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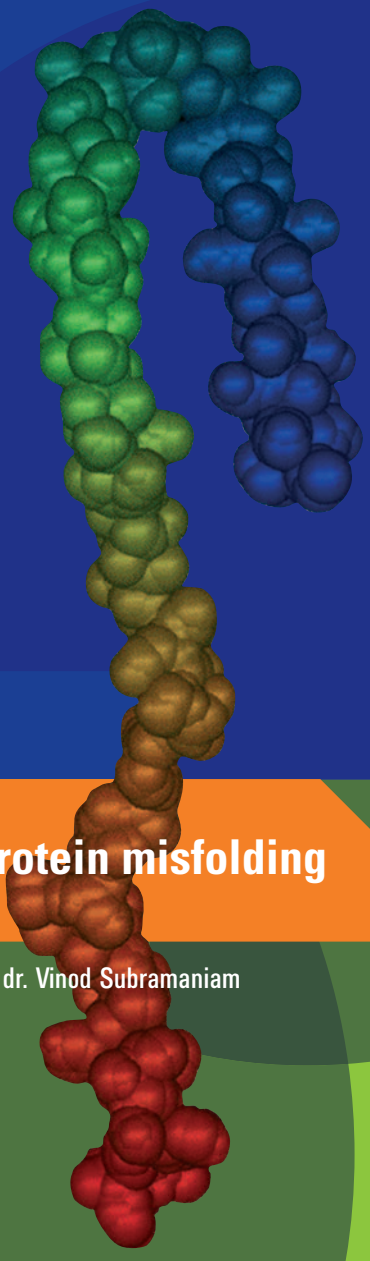

Biophysics of protein misfolding

by Prof. dr. Vinod Subramaniam

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Lecture presented at the occasion of the appointment as professor for

Biophysical Engineering

at the Faculty of Science and Technology
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by

Prof. dr. Vinod Subramaniam

Cover illustration courtesy of Dr. Tom Groothuis

Prelude

Mijnheer de Rector Magnificus, Ladies and Gentlemen,

As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.

Ithaka, C. P. Cavafy, trans. Edmund Keeley & Philip Sherrard

I begin by quoting the first luminous line of a poem by the great modern Greek poet Constantine Cavafy, about a voyage of discovery.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbors you're seeing for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind-
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

I embarked on my personal voyage of discovery some 22 years ago when I boarded a PanAm flight from New Delhi to New York. This voyage has had me entering many harbors – Ithaca, New York; Ann Arbor, Michigan; Göttingen, Germany; Loughborough, England; and now Enschede.

Laistrygonians, Cyclops,
angry Poseidon-don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.

Today I want to share with you my scientific *Ithaka*, the goal that has to a lesser or greater extent occupied my intellectual attention over the last few years, and imparted the rare excitement that stirs my spirit and body: the challenge of visualizing and understanding aspects of protein conformational dynamics, folding and misfolding. I invite you to join me in this journey, and to share my sense of wonder at the riches that I have found along the way and hope to keep discovering as the journey continues.

Introduction

Almost fifty years ago Francis Crick enunciated the principles underlying the central dogma of molecular biology (1, 2), a concept dealing with the sequential transfer of information from DNA to RNA to protein. The processes of DNA replication, DNA transcription, and translation of RNA all involve proteins, and the very action of weaving the web of life is intimately influenced by the motions and conformational dynamics of proteins.

The primary sequence of a protein is a linear chain of amino acids, akin to a string of pearls. The complex and fascinating ribosomal machinery of the cell translates the genetic information encoded by the DNA to add the correct amino acid in the sequence encoding the gene, thus adding just the right pearl in the string during the process of fabricating the necklace. Each successive amino acid elongates the growing nascent polypeptide chain, which begins folding into the correct conformation.

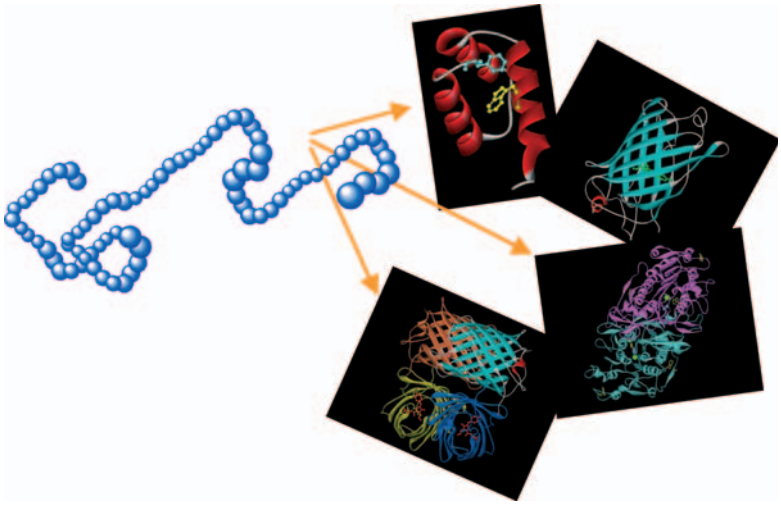

Figure 1: Schematic representation of primary amino acid sequence and three-dimensional folded protein structures.

Normal cellular function of proteins depends critically on their productive folding into active functional forms. This folding into the 'native' state is determined by the primary amino acid sequence and the specific environment of the protein. Nature has evolved a complex set of mechanisms, including helper molecules called molecular chaperones, to aid in the correct folding of proteins. Correctly folded proteins can be induced to unfold by changing physical parameters such as pH, temperature, or solution conditions, yielding a state in which the protein loses its function.

