materials

from

to

disease

HIERARCHICAL SELF-ASSEMBLY
OF

ALPHA-SYNUCLEIN

by
Slay Semerdzhiev

**HIERARCHICAL SELF - ASSEMBLY OF
ALPHA-SYNUCLEIN:
FROM DISEASE TO MATERIALS**

Slav Semerdzhiev

2016

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To my family

CHAPTER 1

INTRODUCTION

1.1 Function of alpha-synuclein

Alpha-synuclein (α S) is a 140 amino acid protein that is most abundant in neuronal cells but is also found in other types of tissues.¹⁻³ The function of α S remains unknown. Due to its abundance in the presynaptic regions of nerve cells, the function of α S is assumed to be related to synaptic transmission.⁴ A study showing binding of α S to the vesicle bound SNARE protein Synaptobrevin 2 and facilitated SNARE-complex assembly in the presence of α S support involvement of α S in neuron signaling (synaptic transmission).⁵ Other biochemical investigations show significant binding of α S to microtubules and a subsequent enhancement of tubulin polymerization kinetics. α S has therefore also been suggested to be a potential functional microtubule-associated protein.⁶ Interactions of α S with tau imply a function of the former associated with the cytoskeleton.⁷ Studies on α S-membrane interactions propose that α S might act as shuttle protein on mitochondrial membranes that participates in the removal of highly toxic peroxidised lipids from the membrane.⁸ Despite the large number of putative functions of α S, there is no consensus on what the real functional role of this protein is.

1.2 Pathological role

The scientific interest in α S was first sparked by its relation to the pathology of neurodegenerative diseases rather by its function. Fibrillar α S has been identified as the main constituent of amyloid inclusions such as Lewy bodies and Lewy neurites, which are the pathological signature of Parkinson's disease and other synuclein related disorders called synucleopathies.⁹⁻¹¹ The role of α S in the etiology of these disease conditions remains however unknown despite the intensive research that has been performed on this topic and the many putative mechanisms that have been put forward. Some of the proposed toxicity mechanisms are based on the hypothetical functional assignments of α S mentioned earlier.

It has been speculated that upon aggregation into amyloids, the protein loses its function and by that exerts toxicity. Other hypotheses assign toxicity to the aggregate species rather than to the loss of function. Many reports indicate that the toxic effect of α S is imposed by prefibrillar aggregate species, i.e. oligomers. The mechanisms through which oligomers may induce harmful effects to the cell is a very rich topic on its own. The exposure of hydrophobic (HP) patches on the oligomer surface has been linked to aberrant binding of multifunctional proteins and increased formation of reactive oxygen species.¹²⁻¹⁵ Numerous *in vitro* studies show that α S oligomers have a membrane permeabilization ability.¹⁶⁻¹⁹ This permeabilization effect is most often rationalized in the context of the popular – though also controversial – “amyloid pore hypothesis” which stipulates that the pore-like structure of the oligomers is prescribing membrane permeabilizing properties to this type of aggregates.²⁰⁻²² Alternatively, membrane “thinning” as a consequence of the insertion of many hydrophobic residues has been proposed to be the cause of the membrane permeabilization.²³ Without going into details, a few other toxic effects of oligomers have been also suggested, such as: changed cytoskeleton organization and dynamics, mitochondrial dysfunction, endoplasmic reticulum stress, impaired protein degradation systems, and enhanced formation of reactive oxygen species and neuroinflammation.²⁴

The amyloid fibrils that form in the α S aggregation process have been for long time dismissed as a carrier of cell toxicity. However, recent studies have reanimated the idea that fibrils may be a toxic species on their own.^{19, 25-27} Some investigations even show that stoichiometrically one fibril can be more toxic than one oligomer which dismisses the notion that fibrils are inert proteinaceous aggregates.²⁶ Mechanisms such as membrane permeabilization, proteasomal impairment and glial activation via TLR2 (Toll-like receptor 2) have been linked to the presence of α S fibrils.^{19, 26, 28-29} Nevertheless, similar to oligomers, uncertainty veils the mechanistic details through which α S fibrils perturb the normal functioning of the cell. A hypothesis inspired by experimental findings suggests that a population of oligomers - part of which toxic - co-exist in a dynamic equilibrium with fibrils and monomers.¹⁹ An interesting hypothesis - claiming that the elongation process (i.e. fibril growth) is toxic rather the fibrils themselves - also gathers momentum.³⁰ This hypothesis is based on the phenomenon of aggregation induced lipid extraction that was originally

observed for human islet amyloid polypeptide (IAPP).³¹⁻³² Adopting this hypothesis for α S would explain the co-localization of lipids with fibrillar α S in Lewy bodies.³³ Experimental findings showing the recruitment of lipids by α S fibrils elongating on the surface of a flat lipid bilayer support this idea.³⁴ Additionally, work demonstrating the synchronous membrane disruption and fibril elongation followed by α S fibrils/lipid co-localization, further supports the lipid extraction concept.³⁵

1.3 Structure

1.3.1 Primary

α S is comprised of 140 amino acids (AAs). The polypeptide chain shows similarities to block-copolymers and is often divided in three segments: the N-terminal region, the middle non-amyloid beta component (NAC) part and the C-terminal region, corresponding to the amino-acid stretches 1-60, 61-94 and 95-140 respectively. The N-terminal region resembles a polyampholyte as it carries both negative and positive charges (Figure 1.1). The presence of several KTKEGV motifs, which are also observed in apolipoproteins, encode lipid membrane binding properties for this domain. Numerous experimental findings confirm the

Figure 1.1 Schematic representation of the amino acid composition of α S. Blue, grey and red segments designate the N-terminal region, NAC and C-terminal region respectively. Hydrophobic amino acids are in blue colour while the hydrophilic ones are in black. The charged amino acids have their corresponding charge assigned above them.

important role that the N-terminal region plays in the interaction of α S with different lipid and surfactant substrates. The NAC region consists primarily of hydrophobic amino acid residues and is believed to be the main culprit for the aggregation propensity of α S. The

observation that the amyloidogenic properties of α S are very sensitive to compositional changes in this part of the sequence support this role for the NAC region. Omitting AAs from this region can abrogate the aggregation susceptibility of α S.³⁶ Beta-synuclein, a homologue of α S that misses 11 AAs in the NAC region, has significantly lower propensity to aggregate and can only be ‘forced’ to do so by introducing special conditions.³⁷ Finally, the C-terminal region has a characteristic high negative charge density at physiological pH. In contrast to the NAC, the C-terminal region has an inhibitory effect on the aggregation of α S. The enhanced aggregation kinetics observed for α S mutants with a truncated C-terminal region clearly demonstrate the suppressive effect that the electrostatic repulsion between C-terminal regions of multiple proteins has on the aggregation behavior of the protein.³⁸ Besides the aggregation kinetics, interactions with the C-terminal region influence the morphology of amyloid structures formed by the protein.³⁹⁻⁴¹

1.3.2 Conformation

α S belongs to the family of intrinsically disordered proteins. It is widely perceived that in the absence of binding partners and at physiological conditions, this protein lacks secondary and tertiary structure. It is worth to note however, that recent reports indicate tetrameric helical and multimeric native forms of α S in red blood cells and brain tissue respectively.⁴² Since these findings are an object of controversy among scientists in the field, the most widely accepted native form of α S in the community remains the unstructured protein.⁴³ In the presence of lipid molecules (below the CMC and at the right stoichiometry), the N-terminal and NAC regions can undergo a conformational switch and form an amphipathic α -helix.⁴⁴ Upon binding to lipid membrane or surfactant vesicles, a similar change in conformation is observed.⁴⁴⁻⁴⁸ Depending on the curvature of the lipid substrate to which α S binds, the helix can be broken or extended.

