM cantilever). Scale bar 50 μm .

(b) Tapping mode AFM height image and height profile of a neuroblastoma cell in air (logarithmic height color scale). Horizontal scale bar 20 μm , vertical scale bar 500 nm. *Dashed line* indicates location of line profile. *Dotted rectangle* indicates site of image (c).

(c) Zoom-in on one dendrite of the same cell. Horizontal scale bar 5 μm , vertical scale bar 100 nm. *Dashed line* indicates location of line profile. When such cells are made to express α -synuclein, Lewy body formation may be characterized by AFM within the cell. *Image size, resolution, scan speed:* (b) 80 μm , 512 *pix/line*, 32 $\mu\text{m/s}$; (c) 20 μm , 512 *pix/line*, 40 $\mu\text{m/s}$. In collaboration with Tom Groothuis.

high spatial resolution Raman imaging may identify which α -synuclein aggregate species end up in Lewy bodies. Once the physical origin of AFM phase contrast on protein aggregates has been elucidated, the characterization of oligomers with phase contrast AFM may shed light on the essential differences between oligomers and fibrils that make one species toxic to neurons and renders the other harmless.

7.3.5 α -Synuclein aggregation in living cells

In this work, as in most *in vitro* work on amyloid formation, the protein of interest is expressed and purified and induced to aggregate in a buffer solution containing a minimum of other compounds. The reality of the living cell is far from such a setting: a cell contains high concentrations of soluble macromolecules, such as proteins and DNA, and also structures like membranes and organelles. Cells feature concentration differences, pH gradients, and assembly and disassembly processes: a living cell is a dynamic environment, with,

as Zhang (2003) puts it, a density thicker than a bowl of oatmeal. Molecular crowding not only affects protein diffusion (Banks and Fradin, 2005) and thus the availability of protein molecules for aggregation, but also the rate of α -synuclein aggregation *in vitro* (Munishkina *et al.*, 2004; Fink, 2006).

If one is interested in the kinetic and morphologic properties of α -synuclein aggregation as it occurs *in vivo*, expressing (mutant) α -synuclein and inducing it to aggregate inside the cell necessary and possible (Pandey *et al.*, 2006; Roberti *et al.*, 2007). The methods developed in this thesis would be translatable to the cellular system: AFM imaging can probe surface and sub-surface morphology and mechanical properties (fig. 7.3) while Raman spectroscopy will give insight into the protein distribution and secondary structure content inside the fixed or living cell. Once aggregates have been formed inside the cell, freeze-fracturing may make the inner surface accessible to both AFM and Raman imaging, as well as to other characterization techniques such as electron microscopy. Additionally, the aggregation of α -synuclein may be studied in living cells using Raman spectroscopy (van Manen *et al.*, 2005) or coherent anti-Stokes Raman scattering (CARS) microscopy, effectively making the cells living test tubes (Xie *et al.*, 2006).

7.4 References

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Appendix A.

AFM image plane correction

The AFM images presented in this thesis have been corrected for three types of image artifacts due to the process of AFM imaging: *sample tilt* due to the sample not being in the same plane as the motion of the scanner; *scanner bow* due to the nature of piezo scanners; and *line jumps* due to the reversal of the scan direction at the end of each line.

This appendix outlines a plane correction procedure that removes these artifacts yet retains the height information of objects present in the AFM image. Images were processed using Scanning Probe Image Processor (SPIP) (Image Metrology, Hørsholm, Denmark).

Figure 1. Raw data. The *top left* panel displays the raw height image. The *white lines* indicate the locations of the *x* and *y* profiles shown in the *right-hand* panels. The profiles indicate a substantial tilt is present in both *x* and *y* directions. The *bottom left* panel shows the height histogram.

Figure 2. Manual tilt corrected. Manual tilt is corrected by tilting the image until *x* and *y* profiles are horizontal. A typical 2^{nd} order polynomial scanner bow remains in *x* (*top right* panel), and a sine-like distortion in *y* (*bottom right* panel).

Figure 3. Scanner bow corrected. The z range which is taken into account for scanner bow correction is limited by setting limits on the color scale bar (*red circle*). This excludes the objects in the image from the procedure and minimizes distortion to measured object heights. After scanner bow correction both x and y profiles are almost flat.