Most proteins are poised on the brink of conformational instability and exhibit intrinsic structural dynamics reflecting the delicate balance between the stabilizing and destabilizing forces to which they are subject. In addition, proteins also exhibit conformational dynamics associated with folding and unfolding transitions and with interactions with ligands and nucleic acids. In general, conformational change can provide the molecular mechanism for the biological function of a protein, or for switching a protein between different functional forms, including those that lead to disease states, such as in the neurodegenerative diseases characterized by amyloid. Protein flexibility is critical to understanding the ways in which drugs interact with their specific therapeutic targets, and have a deep implication for pharmaceutical design and the comprehension of drug affinity and specific modes of action. Thus, understanding the molecular origin and detailed nature of protein conformational dynamics and stability is a goal of fundamental biophysical and biochemical importance.

A Twist in the Tale

Our understanding of the basis of protein function has long relied on the structure-function paradigm, which closely couples the function of a protein with the folded structure coded by the amino acid sequence. In recent years a class of proteins called intrinsically disordered proteins, exhibiting little or no secondary and tertiary structure[1] in physiological conditions has been rapidly growing.[2] These proteins thus lack fixed three-dimensional structure in their putatively native states and challenge the correlation of structure with

1 *secondary structure is the general three-dimensional form of local segments of biopolymers such as proteins, and include for example, alpha-helical or beta-sheet structures; tertiary structure of a protein is its three-dimensional structure, as defined by the atomic coordinates.*

2 *The DisPROT database (www.disprot.org) contains 469 proteins as of the last release in December 2006.*

function. What is the role of a disordered protein, and how do we understand the role that disorder plays in the *function* of such proteins? A growing consensus suggests that these 'natively unfolded' or 'intrinsically disordered' proteins could exploit their unique properties in the molecular recognition of multiple targets, the formation of large interaction surfaces, and potentially increased association and dissociation rates, to effectively increase the functional diversity of the proteome (3). How do these proteins fit in with our ideas about structure, folding, function, and the consequences of incorrect folding?

What happens when folding goes wrong?

When the folding process goes wrong, the cell's protein degradation machinery goes into action and identifies and removes improperly folded proteins. One consequence of a failure of the cellular 'quality control' system is the accumulation of misfolded proteins in the cell. This failure could be due either to genetic mutations in relevant proteins or to age-related phenomena. A rapidly growing number of diseases is associated with protein misfolding. Examples of mutation induced misfolding diseases include cystic fibrosis, where a single mutation causes insufficient production of an important ion channel protein, and in certain types of cancer, where mutations in the p53 protein are implicated. The neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are correlated with aging, and may be associated with impairment of the protein degradation machinery with age.

Protein misfolding, aggregation, and neurodegenerative disease

Many neurodegenerative diseases are characterized by the aggregation and accumulation of proteins as fibrillar deposits in the brain tissues of affected patients. It is estimated that 18 million people worldwide have Alzheimer's disease (4) and that about 3 million people suffer from Parkinson's disease (5). These numbers are likely to double in twenty years. Fibrillar protein aggregates are also found in a host of other neurodegenerative diseases as well as in the prion diseases (6). These ordered deposits are generally referred to as amyloid or amyloid-like, and have distinct ultrastructural and dye staining properties. Many of the proteins involved in fibrillar aggregation in neurodegenerative disease are intrinsically disordered, including the human α-synuclein protein, implicated in Parkinson's disease. A simplified schematic of aggregation pathways including images of early intermediates for

α-synuclein is shown in the figure below.
The origin and detailed mechanisms of these diseases are not understood, but a universal factor is the failure of critical proteins to fold into their correct

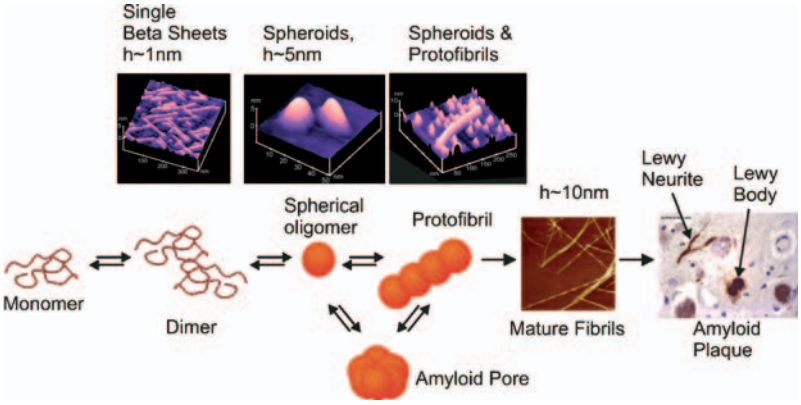

Figure 2: Schematic representation of protein aggregation process showing different intermediate species that form during fibril formation in vitro (scheme after Lashuel (7); upper AFM images from (8)). Amyloid plaque image courtesy of Rob de Vos, Laboratorium Pathologie Oost, Enschede. The dark brown patches are characteristic of α-synuclein in Lewy bodies and Lewy neurites in the brain of a Parkinson's disease patient.

structures and the resultant aggregation of these misfolded proteins. The physics governing protein misfolding and aggregate formation has not been elucidated and is a key problem in contemporary biophysics.