1.4 Self-assembly pathway

The phase behavior of amyloidogenic proteins, α S included, is very complex. At the right conditions α S self-assembles (aggregates) into amyloid fibrils. What triggers the onset of this process *in vivo* is not clear yet. *In vitro* studies however, have been invaluable tools

for studying the physico-chemical triggers that set off the formation of amyloid fibrils. Besides the initiation, the physico-chemical conditions strongly influence the progression of the aggregation process itself (kinetics, composition and morphology of the aggregation species). A range of physico-chemical factors including presence of simple salts or polyelectrolytes in the solution, changes in pH, mutations in the original sequence, agitation, temperature changes, protein concentration, strongly influence how the aggregation process evolves. The self-assembly pathway of α S is pictorially represented in (Figure 1.2). Albeit being in simplified form, this schematic captures the main stages of the self-assembly phenomenon. However, there are still pieces of the puzzle missing and we don't know in detail all the molecular events that α S undergoes on its way to the fibrillar form. It is believed that first step of the self-assembly process is accompanied by conformational changes in the protein monomer. The disordered state of the peptide chain is believed to be promoted by the repulsive intramolecular electrostatic interactions. The balance between the net charge and the hydrophobicity of the protein makes the predominance of the electrostatic interactions only marginal.⁴⁹ Since this balance is only slightly dominated by electrostatic repulsion, introducing a stimulus that can suppress the electrostatics or enhance the hydrophobic interactions can tip the balance in favor of the latter. Such conditions would increase the probability for conformational excursions of the protein chain to a more compact state. Indeed, compaction in the α S molecule has been observed experimentally in aggregation-favorable conditions.⁵⁰⁻⁵² A low beta-sheet content has been also detected in this compacted partially folded intermediate.⁵² The rate of this reconfiguration of the protein molecule seems to also be very important for the overall kinetics of the aggregation process of α S.⁵⁰

The partially folded intermediate seems to have a higher aggregation propensity and its appearance essentially marks the beginning of the second stage of the self-assembly process, i.e. the formation of α S oligomers. This is not surprising because the interactions that drive the collapse of proteins also operate on the intermolecular level. This stage of the self-assembly is the most elusive one. It is experimentally challenging to follow the ongoing dynamics in detail. This is due to the transient nature of the oligomers along with the appearance of multiple oligomeric species during the aggregation process.^{19, 53-54}

Nevertheless, through customized protocols, kinetically trapped α S oligomers were successfully isolated and subsequently subjected to a thorough investigation.^{53,55} In several oligomer preparations characterized with different types of techniques, two main populations of aggregates seem to recur. With small variations, the established mean

Figure 1.2 Simplified representation of the self-assembly (aggregation) pathway of α S.

aggregation number for the two populations of oligomers were: ≈ 18 and ≈ 29 monomers per oligomer; 15-19 and 34-38 monomers per oligomer.^{53,55} In other studies, only the bigger oligomeric species of ≈ 30 α S monomers was identified (possibly due to the different oligomer preparation protocol used), but this number is still in good agreement with the aggregation number obtained in the previously mentioned investigations.^{19,56} In terms of secondary structure, reports indicate fractional folding of $\approx 35\%$ which is lower than what is observed for α S amyloid fibrils ($\approx 65\%$).⁵³ There is also a certain consistency in the recovered morphology of the oligomers. Findings originating from several structural studies revolve, with some variations, around a model describing an oligomer as a particle with toroidal-like (wreath, annular, doughnut) morphology.^{18,53,57} Even with all the structural information available, there is still no clear mechanistic view on how the α S oligomers transform into actively growing fibrils and if all of the different oligomer populations actually have the ability to do so. Despite the fact that the whole self-assembly process is considered a classical nucleation and growth reaction, the oligomers that are the critical nuclei have not yet been identified.

Presumably after some compositional (monomer number) and conformational rearrangements, the oligomers turn into potent amyloid fibrils that continuously recruit monomeric protein (Figure 1.2). Part of the recruited monomer folds into strands that interconnect with their counterparts from adjacent α S molecules through multiple hydrogen bonds running in parallel with the longitudinal fibril axis. Such molecular arrangement gives rise to the structural signature of amyloid fibrils, the cross-beta sheets. The order in this cross-beta sheet amyloid core is so high that it gives rise to crystalline reflections when studied with diffraction techniques.^{27, 58} The intra- and inter-molecular distances between the beta-strands are approximately 0.47 nm and 1.1 nm respectively.⁵⁸⁻⁶⁰ Most structural studies report the amino acid fragments 1-30 and 110-140 to remain unstructured in the fibril, implying that N- and C-terminal regions do not participate in the construction of the amyloid core. The exact set of amino acid stretches that attain a beta-strand conformation and the length of these strands varies within the different reports.⁵⁹⁻⁶² Due to their chiral nature, the monomers do not stack in parallel to each other but slightly rotate (along the central longitudinal axis of the fibril) to give the fibril a helical twist with a certain periodicity (pitch).⁶³ The number of filaments that intertwine into a mature α S fibril are believed to be 4, i.e. ≈ 8 monomers per nanometer length of fibril.⁶⁰ A recent report proposed a structure for α S fibril comprised of 3 filaments and with a different internal organization of the beta-strands.⁶⁴ Instead of in register beta-sheets, the model conceives a “Greek key” configuration for the amyloid core. This difference in the proposed architectures of the amyloid fibrils could be due to the polymorphic nature of amyloid fibrils. Polymorphism could also be traced back to the difference in periodicity that amyloid fibrils often exhibit. As a morphological feature of fibrils, the helical pitch seems to be sensitive to the physico-chemical environment. Ionic strength for example, seems to be an efficient parameter to control the periodicity of beta-lactoglobulin fibrils.⁶⁵ A similar sensitivity of the fibril morphology towards the solution conditions has been also demonstrated for α S.⁶⁶ At elevated NaCl concentrations, there is a significant spread in the measured fibril periodicities. In contrast, the fibrils grown at lower ionic strength or in the presence of EDTA exhibited a significantly lower degree of polymorphism in terms of periodicity. Thus, the mismatch between the architecture of α S fibrils observed in the

different structural studies could be interpreted in the context of the different experimental conditions that were used in these investigations.

1.5 Roadmap for this thesis

The monomeric, oligomeric and fibrillar forms of α S have been heavily studied and are still subjects of intensive investigations. Although we have partially understood the self-assembly of α S, we still lack insights about the fate of the fibrils once they are formed. The scope of most studies (if not none) has barely gone beyond the fibril stage of the self-assembly process. However, if higher order amyloid assemblies like Lewy bodies and Lewy neurites are to be understood, we need to expand the field of view of our investigations to larger length scales that remain yet (at least for α S) unexplored. Moreover, to successfully utilize amyloid fibrils as a basis for novel nano-structured functional materials, we need to better understand their phase behavior and the forces that determine it. In **Chapter 2**, inspired by the self-organization of other semi-flexible biopolymers, we start unraveling the higher order organization of α S fibrils into supra-fibrillar aggregates (SFAs). We also outline the interactions that drive this higher order assembly and interpret the findings in the context of disease conditions and materials. In **Chapter 3** we extend the range of conditions at which we form SFAs, and demonstrate that SFAs can attain an even larger diversity of morphologies. We study the effect of mono- and divalent ions; and the result of formation of SFAs on the overall kinetics of α S aggregation. Additionally, we use these findings to explain the morphology of one of the SFAs species in more detail. **Chapter 4** investigates the internal organization and architecture of SFAs with cylindrical morphology using a set of different techniques such as Polarized Light Microscopy, Atomic Force Microscopy and Confocal Laser Scanning Microscopy. We also use an environment sensitive dye to probe the internal structure of the aggregates. Chapter 5 draws inspiration from the qualitative findings in Chapter 2 regarding the attractive interfibrillar attractions and scrutinizes them by studying the thermal response of α S amyloid networks via oscillatory rheology. We describe the temperature response of the network in the context of a theoretical framework originally devised to describe the mechanical response of semi-flexible polymer networks. Chapter 6 focuses on the effect that the valence of counterions has on the

interfibrillar interactions. While Chapters 2 and 3 give indications that multivalent ions provide an additional effect (different from charge screening) to the interfibril interaction, in this chapter we quantify these findings by probing the mechanical response of α S fibril networks subjected to oscillatory shear. In both **Chapters 5 and 6** we discuss the results in the light of disease conditions and responsive amyloid based materials. Finally, in **Chapter 7** we summarize the work done in this thesis and highlight the findings that stem from it. We also expose our recommendations for future investigations.