Figure 4. Line jumps corrected. Any line jumps that may be present are corrected by a line-by-line subtraction of the line-average height, again excluding any objects in the image from the calculation. The resulting height image has a typical noise level of a few Ångströms. Note that the height histogram now shows a sharp peak at the intensity corresponding to the substrate height (*bottom left panel*).

Figure 5. Resulting image. The resulting image is nearly free of scanner artifacts and still contains accurate height information.

Summary

The misfolding and aggregation of the protein α -synuclein is intimately linked to the pathology of Parkinson's disease (PD). To successfully develop drugs and therapies that act on the cause of the disease instead of on the symptoms, the mechanisms of α -synuclein misfolding, aggregation, and damage to neurons must be understood at the molecular level.

This thesis describes our use of advanced biophysical nanometrology instrumentation in an effort to understand the nanoscale morphology and secondary structure of aggregates of α -synuclein both *in vitro* and in the brain tissue of Parkinson's patients.

Chapter 1 introduces the biophysical and pathological context of the research described in this thesis. Some of the most puzzling features of *in vitro* α -synuclein fibril formation are the degree of polymorphism displayed by the aggregates, and the parameters that control under which conditions aggregation does or does not occur, such as the critical monomeric protein concentration.

Atomic force microscopy or AFM uses a sharp tip to scan along a surface to map the nanometer scale topography of a sample. **Chapter 2** explores the possibilities of AFM in the context of amyloid fibril formation, discusses various modes of operation, and reviews the literature. As an imaging tool, tapping mode AFM in liquid gives the most accurate representation of the fibril morphology. When the goal is investigating the mechanical properties of protein aggregates, AFM force spectroscopy data give insight into the binding strengths and assembly kinetics of amyloid fibrils.

Any measurement influences the system being studied, and this is especially true at the nanoscale. **Chapter 3** quantifies and discusses the influence of the imaging environment in which AFM images are made on the apparent height of protein aggregates. Imaging in air as opposed to in aqueous buffer results in a $\sim 25\%$ lower apparent height, while the use of sharper tips results in only slightly better images and much more handling difficulty.

Chapter 4 proves that single-point mutations in the α -synuclein sequence do not alter the fibril morphology to such an extent that it would rationalize the hypothesis that point mutations in the α -synuclein gene lead to different assembly mechanisms. The diameter of the fibrils is in the 10 nm range, with the

disease-related mutants forming slightly thinner fibrils. There is considerable variability in the periodicity of the fibrils formed by disease-related α -synuclein mutants: fibrils formed by E46K α -synuclein have a narrow distribution of periodicity, close to that of wildtype α -synuclein, whereas the A30P α -synuclein mutant produces fibrils with a wider range of periodicities.

The initial monomeric protein concentration is a critical parameter in *in vitro* α -synuclein fibril formation. **Chapter 5** presents predictions of fibril length as a function of initial concentration made by a statistical-mechanical model of protein aggregation. We compare these predictions to an experimental analysis of α -synuclein fibril length distributions measured by AFM. This experimental verification of the model yields quantitative values for the free energies of the interactions that occur in a fibril formed by α -synuclein. We found that the energy binding monomers in an α -synuclein filament is ~ 16 kJ/mol, and the bond between filaments is ~ 10 kJ/mol, both of which are in the range of the strength of only a few hydrogen bonds. We also determined that the critical concentration of α -synuclein aggregation is 15 ± 5 μ M, which is considerably lower than the concentration of α -synuclein present in healthy neurons.

Because the *in vitro* appearance of a biological structure is not per definition the same as that found in nature, it is imperative to compare the *in vitro* morphological and structural properties of α -synuclein to those found in brain tissue of PD patients. **Chapter 6** reports that the surface morphology of Lewy pathology and surrounding tissue in nerve tissue sections from PD patients does not resemble the fibrillar structures described in chapters 2–5. Instead, fibril structures appear to be coated with amorphous material. Raman spectroscopic data prove however that the protein secondary structure content of the Lewy bodies is mainly characterized by β -sheet structure, similar to *in vitro* α -synuclein fibrils, hinting at the presence of aggregated α -synuclein.