Protein misfolding and aggregation is however not limited to those proteins involved in neurodegenerative or other diseases. Recent work has shown that proteins, and even small peptides, that are completely unrelated to disease also exhibit a propensity to aggregate under appropriate conditions (9-11). Indeed Dobson (9, 12) has suggested that the potential to self-assemble into fibrillar aggregates may be a generic feature of many (if not all) proteins and polypeptide chains. Protein aggregation is also a key issue in the food, biotechnology, and pharmaceutical industries (13, 14). Aggregation of proteins is an important method of providing texture in food products, while the

phenomenon represents an undesirable effect with significant economic consequences in the large-scale production of proteins and peptides for biotechnological or therapeutic purposes. Interestingly, recent results have shown that amyloid-like proteins occur naturally in bacteria and are important for adhesion to surfaces, cell aggregation, and biofilm formation (15, 16). The evidence for the enhanced ability of intrinsically disordered proteins to form fibrillar aggregates is also growing, and we have demonstrated that the intrinsically disordered human protein prothymosin-α is also capable of forming amyloid fibrils (Figure 3) (17).

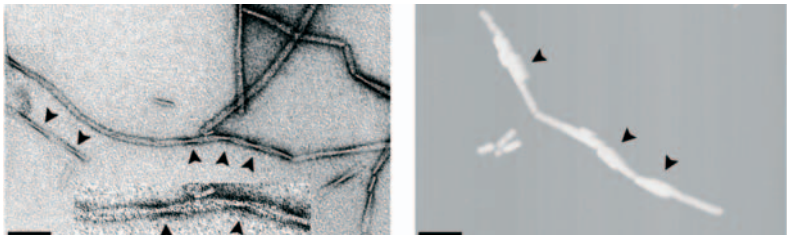

Figure 3: *The structure of fibrils assembled from prothymosin-α.*
Left panel: Transmission electron micrograph of negatively stained sample. Arrowheads indicate narrow regions of fibrils. inset, magnified view of fibril segment with modulated width.
Right panel: Atomic force microscopy image (height). Arrowheads indicate narrow regions of fibrils with increased height.
Scale bars represent 100 nm.

Amyloid fibrils: what do we know, and where are the gaps?

Fibrillar aggregates derived from proteins and peptides of vastly different origins and amino-acid sequences show a remarkably similar morphology and ultrastructure. The insolubility and inability of fibrils to crystallize limits the use of x-ray crystallography and solution nuclear magnetic resonance (NMR) spectroscopy for structure determination. Cryoelectron microscopy and X-ray diffraction have yielded insights into the structure of mature fibrils (18, 19), but these provide lower-resolution structural constraints. Solid-state NMR approaches are beginning to make major contributions to understanding the molecular structure of mature amyloid fibrils (20-23). All amyloid fibers are

composed of beta-strands that are stacked perpendicular to the long axis of the fibers, regardless of the structural origins of the precursor. Electron and scanning probe microscopy of different fibrillar aggregates show nanoscale structures that are on the order of 10 nm in diameter and capable of forming fibrils several micrometers in length (see Figure 2).

The process of fibrillar aggregate formation is described as a nucleation-polymerization process, and is typically manifested in a sigmoidal growth curve (Figure 4) with clear lag, growth, and steady-state phases. The kinetics of the process can be influenced by seeding; indeed the potential to trigger fibril formation by seeding is a characteristic of amyloid protein fibrils. An intriguing open question is whether seeding by chemically modified protein

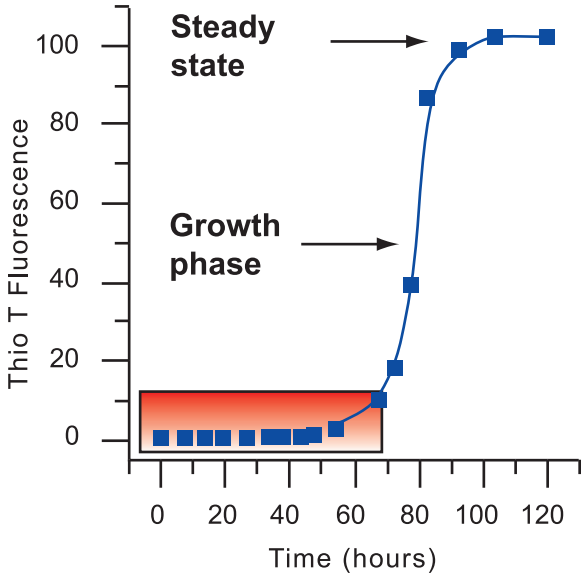

Figure 4: *Fibril growth is a nucleation-polymerization process, here measured by the fluorescence of Thioflavin T, which binds to beta sheet structures. Early aggregates occur during the lag phase (shaded red box). Data for wild-type α-synuclein aggregation.*

species or cross-seeding by nuclei of different proteins is an important trigger for aggregation and the onset of disease. The first phase of amyloid formation clearly involves the association of monomers of protein into oligomeric species. These oligomeric species form spheroids and early prefibrillar aggregates which in turn self-organize into protofibrillar species with distinct morphologies. Although the prefibrillar and protofibrillar species can be visualized with electron microscopy and atomic force microscopy, very little is yet known about the molecular details of these early aggregates.