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References

1. Maroteaux, L.; Campanelli, J. T.; Scheller, R. H., Synuclein - a Neuron-Specific Protein Localized to the Nucleus and Presynaptic Nerve-Terminal. *J. Neurosci.* **1988**, *8*, 2804-2815.
2. Ueda, K.; Saitoh, T.; Mori, H., Tissue-Dependent Alternative Splicing of Messenger-Rna for Nacp, the Precursor of Non-a-Beta Component of Alzheimers-Disease Amyloid. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1366-1372.
3. Beyer, K.; Domingo-Sabat, M.; Lao, J. I.; Carrato, C.; Ferrer, I.; Ariza, A., Identification and Characterization of a New Alpha-Synuclein Isoform and Its Role in Lewy Body Diseases. *Neurogenetics* **2008**, *9*, 15-23.
4. Goedert, M., Alpha-Synuclein and Neurodegenerative Diseases. *Nat. Rev. Neurosci.* **2001**, *2*, 492-501.
5. Burre, J.; Sharma, M.; Tsetsenis, T.; Buchman, V.; Etherton, M. R.; Sudhof, T. C., Alpha-Synuclein Promotes Snare-Complex Assembly in Vivo and in Vitro. *Science* **2010**, *329*, 1663-1667.
6. Alim, M. A.; Ma, Q. L.; Takeda, K.; Aizawa, T.; Matsubara, M.; Nakamura, M.; Asada, A.; Saito, T.; Kaji, H.; Yoshii, M., *et al.*, Demonstration of a Role for Alpha-Synuclein as a Functional Microtubule-Associated Protein. *J Alzheimers Dis* **2004**, *6*, 435-442.
7. Esposito, A.; Dohm, C. P.; Kermer, P.; Bahr, M.; Wouters, F. S., Alpha-Synuclein and Its Disease-Related Mutants Interact Differentially with the Microtubule Protein Tau and Associate with the Actin Cytoskeleton. *Neurobiol Dis* **2007**, *26*, 521-531.
8. Maltsev, A. S.; Chen, J.; Levine, R. L.; Bax, A., Site-Specific Interaction between Alpha-Synuclein and Membranes Probed by Nmr-Observed Methionine Oxidation Rates. *J. Am. Chem. Soc.* **2013**, *135*, 2943-2946.
9. Takeda, A.; Mallory, M.; Sundsmo, M.; Honer, W.; Hansen, L.; Masliah, E., Abnormal Accumulation of Nacp/Alpha-Synuclein in Neurodegenerative Disorders. *Am. J. Pathol.* **1998**, *152*, 367-372.
10. Spillantini, M. G.; Crowther, R. A.; Jakes, R.; Hasegawa, M.; Goedert, M., Alpha-Synuclein in Filamentous Inclusions of Lewy Bodies from Parkinson's Disease and Dementia with Lewy Bodies. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 6469-6473.
11. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., Alpha-Synuclein in Lewy Bodies. *Nature* **1997**, *388*, 839-840.
12. Zampagni, M.; Cascella, R.; Casamenti, F.; Grossi, C.; Evangelisti, E.; Wright, D.; Becatti, M.; Liguri, G.; Mannini, B.; Campioni, S., *et al.*, A Comparison of the Biochemical

Modifications Caused by Toxic and Non-Toxic Protein Oligomers in Cells. *J Cell Mol Med* **2011**, *15*, 2106-2116.

13. Bolognesi, B.; Kumita, J. R.; Barros, T. P.; Esbjorner, E. K.; Luheshi, L. M.; Crowther, D. C.; Wilson, M. R.; Dobson, C. M.; Favrin, G.; Yerbury, J. J., Ans Binding Reveals Common Features of Cytotoxic Amyloid Species. *ACS Chem. Biol.* **2010**, *5*, 735-740.
14. Olzscha, H.; Schermann, S. M.; Woerner, A. C.; Pinkert, S.; Hecht, M. H.; Tartaglia, G. G.; Vendruscolo, M.; Hayer-Hartl, M.; Hartl, F. U.; Vabulas, R. M., Amyloid-Like Aggregates Sequester Numerous Metastable Proteins with Essential Cellular Functions. *Cell* **2011**, *144*, 67-78.
15. van Rooijen, B. D.; Claessens, M. M. A. E.; Subramaniam, V., Lipid Bilayer Disruption by Oligomeric Alpha-Synuclein Depends on Bilayer Charge and Accessibility of the Hydrophobic Core. *Bba-Biomembranes* **2009**, *1788*, 1271-1278.
16. Kostka, M.; Hogen, T.; Danzer, K. M.; Levin, J.; Habeck, M.; Wirth, A.; Wagner, R.; Glabe, C. G.; Finger, S.; Heinzlmann, U., *et al.*, Single Particle Characterization of Iron-Induced Pore-Forming Alpha-Synuclein Oligomers. *J. Biol. Chem.* **2008**, *283*, 10992-11003.
17. Danzer, K. M.; Haasen, D.; Karow, A. R.; Moussaud, S.; Habeck, M.; Giese, A.; Kretzschmar, H.; Hengerer, B.; Kostka, M., Different Species of Alpha-Synuclein Oligomers Induce Calcium Influx and Seeding. *J. Neurosci.* **2007**, *27*, 9220-9232.
18. Lashuel, H. A.; Petre, B. M.; Wall, J.; Simon, M.; Nowak, R. J.; Walz, T.; Lansbury, P. T., Alpha-Synuclein, Especially the Parkinson's Disease-Associated Mutants, Forms Pore-Like Annular and Tubular Protofibrils. *J. Mol. Biol.* **2002**, *322*, 1089-1102.
19. Lorenzen, N.; Nielsen, S. B.; Buell, A. K.; Kaspersen, J. D.; Arosio, P.; Vad, B. S.; Paslawski, W.; Christiansen, G.; Valnickova-Hansen, Z.; Andreasen, M., *et al.*, The Role of Stable Alpha-Synuclein Oligomers in the Molecular Events Underlying Amyloid Formation. *J. Am. Chem. Soc.* **2014**, *136*, 3859-3868.
20. Zhang, H. Y.; Griggs, A.; Rochet, J. C.; Stanciu, L. A., In Vitro Study of Alpha-Synuclein Protofibrils by Cryo-Em Suggests a Cu²⁺-Dependent Aggregation Pathway. *Biophys. J.* **2013**, *104*, 2706-2713.
21. Schmidt, F.; Levin, J.; Kamp, F.; Kretzschmar, H.; Giese, A.; Botzel, K., Single-Channel Electrophysiology Reveals a Distinct and Uniform Pore Complex Formed by Alpha-Synuclein Oligomers in Lipid Membranes. *PLoS One* **2012**, *7*.

22. Yoshiike, Y.; Kaye, R.; Milton, S. C.; Takashima, A.; Glabe, C. G., Pore-Forming Proteins Share Structural and Functional Homology with Amyloid Oligomers. *NeuroMol. Med.* **2007**, *9*, 270-275.
23. Stockl, M. T.; Zijlstra, N.; Subramaniam, V., Alpha-Synuclein Oligomers: An Amyloid Pore? Insights into Mechanisms of Alpha-Synuclein Oligomer-Lipid Interactions. *Mol. Neurobiol.* **2013**, *47*, 613-621.
24. Roberts, H. L.; Brown, D. R., Seeking a Mechanism for the Toxicity of Oligomeric Alpha-Synuclein. *Biomolecules* **2015**, *5*, 282-305.
25. Stefani, M.; Dobson, C. M., Protein Aggregation and Aggregate Toxicity: New Insights into Protein Folding, Misfolding Diseases and Biological Evolution. *J Mol Med-Jmm* **2003**, *81*, 678-699.
26. Pieri, L.; Madiona, K.; Bousset, L.; Melki, R., Fibrillar Alpha-Synuclein and Huntingtin Exon 1 Assemblies Are Toxic to the Cells. *Biophys. J.* **2012**, *102*, 2894-2905.
27. Rodriguez, J. A.; Ivanova, M. I.; Sawaya, M. R.; Cascio, D.; Reyes, F. E.; Shi, D.; Sangwan, S.; Guenther, E. L.; Johnson, L. M.; Zhang, M., *et al.*, Structure of the Toxic Core of Alpha-Synuclein from Invisible Crystals. *Nature* **2015**, *525*, 486-+.
28. Lindersson, E.; Beedholm, R.; Hojrup, P.; Moos, T.; Gai, W. P.; Hendil, K. B.; Jensen, P. H., Proteasomal Inhibition by Alpha-Synuclein Filaments and Oligomers. *J. Biol. Chem.* **2004**, *279*, 12924-12934.
29. Kim, C.; Ho, D. H.; Suk, J. E.; You, S.; Michael, S.; Kang, J.; Lee, S. J.; Masliah, E.; Hwang, D.; Lee, H. J., *et al.*, Neuron-Released Oligomeric Alpha-Synuclein Is an Endogenous Agonist of Tlr2 for Paracrine Activation of Microglia. *Nat Commun* **2013**, *4*.
30. Butterfield, S. M.; Lashuel, H. A., Amyloidogenic Protein Membrane Interactions: Mechanistic Insight from Model Systems. *Angew Chem Int Edit* **2010**, *49*, 5628-5654.
31. Sparr, E.; Engel, M. F. M.; Sakharov, D. V.; Sprong, M.; Jacobs, J.; de Kruijff, B.; Hoppener, J. W. M.; Killian, J. A., Islet Amyloid Polypeptide-Induced Membrane Leakage Involves Uptake of Lipids by Forming Amyloid Fibers. *FEBS Lett.* **2004**, *577*, 117-120.
32. Domanov, Y. A.; Kinnunen, P. K. J., Islet Amyloid Polypeptide Forms Rigid Lipid-Protein Amyloid Fibrils on Supported Phospholipid Bilayers. *J. Mol. Biol.* **2008**, *376*, 42-54.
33. Gai, W. P.; Yuan, H. X.; Li, X. Q.; Power, J. T. H.; Blumbergs, P. C.; Jensen, P. H., In Situ and in Vitro Study of Colocalization and Segregation of A-Synuclein, Ubiquitin, and Lipids in Lewy Bodies. *Exp. Neurol.* **2000**, *166*, 324-333.