Chapter 7 concludes this thesis with a discussion of the results summarized above, suggesting that essential gaps in our understanding of the pathology of the amyloid diseases may be filled by further exploration of the nanoscale properties of aggregating proteins both *in vitro* and *ex vivo*. Specifically, the methods developed and applied in this work allow for a more precise characterization of the nanoscale morphology and protein structure content of pathological tissues, the interactions of disease-related proteins among themselves and with other cellular components, and the properties of aggregating proteins inside living cells. Such investigations will shed new light on the processes causing the neurodegeneration taking place in the neurons of PD patients, and will perhaps point to successful strategies for the treatment of the disease.

Samenvatting

In de hersenen van mensen met de ziekte van Parkinson treffen pathologen opeenhopingen van het eiwit α -synucleïne aan. Dit eiwit heeft in een gezonde hersencel geen vaste secundaire en tertiaire structuur, zoals de meeste andere eiwitten die wel hebben, en kan onder bepaalde omstandigheden op een specifieke manier misvouwen en samenklonteren ('aggregeren') in lange draden. Het grootste obstakel dat de ontwikkeling van medicijnen tegen de ziekte van Parkinson in de weg staat, is onze onvolledige kennis van de moleculaire processen die ten grondslag liggen aan het misvouwen en het aggregeren van α -synucleïne en vergelijkbare eiwitten, en de manieren waarop hersencellen daarvoor beschadigd raken en afsterven. Dit proefschrift beschrijft de resultaten van ons onderzoek naar de nanoschaal morfologie en de secundaire structuur van α -synucleïne fibrillen, zowel *in vitro* als in zenuwweefsel van Parkinson-patienten.

Hoofdstuk 1 introduceert de biologische en biofysische context van het onderzoek. Enkele van de belangrijkste open vragen op het gebied van α -synucleïne aggregatie zijn: wat is de reden voor de grote verscheidenheid aan vormen/structuren die de α -synucleïne aggregaten kunnen aannemen, en: welke (biochemische) omstandigheden bepalen of de schadelijke processen optreden.

We gebruiken *atomic force microscopy* (AFM) om de oppervlaktemorfologie van de eiwitdraden te karakteriseren op de schaal van nanometers. AFM maakt gebruik van een scherpe naald (~ 10 nm) die over een oppervlak bewogen wordt, en zo de topografie van het *sample* in kaart brengt. In **hoofdstuk 2** onderzoeken we de mogelijkheden van AFM bij het bestuderen van eiwit-aggregaten. Het blijkt dat de meest waarheidsgetrouwe weergave van de fibrilmorfologie wordt behaald met *tapping mode* AFM in vloeistof. Uit literatuuronderzoek blijkt verder dat AFM-krachtspectroscopie belangrijke informatie geeft over de bindingssterkte en aggregatiekinetiek van eiwitdraden.

Bij het doen van metingen op nanometerschaal aan biologische *samples* beïnvloedt het meetstelsel altijd het te bemeten *sample*. In **hoofdstuk 3** kwantificeren we de invloed van het meetmilieu op de gemeten *sample*-eigenschappen: meten in lucht geeft een $\sim 25\%$ lagere fibrildiameter dan in vloeistof, terwijl het gebruik van scherpere naalden weliswaar een hogere resolutie geeft,

maar geen extra informatie over de fibrilstructuur oplevert.

In **hoofdstuk 4** onderzoeken we de invloed van mutaties in het α -synucleïne gen, zoals die voorkomen in bepaalde families met een genetische variant van de ziekte van Parkinson, op de morfologie van de aggregaten. Het blijkt dat door deze mutaties de structuur van de eiwitdraden weliswaar iets verandert (kleinere diameter, andere periodiciteit), maar niet zo sterk dat het de hypothese rechtvaardigt dat genetische afwijkingen leiden tot andere aggregatie-mechanismen.