There is a wide gap in our knowledge of the molecular structures and morphologies of the early aggregate species. This gap presents a unique opportunity for biophysical and high-resolution imaging methodologies to understand their structure, composition, and role in seeding and in the aggregation process. These early aggregates are the focus of my research.

The structural and biophysical details of early aggregate species are particularly important in light of the building consensus that the mature amyloid fibril species are very likely not responsible for cell damage and disease. Indeed it has been postulated that mature fibrillar species may be a cellular defense mechanism to prevent the highly toxic early intermediates from causing wanton cell damage. The very fact that many different proteins form remarkably stable fibrillar structures of similar morphology also suggests that alternative entities are the true culprits in cell death. Recent work has also demonstrated that granular and amorphous aggregates formed from non-disease-related amyloid-forming proteins have a much higher potential to impair cell viability in comparison to mature fibrils (24). The spotlight is turning inexorably towards early aggregation intermediates although the structures and mechanisms of action of these early intermediates are unknown (25-27). There is evidence that amyloid toxicity may be caused by membrane permeabilization by pore-like early intermediates, analogous to the bacterial pore-forming toxins, leading to disruption of calcium regulation and cell-death (7, 28-33).

Alpha-synuclein and Parkinson's Disease

The human α-synuclein protein has occupied my attention for the last several years, and serves as the model system for our studies on protein misfolding and aggregation.

α-synuclein plays a central role in the etiology of Parkinson's disease (34), and forms fibrillar aggregates that are found in Lewy bodies in the brain, structures which are the hallmark of the disease (35-37). Three point mutations (A30P, A53T, and E46K)[3] are associated with early-onset Parkinson's disease (38-40). α-synuclein is also associated with other diseases, including diffuse Lewy bodies disease, dementia with Lewy bodies, Lewy bodies variant of Alzheimer's disease, and multiple system atrophy. The diseases associated with α-synuclein are called the synucleinopathies. Overexpression of the wild-type or disease mutant protein in animal models (transgenic mice or the fruitfly Drosophila) leads to symptoms, including motor deficits and neuronal inclusions, characteristic of Parkinson's disease.

α-synuclein displays remarkable structural versatility: it is an intrinsically disordered or 'natively unfolded' protein at physiological conditions, but can readily adopt beta-sheet structure in aggregates or alpha-helical structure when bound to lipids (41-44). The function of α-synuclein is unknown, but it is thought to involve lipid-binding in vesicles and synaptic membranes (45-50). A ground-breaking report in 2006 has indicated that in a yeast model, α-synuclein blocks endoplasmic reticulum to Golgi vesicular trafficking, suggesting that disruption of basic cellular functions is the cause of the synucleinopathies (51).

Several reports have studied the effect of physicochemical factors upon the aggregation of α-synuclein (52-60). We have investigated the effect of solution pH and salts on the aggregation of α-synuclein. Our studies revealed that decreasing the pH decreased the lag phase of the aggregation, and increased the growth rate (61). These changes in the aggregation kinetics were reproduced by increasing the salt concentration at neutral pH, suggesting that the mechanism by which these two effects (low pH, high salt concentration)

3 *A30P – residue alanine in position 30 replaced by proline; A53T – alanine in position 53 replaced by threonine; E46K – glutamic acid in position 46 replaced by lysine.*

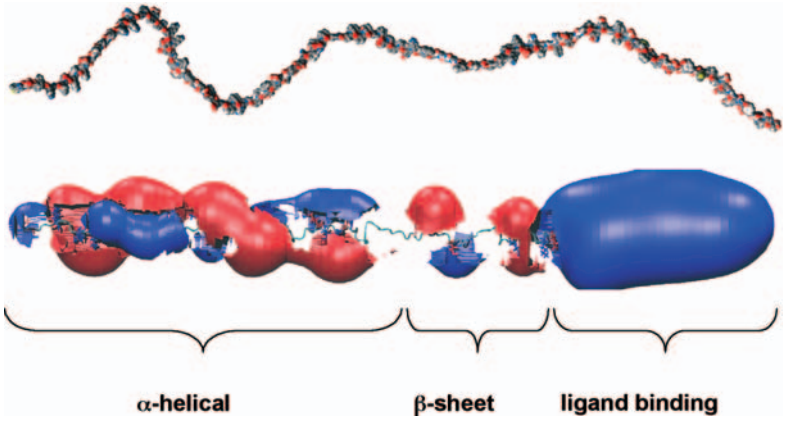

Figure 5: Molecular architecture of α-synuclein. Upper image depicts the α-synuclein primary sequence, while the lower image indicates the location of charged residues. Positively charged residues are colored red, while negatively charged residues are colored blue. Images courtesy of Dr. Gertjan Veldhuis and Reinhard Klement.

act upon the aggregation is by electrostatic shielding of the C-terminal region of α-synuclein. In addition to the changes in aggregation kinetics, we also discovered that the changes in solution conditions yielded morphologically distinct aggregates ranging from ordered fibrils at pH 7 to large amorphous clumps at pH 4 (see Figure 6). The importance of aggregate morphology for protein deposition diseases has been clearly demonstrated for other misfolding diseases. It has been shown that granular and amorphous aggregates formed from non-disease-related amyloid-forming proteins have a much higher potential to impair cell viability than do mature fibrils (24). Thus, it is very likely that the structural diversity of α-synuclein is a key element in the pathology of the synucleinopathies.

The provocative hypothesis that amyloid toxicity may be caused by membrane permeabilization by pore-like early intermediates has also been postulated for α-synuclein (31, 62-66), but remains to be unequivocally verified.