-
34. Reynolds, N. P.; Soragni, A.; Rabe, M.; Verdes, D.; Liverani, E.; Handschin, S.; Riek, R.; Seeger, S., Mechanism of Membrane Interaction and Disruption by Alpha-Synuclein. *J. Am. Chem. Soc.* **2011**, *133*, 19366-19375.
 35. Chaudhary, H.; Stefanovic, A. N. D.; Subramaniam, V.; Claessens, M. M. A. E., Membrane Interactions and Fibrillization of Alpha-Synuclein Play an Essential Role in Membrane Disruption. *FEBS Lett.* **2014**, *588*, 4457-4463.
 36. Giasson, B. I.; Murray, I. V. J.; Trojanowski, J. Q.; Lee, V. M. Y., A Hydrophobic Stretch of 12 Amino Acid Residues in the Middle of Alpha-Synuclein Is Essential for Filament Assembly. *J. Biol. Chem.* **2001**, *276*, 2380-2386.
 37. Yamin, G.; Munishkina, L. A.; Karymov, M. A.; Lyubchenko, Y. L.; Uversky, V. N.; Fink, A. L., Forcing Nonamyloidogenic Beta-Synuclein to Fibrillate. *Biochemistry* **2005**, *44*, 9096-9107.
 38. Hoyer, W.; Cherny, D.; Subramaniam, V.; Jovin, T. M., Impact of the Acidic C-Terminal Region Comprising Amino Acids 109-140 on Alpha-Synuclein Aggregation in Vitro. *Biochemistry* **2004**, *43*, 16233-16242.
 39. Sweers, K. K. M.; van der Werf, K. O.; Bennink, M. L.; Subramaniam, V., Atomic Force Microscopy under Controlled Conditions Reveals Structure of C-Terminal Region of Alpha-Synuclein in Amyloid Fibrils. *ACS Nano* **2012**, *6*, 5952-5960.
 40. Hoyer, W.; Antony, T.; Cherny, D.; Heim, G.; Jovin, T. M.; Subramaniam, V., Dependence of A-Synuclein Aggregate Morphology on Solution Conditions. *J. Mol. Biol.* **2002**, *322*, 383-393.
 41. Semerdzhiev, S. A.; Dekker, D. R.; Subramaniam, V.; Claessens, M. M., Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. *ACS Nano* **2014**, *8*, 5543-5551.
 42. Bartels, T.; Choi, J. G.; Selkoe, D. J., Alpha-Synuclein Occurs Physiologically as a Helically Folded Tetramer That Resists Aggregation. *Nature* **2011**, *477*, 107-U123.
 43. Lashuel, H. A.; Overk, C. R.; Oueslati, A.; Masliah, E., The Many Faces of Alpha-Synuclein: From Structure and Toxicity to Therapeutic Target. *Nat. Rev. Neurosci.* **2013**, *14*, 38-48.
 44. Ferreon, A. C. M.; Gambin, Y.; Lemke, E. A.; Deniz, A. A., Interplay of Alpha-Synuclein Binding and Conformational Switching Probed by Single-Molecule Fluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 5645-5650.
 45. Trexler, A. J.; Rhoades, E., Alpha-Synuclein Binds Large Unilamellar Vesicles as an Extended Helix. *Biochemistry* **2009**, *48*, 2304-2306.
-

46. Georgieva, E. R.; Ramlall, T. F.; Borbat, P. P.; Freed, J. H.; Eliezer, D., Membrane-Bound Alpha-Synuclein Forms an Extended Helix: Long-Distance Pulsed ESR Measurements Using Vesicles, Bicelles, and Rodlike Micelles. *J. Am. Chem. Soc.* **2008**, *130*, 12856-+.
 47. Ulmer, T. S.; Bax, A.; Cole, N. B.; Nussbaum, R. L., Structure and Dynamics of Micelle-Bound Human Alpha-Synuclein. *J. Biol. Chem.* **2005**, *280*, 9595-9603.
 48. Bussell, R.; Eliezer, D., A Structural and Functional Role for 11-Mer Repeats in Alpha-Synuclein and Other Exchangeable Lipid Binding Proteins. *J. Mol. Biol.* **2003**, *329*, 763-778.
 49. Uversky, V. N.; Gillespie, J. R.; Fink, A. L., Why Are "Natively Unfolded" Proteins Unstructured under Physiologic Conditions? *Proteins-Structure Function and Genetics* **2000**, *41*, 415-427.
 50. Ahmad, B.; Chen, Y. J.; Lapidus, L. J., Aggregation of Alpha-Synuclein Is Kinetically Controlled by Intramolecular Diffusion. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 2336-2341.
 51. Dedmon, M. M.; Lindorff-Larsen, K.; Christodoulou, J.; Vendruscolo, M.; Dobson, C. M., Mapping Long-Range Interactions in Alpha-Synuclein Using Spin-Label NMR and Ensemble Molecular Dynamics Simulations. *J. Am. Chem. Soc.* **2005**, *127*, 476-477.
 52. Uversky, V. N.; Li, J.; Fink, A. L., Evidence for a Partially Folded Intermediate in Alpha-Synuclein Fibril Formation. *J. Biol. Chem.* **2001**, *276*, 10737-10744.
 53. Chen, S. W.; Drakulic, S.; Deas, E.; Ouberaï, M.; Aprile, F. A.; Arranz, R.; Ness, S.; Roodveldt, C.; Guillems, T.; De-Genst, E. J., *et al.*, Structural Characterization of Toxic Oligomers That Are Kinetically Trapped During Alpha-Synuclein Fibril Formation. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E1994-E2003.
 54. Paslawski, W.; Mysling, S.; Thomsen, K.; Jorgensen, T. J. D.; Otzen, D. E., Co-Existence of Two Different Alpha-Synuclein Oligomers with Different Core Structures Determined by Hydrogen/Deuterium Exchange Mass Spectrometry. *Angew Chem Int Edit* **2014**, *53*, 7560-7563.
 55. Zijlstra, N.; Claessens, M. M. A. E.; Blum, C.; Subramaniam, V., Elucidating the Aggregation Number of Dopamine-Induced Alpha-Synuclein Oligomeric Assemblies. *Biophys. J.* **2014**, *106*, 440-446.
 56. Zijlstra, N.; Blum, C.; Segers-Nolten, I. M. J.; Claessens, M. M. A. E.; Subramaniam, V., Molecular Composition of Sub-Stoichiometrically Labeled α -Synuclein Oligomers Determined by Single-Molecule Photobleaching. *Angew Chem Int Edit* **2012**, *51*, 8821-8824.
-