Om de invloed van de beginconcentratie van het eiwit op de lengte van de gevormde fibrillen te karakteriseren hebben we een statistisch-mechanisch model van eiwitfibrillatie aangepast voor het specifieke geval van α -synucleïne (**hoofdstuk 5**). We vergelijken de voorspellingen van het model met experimentele lengtedistributies gemeten met AFM. Dit maakt het mogelijk om de vrije energie te bepalen die geassocieerd wordt met de verschillende interacties tussen de monomeren in een fibril: de bindingsenergie tussen twee monomeren in een filament is ~ 16 kJ/mol, en de bindingsenergie tussen twee filamenten is ~ 10 kJ/mol. Beide waarden zijn in de ordegrrootte van enkele waterstofbruggen. De kritische concentratie van α -synucleïne fibrillatie is met 15 ± 5 μ M beduidend lager dan de concentratie die in gezonde hersencellen aanwezig is.

De morfologie en structuur van eiwitaggregaten *in vitro* is niet per definitie hetzelfde als de structuur die in hersencellen voorkomt. Daarom is het van belang de resultaten die behaald zijn op laboratoriumschaal, te vergelijken met het hersenweefsel van Parkinson-patienten. Uit de resultaten gepresenteerd in **hoofdstuk 6** blijkt dat de oppervlaktemorfologie van Lewy pathologie niet overeenkomt met het beeld dat de eiwitdraden *in vitro* laten zien. Wij vermoeden dat dat komt doordat de draden *in vivo* bedekt worden met een laag amorf ge-aggregeerd eiwit. Raman spectroscopische data tonen echter aan dat de secundaire structuur van de eiwitten in het weefsel wel degelijk β -sheet is, en dus overeenkomt met de structuur van *in vitro* gevormde eiwitdraden.

Hoofdstuk 7 sluit dit proefschrift af met een discussie van de hierboven samengevatte resultaten. De gaten in ons begrip van de moleculaire processen die ten grondslag liggen aan neurodegeneratieve ziekten zoals de ziekte van Parkinson kunnen gedeeltelijk worden gedicht door verder onderzoek aan de nanometerschaal morfologie en structuur van eiwitaggregaten. De methoden die we in dit onderzoek ontwikkeld en beschreven hebben zijn ook toepasbaar om de morfologie en structuur van de componenten van (hersens-)weefsel nader te karakteriseren. Tenslotte kunnen ook de interacties tussen verschillende eiwitten en andere moleculen in de cel, zowel in een reageerbuis als in levende gekweekte cellen, worden bemeaten om de misvouwing en aggregatie van α -synucleïne te karakteriseren. Zulk onderzoek kan nieuw licht werpen op de moleculair-biofysische processen die neurodegeneratie veroorzaken, en zal wellicht de effectieve behandeling van aandoeningen als de ziekte van Parkinson een stap dichterbij brengen.

Dankwoord

Ik ben er! Er bestaat niet zoiets als een onderzoek dat af is, maar hier ligt een proefschrift dat tenminste mijn deel van ons werk afmaakt. Ik schrijf *ons* werk, want hoewel de wetenschappelijke verantwoordelijkheid voor de tekst van dit proefschrift geheel de mijne is, is het feitelijke werk dat het beschrijft mede gedaan door vele BPE'ers, studenten en collega's van andere vakgroepen. Ik kan jullie niet genoeg bedanken:

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A handwritten signature in black ink, reading 'Mark van Raay'. The signature is written in a cursive, flowing style. Below the name, there is a long, horizontal, slightly wavy line that extends across the width of the signature.

Curriculum Vitae and publications list

About the author

Martijn van Raaij was born on July 7th, 1979 in Ede (The Netherlands). After graduating from high school in Wageningen in 1997 he went to study Applied Physics at the University of Twente (UT) in Enschede, The Netherlands. During the master's phase of that program he did an internship at Immunicon, Philadelphia, PA, USA with Leon Terstappen and a research project at the Biophysical Engineering Group at the UT under supervision of Hans Kanger, Carl Figdor and Jan Greve. He obtained his MSc. in Applied Physics in 2003 and gained an additional master's degree in Science Education from the same university in the following year. After working as a physics teacher at a local high school he returned to the Biophysical Engineering Group to do a Ph. D. project on the biophysical characterization of protein aggregates. That project, supervised by Ine Segers-Nolten and Vinod Subramaniam, resulted in this thesis. Martijn van Raaij will start a postdoctoral fellowship at the Imaging department of Sunnybrook Health Sciences Centre in Toronto, Canada, in February 2009.

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