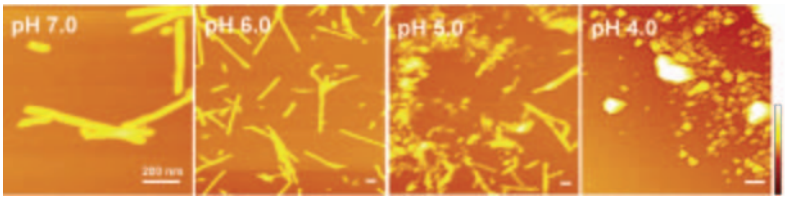

Figure 6: *Scanning force microscopy images of a-synuclein aggregates. Aggregate morphology changes from fibrillar to amorphous as pH is decreased. Data adapted from Hoyer et al. (61).*

Studying the biophysics of protein misfolding in vitro

In vivo studies in humans aimed at revealing the relationship between protein aggregation and disease are not easy given the lack of appropriate biomarkers and limitations in brain imaging methods. Accurate diagnosis of neurodegenerative diseases is only possible with appropriate tissue biopsies, which can only be performed post-mortem in humans. In vitro studies of protein aggregation thus have a significant role in yielding insights into the biophysics of protein misfolding that is relevant to disease. Aggregation induced in the test tube reflects mechanistic and structural characteristics of the in vivo process, although it cannot reproduce the complex cellular environment. Amyloid fibrils obtained from incubations of the isolated proteins are indistinguishable from those derived from patients' brains. The absence of in vivo influences allows for the detailed, controlled, investigation of the aggregation kinetics and the structures of the involved species. This property facilitates the identification of factors that modulate and influence the physics of amyloid formation. These factors include protein-ligand interactions, solution conditions, point mutations, post-translational chemical modifications and proteolytic processing. Furthermore, oligomers and aggregates formed in vitro can be isolated and investigated in regard to their toxicity in cell culture, an approach that is particularly interesting for identifying the mechanisms of cell-damage.

Where do we go from here?

My goal is to use a wide repertoire of innovative microscopy and spectroscopy approaches to

- glean insights into the biophysics of α-synuclein aggregation,
- quantitatively visualize the morphology and interactions of early aggregates of α-synuclein with lipid bilayers, other proteins, and cell membranes,
- establish and test different hypotheses for aggregate toxicity,
- gain insights into the roles of intrinsically disordered proteins in biology.

The palette of biophysical tools available to us allows us to measure:

- the forces driving protein misfolding and aggregation,
- the magnitude of the inter- and intramolecular forces and energy barriers,
- the thermodynamics and kinetics of aggregation and of binding to lipids and cell membranes of α-synuclein and other proteins.

These data will inform and validate theoretical models of protein aggregation.

Nanoscale characterization of early aggregate species

It is evident that fibrillar protein aggregates are nanometer scale structures with unique self-assembly and material properties. Although extensive work has been carried out on protein aggregation, there is still a huge gap in our understanding of the basic biophysics of the nucleation-polymerization process that underlies amyloid formation and its consequences for disease. Our early work has shown that α-synuclein can be seeded with aggregates preformed at pH 4 and pH 7 (Figure 7). Addition of fibrillar pH 7 seeds abolished the lag phase almost completely in contrast to the amorphous pH 4 aggregates. The fibrillar species thus appear to function far better than the amorphous species as fibrillization nuclei.

What then are the detailed structures of the aggregates that can effectively template α-synuclein fibril formation? Can we visualize these and characterize their mechanical properties? Does seeding with nuclei of different morphologies generate fibrils that reflect the morphology of the precursor seed, or of the solution conditions? The last question is particularly interesting in light of a recent report indicating a 'shape memory' for the beta-amyloid peptide of Alzheimer's disease (67). In this case, different fibril

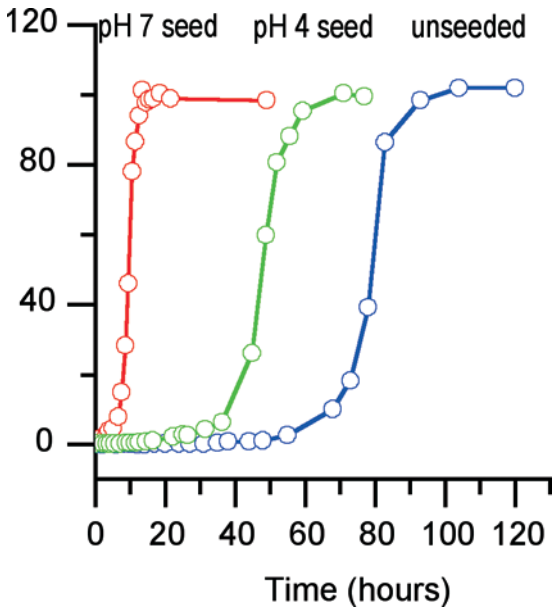

Figure 7: *Seeding accelerates fibril formation. Fibrillar pH 7 aggregates abolish the lag-phase of α-synuclein aggregation, while amorphous pH 4 aggregates are less efficient. Data adapted from Hoyer et al. (61).*

morphologies had distinct underlying molecular structures that were influenced by subtle variations in fibril growth conditions. Both morphology and molecular structure were self-propagating when fibrils were grown from preformed seeds. The issue of cross-seeding by nuclei of different proteins is particularly relevant, and is in fact a terrifying prospect for the initiation and propagation of disease (68).