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57. Giehm, L.; Svergun, D. I.; Otzen, D. E.; Vestergaard, B., Low-Resolution Structure of a Vesicle Disrupting Alpha-Synuclein Oligomer That Accumulates During Fibrillation. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 3246-3251.
58. Serpell, L. C.; Berriman, J.; Jakes, R.; Goedert, M.; Crowther, R. A., Fiber Diffraction of Synthetic Alpha-Synuclein Filaments Shows Amyloid-Like Cross-Beta Conformation. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 4897-4902.
59. Chen, M.; Margittai, M.; Chen, J.; Langen, R., Investigation of Alpha-Synuclein Fibril Structure by Site-Directed Spin Labeling. *J. Biol. Chem.* **2007**, *282*, 24970-24979.
60. Vilar, M.; Chou, H. T.; Luhrs, T.; Maji, S. K.; Riek-Loher, D.; Verel, R.; Manning, G.; Stahlberg, H.; Riek, R., The Fold of Alpha-Synuclein Fibrils. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 8637-8642.
61. Heise, H.; Hoyer, W.; Becker, S.; Andronesi, O. C.; Riedel, D.; Baldus, M., Molecular-Level Secondary Structure, Polymorphism, and Dynamics of Full-Length Alpha-Synuclein Fibrils Studied by Solid-State Nmr. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 15871-15876.
62. Gath, J.; Bousset, L.; Habenstein, B.; Melki, R.; Meier, B. H.; Bockmann, A., Yet Another Polymorph of Alpha-Synuclein: Solid-State Sequential Assignments. *Biomol Nmr Assign* **2014**, *8*, 395-404.
63. Aggeli, A.; Nyrkova, I. A.; Bell, M.; Harding, R.; Carrick, L.; McLeish, T. C. B.; Semenov, A. N.; Boden, N., Hierarchical Self-Assembly of Chiral Rod-Like Molecules as a Model for Peptide Beta-Sheet Tapes, Ribbons, Fibrils, and Fibers. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 11857-11862.
64. Tuttle, M. D.; Comellas, G.; Nieuwkoop, A. J.; Covell, D. J.; Berthold, D. A.; Kloepper, K. D.; Courtney, J. M.; Kim, J. K.; Barclay, A. M.; Kendall, A., *et al.*, Solid-State Nmr Structure of a Pathogenic Fibril of Full-Length Human [Alpha]-Synuclein. *Nat. Struct. Mol. Biol.* **2016**, *advance online publication*.
65. Adamcik, J.; Mezzenga, R., Adjustable Twisting Periodic Pitch of Amyloid Fibrils. *Soft Matter* **2011**, *7*, 5437-5443.
66. Sidhu, A.; Segers-Nolten, I.; Subramaniam, V., Solution Conditions Define Morphological Homogeneity of Alpha-Synuclein Fibrils. *Bba-Proteins Proteom* **2014**, *1844*, 2127-2134.
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CHAPTER 2

SELF-ASSEMBLY OF PROTEIN FIBRILS INTO SUPRAFIBRILLAR AGGREGATES: BRIDGING THE NANO- AND MESOSCALE

Abstract

We report on *in vitro* self-assembly of nanometer sized α -synuclein amyloid fibrils into well-defined micrometer sized suprafibrillar aggregates with sheet-like or cylindrical morphology depending on the ionic strength of the solution. The cylindrical suprafibrillar structures are heavily hydrated, suggesting swollen gel-like particles. In contrast to higher order structures formed by other negatively charged biopolymers, multivalent ions are not required for the suprafibrillar aggregates to form. Their formation is induced by both mono- and divalent counterions. The self-assembly process is not mediated by protein specific interactions but rather by the cooperative action of long-range electrostatic repulsion and short-range attraction. Understanding the mechanism driving the self-assembly might give us valuable insight into the pathological formation of fibrillar superstructures such as Lewy bodies and neurites - distinct signatures of Parkinson's disease - and will open the possibility to utilize the self-assembly process for the design of novel fibril-based smart nanostructured materials.

2.1 Introduction

The self-assembly of proteins into amyloids appears to be a generic phenomenon.¹⁻
² A common architectural motif of amyloid fibrils is the characteristic cross- β spine comprised of β -strands oriented perpendicularly to the longitudinal fibril axis. The connection of protein monomers *via* multiple hydrogen bonds parallel to the fibril's growth direction results in fibrils with diameters of 1-10 nm and lengths of >100 nm.³ Due to their architecture, amyloid fibrils are extremely robust against chemical destabilization and mechanical stress. Thus, once formed they remain intact and accumulate.

In tissues fibrils accumulate in suprafibrillar assemblies with dimensions up to tens of micrometers. Such suprafibrillar structures are a distinct signature of many neurodegenerative diseases.⁴ In the case of Parkinson's disease, fibrillar α -synuclein (α S) is deposited in suprafibrillar aggregates known as Lewy bodies and neurites.⁵ The pathological significance of these structures and formation mechanisms remain to be elucidated. Cell toxicity has been attributed to both prefibrillar α S aggregates (oligomers) and fibrillar α S but there is no general consensus on which species is most potent in disrupting the cell's normal functioning.⁶⁻⁷ It has been proposed that the formation of α S fibrillar inclusion bodies is a result of a cellular defense machinery responsible for the interception and sequestration of harmful protein aggregates within the cell through active retrograde transport.⁸ However, there is evidence that under certain conditions fibrils of some amyloidogenic proteins and peptides spontaneously assemble into higher order structures suggesting that a more generic physicochemical process could also be involved in the formation of intracellular fibril assemblies.⁹⁻¹²

Amyloid formation and higher order organization is not necessarily associated with a disease condition. Nature has utilized the advantageous features of amyloids to devise functional materials. For example, amyloid networks are used as a storage system for hormones in glands and other organs and as adhesives by barnacles or *E. coli* to deploy their colonies on different surfaces.¹³⁻¹⁵ Some fish and insect species exploit the chemical and mechanical stability of amyloid fibrils – comparable to those of silk and steel – by using them as a reinforcing scaffold protecting their eggs from damage.¹⁶⁻¹⁸ These and many more examples classify amyloids as an attractive candidate for the design of novel bio-inspired

materials. However, to profit from the advantageous properties of amyloids and to construct materials that are ordered at both the nano- and micro-scale a better understanding of the interactions driving the multi-scale self-assembly is required.

In many aspects – high charge density, morphological features and rigidity - α S fibrils resemble other filamentous biopolyelectrolytes such as DNA, F-actin and microtubules. The aforementioned biopolymers form higher order structures in the presence of multivalent counterions through various interaction mechanisms.¹⁹⁻²³ The attraction between rods with the same charge can be mediated through correlated movement of condensed counterions on the rods' charged surfaces resembling van der Waals interactions.²⁴⁻²⁷ It has even been suggested that the positions of condensed counterions on the rods' surfaces can become so strongly correlated that they adopt a Wigner crystal-like arrangement. The cohesive energy of this kind of crystal structure then acts as a source of the attractive interaction.²⁸⁻³¹ Experimental observations have indeed revealed counterion density fluctuations coupled to the twisted topography of the polyelectrolyte's charged surface giving rise to "zipper-like" charge alignment.³²

The self-assembly of α S into nano-sized amyloid fibrils has been studied in the past decades due to its relation with Parkinson's disease. However, the scope of those studies has rarely gone beyond the organization of the protein at the nano-scale.³³ Given the morphological similarity between α S amyloid fibrils and other self-organizing charged rod-like biopolymers we anticipate a higher-order organization of α S fibrils. To understand the self-assembly of fibrils into defined structures and to elucidate the forces driving this phenomenon we investigate the higher order organization of α S fibrils by systematic variation of physico-chemical parameters such as pH, temperature and ionic strength.

2.2 Materials and methods

2.2.1 Preparation and labelling of α S monomers

Expression of the human α S wild type (α SWT), the140C mutant (α S140C) with a single alanine to cysteine substitution at residue 140 and the truncated 1-108 (α S 1-108) mutant missing the last 32 amino acid residues from its original sequence were performed

in *E. coli* B121 (DE3) using the pT7-7 based expression system. Details on the purification procedure for α SWT and α S140C are described elsewhere.³⁴ The α S 1-108 was purified using the Resource S column (GE Healthcare Lifesciences, UK). The start buffer used was 50 mM glycine at pH 3.3. For the elution step the glycine buffer was supplemented with 1M NaCl. Purified protein was concentrated using Centriscart C4 centrifugal microconcentrators (Sigma-Aldrich, USA) with a 5kD cut-off prior to desalting. The desalting step was carried out using PD-10 columns (GE Healthcare Lifesciences, UK). α SWT and α S-A140C were conjugated with AlexaFluor 405 succinimidyl ester or AlexaFluor 647 maleimide (Life Technologies, USA) following the manufacturer's labelling protocols for both fluorescent probes.

2.2.2 Cylindrical suprafibrillar aggregates

If not mentioned otherwise, aggregations were sped up by shaking the solutions at 900 rpm. The cylindrical suprafibrillar aggregates were grown in 10 mM TRIS buffer, pH = 7.4, 37 °C, 100 μ M total protein concentration, and 2 mM CaCl_2 . For the α S 1-108 mutant no salt was added in the aggregation buffer. To study the effect of salt concentration on the formation of aggregates, the 10mM Tris buffer, pH=7.4, was supplemented with different concentrations of NaCl, KCl, CaCl_2 or MgCl_2 . To assess the aggregation state of the fibrils at different salt conditions phase contrast images were taken using a TE2000 microscope (Nikon, Japan) in transmission mode using a PlanFluor 60x Ph1 DLL objective (Nikon, Japan). To get insight into the time required for completion of aggregation, α SWT aggregates were grown in the presence of 5 μ M Thioflavin T (Fluka, Sigma-Aldrich, UK). The Thioflavin T fluorescence intensity at 485 nm was followed in time using Infinite 200 PRO multimode plate reader (Tecan Ltd., Switzerland). For the dual colored aggregates, aggregation was initiated using a mixture of 5000:1 of unlabeled α SWT: α SWT labelled with AlexaFluor 405 succinimidyl ester (α S-AI405). The mixture was then divided in aliquots and incubated at 37° and 900 rpm shaking. Monomers of α S 140C conjugated with AlexaFluor 647 maleimide (α S140C-AI647) were added at different time points to the different aliquots keeping the ratio α SWT: α S140C-AI647 at 5000: 1. The total (labelled and plain α SWT) protein concentration in the aliquots was 100 μ M. When stationary state of the self-assembly

process was reached the aliquots were imaged with using a Nikon Eclipse Ti microscope in confocal laser scanning mode. The AlexaFluor 405 and AlexaFluor 647 were excited using 402 nm (CUBE, Coherent Inc., USA) and 647 nm (2RU-VFL-P-300-647, MPB communications Inc., Canada) lasers respectively. The signal from the blue dye was collected using a 450/50 nm bandpass emission filter and from the red one using a 700/75 nm bandpass filter. To study the effect of electrostatic interaction on the growth of the suprafibrillar structures aliquots of pregrown blue aggregates (α SWT: α S-A1405 = 5000:1) were incubated with preformed red fibrils (α SWT: α S140C-A1647 = 5000:1) grown at 10 mM TRIS, pH=7.4, 0.1 mM CaCl_2 , and shaking with 900 rpm 37 °C. For the low salt conditions prior to the incubation with red fibrils, the pregrown blue aggregates were left at rest for 30 min to sediment and then the supernatant was withdrawn from the sample. The aggregates were re-suspended in 10 mM TRIS buffer. The procedure was repeated 3 times to remove the excess salt from the solution. For the higher salt conditions the concentration of CaCl_2 in the blue aggregate solution was set to 3 mM prior incubation with the preformed fibrils.

2.2.3 Disk-like aggregates

Aggregates with circular morphology were formed in 2 mM Citric acid, pH = 4.3, shaking with 900 rpm at 37 °C. Phase contrast images were taken using TE2000 microscope (Nikon, Japan) in transmission mode using a PlanFluor 20x Ph1 DLL objective (Nikon, Japan).

2.2.4 Temperature induced fibril clustering

100 μ M α SWT was aggregated in 10 mM TRIS buffer, pH = 7.4, 5 μ M Thioflavin T (ThT) 37 °C, shaking at 900 rpm and 10 mM NaCl for 5 days. Then aliquots of the solution were incubated at 60 °C and 80 °C for 3 days. Samples were imaged with a Nikon Ti Eclipse microscope in widefield fluorescence mode. ThT was excited using a mercury lamp (C-HGFIE Intensilight, Nikon) as a light source and the fluorescence signal was collected using 470/70 nm bandpass excitation filter, 495 nm dichroic beam splitter and 525/50 nm bandpass emission filter (49002 - ET – EGFP, Chroma, USA).

2.2.5 Atomic force microscopy

A solution of the suprafibrillar aggregates was deposited on a mica surface and left at rest for 20 min for the aggregates to sediment and adhere to the substrate. The sample was then gently washed with deionized water (MilliQ, Milipore Corp., USA) and dried with a nitrogen flow. Height and phase images were obtained using a Multimode 8 AFM (Bruker, USA) in tapping mode using a MSCT Si₃N₄ tip (Bruker AFM probes, USA).

2.3 Results and discussion

2.3.1 Formation of suprafibrillar aggregates

The formation of α S amyloid fibrils is induced by incubating the protein in buffered solution at 37 °C under constant agitation. The incubation time necessary to obtain the fibrils varies from hours to several days depending on aggregation conditions used such as pH, ionic strength, temperature, protein concentration and seeding. To follow fibril formation in time, α S aggregation is monitored using the amyloid binding dye Thioflavin T (ThT). The time resolved ThT fluorescence intensity trace attains the typical sigmoidal shape characteristic for the nucleation and growth mechanism accompanying the amyloid fibril

Figure 2.1 Supra-fibrillar α -synuclein (α S) aggregates. (A) Phase contrast and (B) fluorescence images of wild type α S (α S WT) cylindrical aggregates formed at 100 μ M protein concentration, 2 mM CaCl₂, 10 mM Tris buffer, pH = 7.4, 37 °C and in the presence of 5 μ M Thioflavin T. (C) Fluorescence and (D) phase contrast images of 1-108 truncated α S (α S 1-108) mutant formed at identical conditions but in a salt free solution (no CaCl₂ added). Scale bars are 100 μ m (A, B) and 20 μ m (C, D).

Figure 2.2 Length and width distribution of the α S WT SFAs. The aggregates formed at 100 μ M α S WT protein concentration, 2 mM CaCl_2 , 10 mM Tris buffer, pH = 7.4, 37 $^{\circ}\text{C}$ and in the presence of 5 μ M Thioflavin T.

formation (not shown here). Under the experimental conditions used in this study (see Materials and Methods section) the sample becomes turbid within 10 hours after the aggregation is initiated indicating the evolution of large structures in the solution. Visual inspection of the aggregated samples with optical microscopy surprisingly revealed the presence of entirely new species of protein aggregates. The sample is densely populated by protein assemblies with a cylindrical morphology and a fairly narrow size distribution (Figures 2.1 and 2.2). The high ThT fluorescence intensity of the self-assembled structures (Figure 2.1 B) indicates amyloid content. Atomic force (Figures 2.3 C, D) and high resolution scanning electron (not shown) micrographs confirm that the structures are indeed comprised of fibrillar α S. It also can be seen from the images that individual and clustered fibrils are still present in the sample indicating that not all of the fibrillar protein is incorporated in the self-assembled structures. 3D reconstructed confocal laser scanning microscope (CLSM) images of suprafibrillar aggregates grown with addition of fluorescently labelled α S140C monomers (see Material and Methods) at different time points during the self-assembly process show how the structures evolve in time (Figure 2.4). The observed layered patterns suggest that growth propagates radially from the central longitudinal axis of the suprafibrillar aggregates towards the periphery. Once dried on a mica surface the

structures collapse to sheets with thickness ~ 640 nm (Figure 2.3 B) and a general shape the same as that observed for the swollen structures in solution. This observation suggests that

Figure 2.3 Mesoscopic α -synuclein aggregates are fibrillar. (A) Tapping mode AFM micrograph of a dried large α S aggregate collapsed on mica. The overall shape of the tube agrees with the shapes observed in solution. The scale bar is $10\mu\text{m}$. (B) Cross sectional height profile of the aggregate taken along the dashed line shown in A. The dried aggregates are ≈ 640 nm high. (C) Amplitude image of the surface of the aggregate and (D) x8 magnification of part of the aggregate's surface. The large aggregates consist of fibrils. Scale bars are $10\mu\text{m}$ and $1\mu\text{m}$ respectively.

these structures are heavily hydrated. Calculations generate a rough estimate for a water content of $\geq 90\%$ (Chapter 4); a significant fraction of the aggregate's volume can be assigned to the solvent. This implies a gel-like architecture of the suprafibrillar aggregates.