We are currently focusing on using all the modes and contrast mechanisms (tapping mode, contact mode, height, phase, adhesion) of atomic force microscopy to:

- quantitatively characterize the morphologies of early aggregates and mature fibrils of α-synuclein (and other amyloid fibril forming proteins)
- achieve ultra-high resolution images of the spherical oligomers, protofibrils and fibrils formed under various conditions

Biological nanostructures require low force imaging to enable non-invasive monitoring of their structure and formation. My colleague Kees van der Werf in the group has spent many years perfecting a custom-designed standalone AFM which can be operated at extremely low tapping amplitudes (1-2 nm), enabling us to achieve single protein resolution. Using this platform and the sharpest AFM tips available we are aiming for the highest possible resolution of these protein nanostructures. Quantitative AFM imaging of 'soft' biological samples is also critically dependent on well-characterized tip-sample interactions, and we will use the data generated to understand and model these interactions.

Atomic force microscopy can operate in physiological solutions, thus enabling a detailed exploration of the fibrillization phase space as a function of time and solution conditions. Martijn van Raaij in the laboratory has been using atomic force microscopy to study in detail the nanoscale morphologies and structures of wild-type alpha-synuclein and disease related mutants. An AFM image of fibrils generated from the E46K disease-related mutant of α-synuclein displaying a range of different morphologies in a single field of view is shown in Figure 8 (69). Key issues include understanding the origin of this structural heterogeneity and correlating the variations with material and physical properties of the respective aggregate species.

We are now implementing a concerted effort focused on the early intermediates found in the lag phase of the fibrillization kinetics curve (Figure 4). This research will give us more insight into the molecular mechanisms underlying the templated formation of these protein nanostructures. Detailed analysis of the kinetics and energetics of the process (by measuring, for example, aggregate growth as a function of temperature and protein concentration) will enable us to map the energy landscape for the process. The extraction of quantitative kinetic parameters will enable us to develop suitable theoretical models for the aggregation process consistent with the physics of these polymer systems (70, 71). In this context it is interesting to note that critical monomer/nucleus concentration fluctuations may trigger the onset of the nucleation process (72), in a manner analogous to that proposed for protein crystallization (73). These studies will yield insights into which of the intermediate species are critical for aggregation, and lead us towards suitable molecules that may inhibit further aggregation or lead to fibril dissolution. For example, are aggregation inhibitors more effective on a particular type of intermediate species?

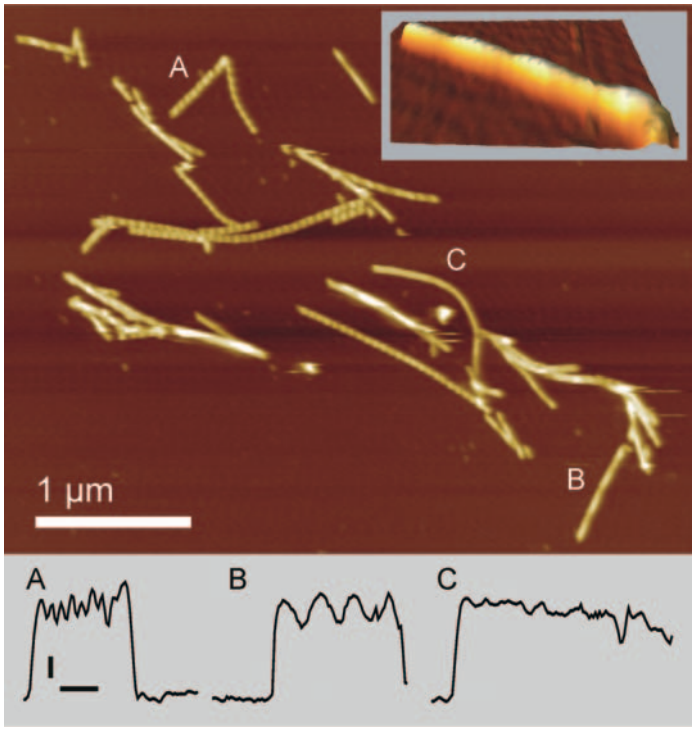

Figure 8: *Tapping mode atomic force micrograph of E46K mutant of α-synuclein in liquid after 72h of aggregation. The color scale represents 17.6 nm in height. The fibril fragments marked A, B and C are profiled along their length in the bottom panel indicating the range of morphologies observed in E46K fibrils. Vertical scale bar: 2 nm; horizontal scale bar: 200 nm. (adapted from(69)).*

Nanomechanics of early aggregates and mature fibrils

The correlation between morphological heterogeneity, structural plasticity, and the mechanical properties of the underlying fibrillar structures has not been explored. Nanomechanical properties of amyloid fibrils have been studied in a very limited way (74-77). A systematic study of amyloid material properties will be important for understanding their role in the disease

process, and for using these nanostructures as novel nanobiotechnological materials.

Recent work in the laboratory has explored the mechanical properties of α-synuclein fibrils by using a 'lateral deformation' approach (Figure 9). In this method, we deform the fibrils by scanning an AFM tip in contact with the sample while applying a relatively high force. Our data indicate that fibril deformation appears to occur at specific points along the fibril that are correlated to an apparent twist in the fibril morphology. However, these results also suggest that an alternative mode of fibril assembly, the so called 'lateral annealing' mode, may be operative here. In this mode, the fibrils are built of smaller individual units that are apparently "stitched together" at the fibril ends, and the deformation may occur preferentially at these interfaces.