2.3.2 Long-range electrostatic repulsion

The highly charged C-terminal part (Figure 2.5) of α S and compact structure of the cross β -sheet fibril core yields amyloid fibrils with a high surface charge density. Attractive

Figure 2.4 Pattern layers revealing the growth propagation direction of the supra-fibrillar aggregates. Addition of labelled α S monomers different in color than those used to initiate the aggregation, at different time points of the self -assembly process, allows following how the structures evolve in time. (A) The 3d image reconstruction (central panel), the longitudinal (1,2,3) and transverse (4,5,6) cross-sections of supra-fibrillar aggregates formed in aliquots to which the α S140C-A1647 monomers were added 15 hours after the aggregation was initiated. (B) Identical aliquot to which the monomers were added 25 hours after the aggregation was started.

interactions between like-charged semi-flexible polyelectrolytes have been observed in multiple systems. At sufficiently high concentrations of multivalent counterions, biopolyelectrolytes such as DNA, microtubules, F-actin, viruses and viral capsid particles are able to self-organize into higher order structures.¹⁹⁻²³ Similarly, α S suprafibrillar assemblies require sufficiently high salt concentrations to form. At low ionic strength the solution mainly contains individual α S fibrils as the inter-fibril repulsion is too strong. With increasing salt concentration electrostatic effects are gradually screened making it possible for the fibrils to come in proximity. As a result, increasing quantities of fibrillar sheet-like structures appear and the fraction of individual fibrils decreases (Figure 2.6). Further increments in the ionic strength gradually lead to the appearance of cylindrical aggregates coexisting with suprafibrillar sheets, and at even higher salt concentrations mainly the former species remains visible in the solution. The formation of the higher order fibrillar assemblies seems

Figure 2.5 The α S molecule. (A) The main domain regions in the sequence of wild type (WT) α S. At pH=7.4 α SWT has a net charge ~ -9 and ~ -12 localized on the C-terminal (acidic region). (B) α S 1-108 truncated mutant missing the last 32 amino acids residues from the original WT sequence. As a consequence the net charge on the molecule at physiological pH is $\sim +3$ and ~ 0 on the remaining C-terminal part. (C) Distribution of charged amino acid residues along the sequence of the protein.

to be invariant to the chemical nature of the counterions used to induce the self-assembly process. Despite the fact that α S has a divalent metal binding site and a calcium binding motif,³⁵⁻³⁷ there are no observable differences in the phase behavior in the presence of Ca^{2+} or Mg^{2+} . Even more surprisingly, and contrary to other biopolyelectrolyte systems, α S fibrils cluster into higher order structures irrespective of the valence of the counterions. Albeit at different concentrations, both the monovalent (NaCl and KCl) and divalent (CaCl_2 and MgCl_2) salts are equally successful in inducing the formation of higher order assemblies (Figures 2.6 E, D). The condensation of fibrils in the presence of monovalent counterions suggests that counterion condensation is not responsible, or at least not the only mechanism giving rise to an attractive inter-fibril potential driving the self-assembly phenomenon. This assumption is in agreement with theoretical predictions for the interactions of two parallel rod-like polyelectrolytes carrying the same charge. In the presence of monovalent salts the onset of attractive interactions between the rods due to correlated fluctuations of condensed counterions is at very short distances where the rods practically start to sterically hinder one another.²⁴ However, one cannot discard the option that counterion

Figure 2.6 Supra-fibrillar organization of α -synuclein as a function of salt concentration. (A) Scanning electron micrograph of fibrils in solution. (B-C) Light microscopy images of fibrils organized into fibrillar sheets or condensed into tube shaped supra-fibrillar structures. (D) Appearance of the different fibrillar phases as a function of α S, NaCl ($\blacktriangle$) and KCl ($*$) concentration. (E) Organization of fibrils as a function of the $CaCl_2$ ($\blacksquare$) and $MgCl_2$ ($\bullet$) concentrations. The colors in (D-E) refer to the structures shown in (A-C). At low ionic strength fibrils remain in solution (red) while at high ionic strength most fibrils are present in tube like aggregates. The intermediate regime (green) is characterized by the presence of sheet like structures (B) but tubes (C) are also present. The data shown for aggregation in $CaCl_2$ and $MgCl_2$ represent an average of 3 different protein aggregation experiments.

condensation contributes to the attractive potential between filaments in the presence of multivalent ions since the interactions in this case are more long-ranged. Given the high charge density at the fibril's surface it is expected that counterion condensation takes place at any salt condition. Therefore, correlated motions of condensed multivalent counterions

along the rods most likely play an important role in the aggregation of fibrils into higher order assemblies.

The independence of the fibril to suprafibrillar aggregate transition on the chemical nature of the counterions and the strong influence of the counterion valence on the transition point suggest that the high salt concentrations are mainly necessary to sufficiently screen electrostatics. This assertion is further confirmed in experiments in which the charge on the fibrils is modulated by pH or use of a α S deletion mutant lacking the charged C-terminal part (Figure 2.5 B). Amyloid fibrils of wild type α S do assemble into suprafibrillar aggregates in absence of salt as long as the pH is set close to the isoelectric point of the protein of 4.7. Increasing the pH to higher values leads to recovery of the negative charges and eventually the repulsion becomes too strong, forcing the preformed aggregates to disassemble into individual α S amyloid fibrils (Figure 2.7). The α S 1-108 truncation mutant (Figure 2.5 B) is slightly positively charged at neutral pH and still able to form amyloid fibrils.³⁸ Interestingly the α S 1-108 fibrils retain their ability to form higher order fibrillar

Figure 2.7 Disassembly of supra-fibrillar α S aggregates. (A) Phase contrast image of disk shaped fibrillar aggregates formed at 100 μ M α S, 2 mM citric acid, and pH 4.3. (B, C) Increasing the pH by the addition of NH_3 up to 70 mM charges the protein. The negative charge on the fibrils becomes overwhelmingly high and the aggregates fall apart to fibrils. (D) AFM images of the sample from C dried on a mica sheet. Scale bars are 100 μ m for A, B, and C and 20 μ m for D.

assemblies throughout the whole salt concentration range used and even in the absence of added salt (Figure 2.1 C). These findings further confirm that the formation of wild type α S suprafibrillar aggregates at low ionic strength is mainly prevented by the excessive interfibrillar electrostatic repulsion. Once the charge is sufficiently screened, short-ranged attraction takes over and drives the fibrils into the assembly. However, the persisting ability

of α S 1-108 to form suprafibrillar aggregates has another important implication. In agreement with what was previously postulated, it hints at the presence of attractive interactions originating from a different source than correlated movements of condensed counterions.

2.3.3 Finite size of the suprafibrillar aggregates

There is another important role in the self-assembly process that can be assigned to the electrostatic interactions. Electrostatic interactions seem to dictate the development of suprafibrillar aggregates into finite sized objects. The experimental findings suggest that the growth of the aggregates is not simply limited by depletion of monomers and fibrils from solution. Increasing the ionic strength of a solution of suprafibrillar aggregates and subsequent addition of pregrown fibrils results in formation of additional layers in the suprafibrillar structures (Figure 2.8 C). On the other hand, reduction of the ionic strength

Figure 2.8 Limited growth of supra-fibrillar aggregates. (A) Supra-fibrillar aggregates were initially grown with α S labeled with AlexaFluor 405 in 10 mM Tris, 2 mM CaCl_2 until the steady state of the self-assembly process has been reached. (B) Decreasing the salt concentration by washing the blue-labeled fibrillar aggregates with 10 mM Tris buffer and incubating the sample with pre-grown red-labeled fibrils does not induce the formation of additional layers, probably due to charge buildup. (C) Increasing the salt concentration up to 3 mM CaCl_2 and incubating fraction of the sample with pre-grown fibrils of α S140C mutant labeled with Alexa Fluor 647 leads to buildup of additional layers in the supra-fibrillar structures.

deteriorates the charge screening and the added fibrils do not condense on the aggregate's surface to form supplementary layers (Figure 2.8 B). These findings allow us to reason that the growth of the aggregates is probably contained by the electrostatic energy build up that cannot be compensated for with newly-established interfibrillar contacts. Such an interplay between long-range repulsion and short-range attraction is not uncommon and has been shown to stabilize aggregating colloidal systems giving rise to the formation of finite-sized aggregates.³⁹⁻⁴¹ Considering the long-range character of electrostatic interactions, the accumulation of charge can indeed result in build-up of excessive electric potential at the surface of the growing aggregates, preventing further addition of α S fibrils. Given the size of the aggregates ($d = 10 - 20 \mu\text{m}$) and assuming a homogeneous distribution of material throughout the aggregate's volume, the latter hypothesis looks unlikely but remains plausible. Another possible explanation of the observed phenomenon is the depletion of "free" counterions from the bulk. Due to their high surface charge densities, α S fibrils most probably concentrate (or even condense) a significant amount of counterions in their ionic atmosphere to sufficiently screen the electrostatic repulsion and consequently to cluster. The latter scenario can also be considered from a slightly different perspective where the growth of the suprafibrillar aggregates is not limited because of counterion depletion but rather because of the significant loss of counterion entropy. It is possible that at some point further cohesive contacts between fibrils cannot energetically compensate the entropy loss originating from the confinement of counterions in the vicinity of the fibril's surface.