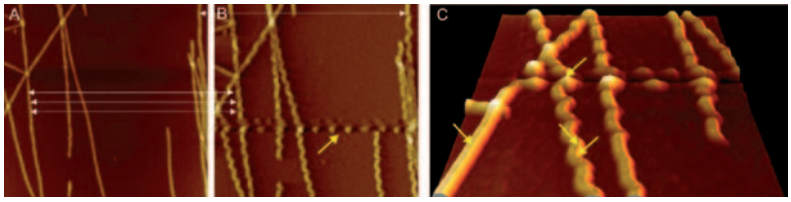

Figure 9: *'Lateral deformation' studies of fibril nanomechanics. Panel A shows the image of a field of α-synuclein fibrils before mechanical disruption. Following initial low-force tapping mode imaging, the field was imaged in contact mode while applying significant force. Panel B depicts a subsequent tapping mode image. The fibrils appear to deform at nodes that correspond to the inherent fibril twist. White lines indicate the corresponding nodes in both images. The yellow arrow indicates a scan line which was repeatedly scanned in contact mode to displace fibrils and deposit amyloid material on the surface. Panel C shows a magnified 3D image of deformed fibrils. Yellow arrows indicate positions for comparative nanoindentation measurements (K. O. van der Werf et al., unpublished).*

I want to further understand the nanomechanical properties of fibrillar aggregates by combining the 'lateral deformation' approach with nanoindentation. In nanoindentation, an AFM tip is used to apply force on the sample at a defined location. Force-displacement curves are recorded for the approach and retraction trajectories, and can be used to calculate the Young's

modulus at each position. Repeating these measurements for different fibrils along the length of the fibrils will yield a wealth of information about the internal structure, packing densities, and local structural heterogeneities of fibrils (Figure 9c). These experiments can be performed on fibrils of different morphologies, as in Figure 8, and on different mutants to understand the role that morphological diversity may have on mechanical properties and the potential link to disease. Nanoindentation can be used with early aggregates, and will be especially useful for understanding their mechanical properties.

α-synuclein lipid interactions at the nanometer scale

Cellular membranes represent a fertile biological interface for protein function and interactions. Many cytosolic proteins are recruited to different cellular membranes to form protein-protein and lipid-protein interactions critical for cell signaling and membrane trafficking. The mechanisms by which these proteins are recruited to, and interact with, various cell membranes are complex. These mechanisms are only now beginning to be elucidated by in vitro membrane binding studies using model membranes, computational approaches, and cellular studies using fluorescent protein-tagged proteins. The interaction of α-synuclein with membranes has been postulated for both the normal function of the protein and in the pathology of Parkinson's disease. A role in synaptic plasticity and regulation has been proposed, potentially involving membrane interactions (78-80). As mentioned earlier, the formation of pore-like structures and subsequent membrane permeabilization may be responsible for the disease state (7, 65, 66, 81). A number of in vitro studies have addressed the nature of the α-synuclein/membrane interactions, but much of the literature is contradictory.

Bart van Rooijen in the lab has shown that protofibrillar α-synuclein is extremely efficient in permeabilizing dye-filled lipid vesicles, as indicated by dye release experiments measured by bulk fluorescence spectroscopy (Figure 7). α-synuclein protofibrils at 1 μM concentration are far more effective at disrupting vesicles than 10 μM monomeric α-synuclein. Mature fibrils elicit the same response as monomeric α-synuclein, indicating that the protofibrillar species interact in substantially different ways than monomeric or fibrillar synuclein, although the details of the mechanisms are still unknown.

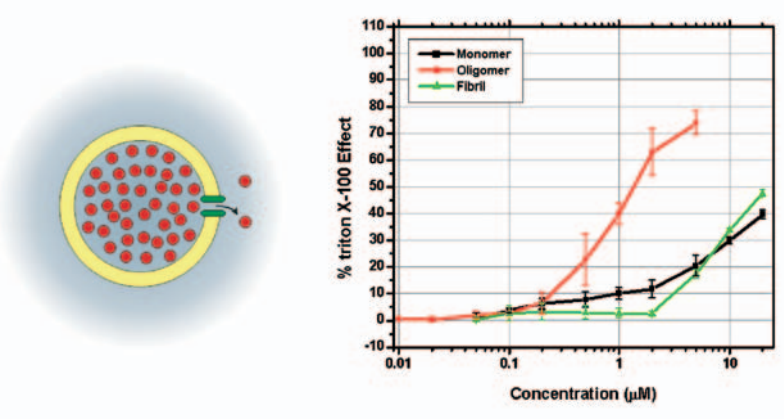

Figure 10: Oligomeric synuclein permeabilizes dye-filled vesicles more efficiently than monomeric synuclein. Phospholipid vesicles containing calcein were incubated for defined periods with the indicated concentration of monomeric or oligomeric synuclein. The fluorescence detected was normalized to the fluorescence intensity achieved after vesicle destruction with the detergent Triton X-100. B. D. van Rooijen et al., unpublished.

Using electron microscopy, Bart has shown that a fraction of the oligomeric species form pore-like structures. Whether these pores are truly responsible for toxicity remains to be unequivocally established, but the indications are certainly tempting. We would like to understand the interactions of monomeric and oligomeric α-synuclein with supported lipid bilayers with nanometer spatial resolution using a combination of quantitative microscopy and spectroscopy techniques. The goal is to

- understand the specificity of α-synuclein interaction with different lipids,
- quantitatively understand the role lipids may play in structural and functional polymorphism of the protein,
- image the effect of protein monomer and oligomer binding on lipid bilayer disruption and reorganization,
- measure and model subtle structural changes in both lipid and protein as a function of lipid, protein concentration, protein mutations or truncations.

Once again, we will focus on the specific interaction of early aggregate species with lipids. Work with the amyloid-beta peptide has shown that the peptide can physically disrupt the bilayer (82-84). The human amylin peptide can induce membrane defects which can spread across the bilayer surface (85), possibly by extraction of lipids into the forming amyloid deposits (86), suggesting an alternative mechanism to the annular pore hypothesis for amyloid-lipid interactions.

To get deeper insights into the physics and mechanisms of α-synuclein-lipid interactions, we propose using a combination of scanning probe and optical techniques to sensitively image lipid domains in the bilayer with nanometer precision. Subsequent partitioning of monomeric or oligomeric protein, or protofibrillar and fibrillar structures, into the lipid bilayer can also be imaged using AFM to provide the nanometer spatial resolution required to image protein oligomers and protofibrils on the surface. However, in order to reveal the dynamics of the lipid-protein interaction and associated lipid domain reorganization, complementary imaging and spectroscopy using combinations of AFM with optical microscopy techniques is required.

Into the cell

Finally, I want to visualize the effect of molecular crowding in the cell on the structure and dynamics of intrinsically disordered proteins. Using judicious combinations of existing or to-be-developed biophysical tools, I want to extend our studies on misfolding and aggregation of relevant disease-related proteins into the complex milieu of the neuron. Exciting possibilities arise! With modern molecular biology, imaging, and spectroscopy methods, we should be able to get a glimpse into these processes as they occur in the cellular context, with major implications for understanding the intrinsic factors that drive protein misfolding and aggregation and potential intervention strategies.

But is it physics?

Emphatically yes! I can think of no better way to answer this question than to quote the prescient words of a consummate physicist, Richard Feynman, no less true today than when they were uttered almost half a century ago:

"Certainly no subject is making more progress on so many fronts than biology, and if we were to name the most powerful assumption of all, which leads one on and on in an attempt to understand life, it is that *all things are made of atoms*, and that everything that living things do can be understood in terms of the jiggling and wiggling of atoms."[4]

4 *Richard Feynman, Lectures on Physics, vol. 1, p. 3-6 (1963)*

A word of thanks

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Research at this University is structured under the aegis of institutes, which serve the role of fostering interaction and collaboration between groups. I have the privilege of participating in both the MESA+ Institute for Nanotechnology and the Institute for Biomedical Technology, both of which have provided essential support for the initiation of my activities here in Enschede. This involvement in both institutes makes it possible to build bridges between disciplines, and has been particularly fruitful.

Science today is a global activity and collaboration is an essential part of science. It is also one of the most fulfilling aspects of being a scientist. I have the privilege of having many collaborators here at the UT, nationally, and internationally, and I thank all them for their contributions to this endeavour.

None of this work that I have spoken about would be possible without the contributions of many talented colleagues and team members, past and present. I thank them all, and in particular all members of the Biophysical Engineering group for their committed engagement to our core business of research and teaching.

I thank the Stichting FOM for their generous support of my research, and for their consummate professionalism as a funding organization. The Stichting Internationaal Parkinsons Fonds has also supported our work, and I am grateful.

Dames en heren, in een instelling zoals de UT, is het onmogelijk dat wij wetenschappers ons werk kunnen uitvoeren zonder de ondersteuning van een groot aantal mensen die hun werk achter de schermen doen. Ik wil een bijzonder woord van dank schenken aan de medewerkers van de faculteit TNW en de UT die ons werk mogelijk maken.

Let me add a few personal notes.

My parents instilled in me a love and respect for knowledge, and gave me the impetus to embark on this voyage 22 years ago. My late father would have been proud to participate in today's celebrations; I am delighted that my mother is sitting front and center today. I dedicate this lecture to them.

My PhD supervisors, Duncan Steel and Ari Gafni provided me with my first taste of interdisciplinary research in a laboratory run jointly by a condensed matter and optical physicist and a biochemist. A crazy enterprise, you might think, but their laboratory remains the archetype of how interdisciplinary research in biophysics should be done. I owe Duncan and Ari a great deal for their patient and insightful guidance, and for the introduction to the world of protein biophysics.

I owe a special debt of gratitude to Dr. Tom Jovin. Tom gave me unfettered freedom and unstinting support in his laboratory at the Max Planck Institute for Biophysical Chemistry in Göttingen. The years I spent in his laboratory were instrumental in further developing my interests in protein biophysics and quantitative imaging, and in the genesis of many of the ideas I have spoken about today. I am honoured that Tom and Donna Jovin are here today, and I thank them for their professional and personal friendship over the years.

I have one final debt of gratitude to acknowledge, and it is perhaps the greatest one. My voyage has taken me to many ports, and sometimes through some very choppy waters. My wife Sowmya has been a steadfast companion, and a source of support, wise advice, and inspiration. Thank you.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.

Ladies and Gentlemen, my voyage is not over. But this lecture is, and it is time for a drink. Thank you for your attention and for sharing this day with me.

Ik heb gezegd.

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