2.3.4 Short-ranged attraction: hydrophobic interactions

The ability of α S 1-108 fibrils to assemble into suprafibrillar aggregates in the absence of salt and the formation of full length α S suprafibrillar structures in the presence of only monovalent salt implies the presence of another source of inter-fibril attraction than counterion condensation. The profound effect that temperature has on the fibril clustering suggests that the short-range attraction is most probably of entropic origin. Higher temperatures shift the transition point from fibrils to higher order structures towards lower salt concentrations. Aggregating 100 μM wild type α S solution with 10 mM CaCl_2 at 37°C results in the formation of a mixture of fibrils and very low number of small aggregates

(Figure 2.9 A). Incubating this solution at 60 °C enhances fibril clustering leading to a reduction in the free fibrils fraction and formation of bigger fibrillar aggregates (Figure 2.9 B, D). Incubating the fibrillar solution (formed at 10 mM CaCl₂ at 37°C) at 80 °C reinforces

Figure 2.9 Effect of temperature on filament condensation. The protein fibrils are stained using the β -sheet specific dye Thioflavin T (ThT). (A) At 37 °C, 100 μ M α S in a 10 mM NaCl and 10 mM Tris buffer at pH 7.4 aggregates into fibrils. The high ThT background fluorescence and the low number of small aggregates indicate that the predominant fraction of fibrils remains free in the solution. (B) Part of the α S solution shown in A is incubated at 60 °C. The amount of free fibrils in the solution is reduced and fibrils cluster and big supra-fibrillar structures emerge. (C) When the solution from A is incubated at 80 °C all the fibrils condense into even bigger supra-fibrillar structures and none remain free in the solution resulting in a background fluorescence as low as that for the control sample. (D) Average values of ThT background fluorescence signal for A, B and C originating from the amyloid fibrils free in the solution. The control sample is identical in composition to those in A, B and C but with only monomeric α S present. Scale bars are 100 μ m.

the effect observed in the 60 °C sample, and as a result all the available fibrils cluster (Figure 2.9 D) to form even bigger aggregates (Figure 2.9 C). The influence of temperature on the clustering of fibrils does not stem from possible temperature induced differences in fibril morphology. The enhancing effect of temperature on the fibril clustering is probably due to the presence of hydrophobic domains in the fibrils that are exposed to unfavorable contacts with the solvent. Such hydrophobic solvation is entropically penalized and thus higher temperature fosters a process that ultimately leads to shielding of the hydrophobic patches on the fibril surface from the surrounding polar medium. The presence of hydrophobic domains on α S fibrils would not be a surprise given the amino acid sequence of the protein and the architecture of the fibrils. The amino acid composition of the central region (NAC region) of the protein sequence is dominated by hydrophobic residues and is believed to be responsible for α S aggregation and fibril formation.⁴² Even though there is no general consensus on the subtle details regarding the exact architecture of the α S fibril, the general structural blueprint that emerges from all the studies concerns a fibril with hydrophobic core including the NAC region and solvent exposed C-terminal part.⁴³ It is possible that yet some surface from the hydrophobic core in the mature fibril remains solvent exposed or is being exposed upon compaction of the C-terminal segment when the charges are abolished at low pH or screened with salt.⁴⁴ Nevertheless, the strong promoting effect of temperature on fibril aggregation remains surprising. Besides reinforcing hydrophobic interactions, elevated temperatures influence charge screening. Considering the dependence of the Debye length on the temperature – the size of the double electric layer scales with $T^{1/2}$ – increasing the temperature should deteriorate charge screening, *i.e.* stabilize the fibrils against clustering. However, the hydrophobic effect seems to be the dominant one in the balance resulting in net increase of the interfibrillar attraction.

2.4 Conclusion

In summary we have shown that α S hierarchically self-assembles into finite sized suprafibrillar aggregates with well-defined morphology spanning the gap between nano- and microscale. The self-assembly process is a consequence of the interplay between long-ranged repulsive and short-ranged attractive interactions. The repulsive part of the

interactions stems from the high net negative charge on the α S fibrils. Additionally, the charge on the fibrils has a regulatory role in the growth process of the aggregates. Rather than only resulting from the correlated movement of condensed counterions, the attractive part of the interactions seems to be of entropic origin. Attraction resulting from counterion correlation cannot be excluded when multivalent ions are present in the aggregating solution. Finally it is important to point out that the observed self-assembly process is not only interesting from a materials science perspective. The interactions governing the fibril clustering may also have physiological implications. The *in vitro* generated suprafibrillar aggregates – no matter what morphology – start appearing at physiologically relevant protein concentrations and ionic strength. Within the brain α S represents 0.5-1% of the total protein content equating to approximately 20-40 μ M.⁴⁵ The magnesium levels within the cell are in the 800 μ M range, while the sodium and potassium levels are around 14 mM and 140 mM respectively. The total ionic strength in the cytosol is most likely in the range where the fibrils have an enhanced propensity to self-aggregate. Thus, once fibrils are formed *in vivo* they may be predisposed to cluster and generate suprafibrillar assemblies such as Lewy bodies and neurites.

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References

1. Chiti, F.; Webster, P.; Taddei, N.; Clark, A.; Stefani, M.; Ramponi, G.; Dobson, C. M., Designing Conditions for in Vitro Formation of Amyloid Protofilaments and Fibrils. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 3590-3594.
2. Chiti, F.; Dobson, C. M., Protein Misfolding, Functional Amyloid, and Human Disease. *Annu. Rev. Biochem.* **2006**, *75*, 333-366.
3. Sunde, M.; Serpell, L. C.; Bartlam, M.; Fraser, P. E.; Pepys, M. B.; Blake, C. C. F., Common Core Structure of Amyloid Fibrils by Synchrotron X-Ray Diffraction. *J. Mol. Biol.* **1997**, *273*, 729-739.
4. Forman, M. S.; Trojanowski, J. Q.; Lee, V. M. Y., Neurodegenerative Diseases: A Decade of Discoveries Paves the Way for Therapeutic Breakthroughs. *Nat Med* **2004**, *10*, 1055-1063.
5. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., Alpha-Synuclein in Lewy Bodies. *Nature* **1997**, *388*, 839-840.
6. Pieri, L.; Madiona, K.; Bousset, L.; Melki, R., Fibrillar Alpha-Synuclein and Huntingtin Exon 1 Assemblies Are Toxic to the Cells. *Biophys. J.* **2012**, *102*, 2894-2905.
7. Winner, B.; Jappelli, R.; Maji, S. K.; Desplats, P. A.; Boyer, L.; Aigner, S.; Hetzer, C.; Loher, T.; Vilar, M.; Campion, S., *et al.*, In Vivo Demonstration That Alpha-Synuclein Oligomers Are Toxic. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 4194-4199.
8. Kopito, R. R., Aggresomes, Inclusion Bodies and Protein Aggregation. *Trends Cell Biol* **2000**, *10*, 524-530.
9. Donald, A. M., Aggregation in Beta-Lactoglobulin. *Soft Matter* **2008**, *4*, 1147-1150.
10. Krebs, M. R. H.; MacPhee, C. E.; Miller, A. F.; Dunlop, L. E.; Dobson, C. M.; Donald, A. M., The Formation of Spherulites by Amyloid Fibrils of Bovine Insulin. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14420-14424.
11. Lara, C.; Adamcik, J.; Jordens, S.; Mezzenga, R., General Self-Assembly Mechanism Converting Hydrolyzed Globular Proteins into Giant Multistranded Amyloid Ribbons. *Biomacromolecules* **2011**, *12*, 1868-1875.
12. Ridgley, D. M.; Barone, J. R., Evolution of the Amyloid Fiber over Multiple Length Scales. *ACS Nano* **2012**, 1006-1015.
13. Barlow, D. E.; Dickinson, G. H.; Orihuela, B.; Kulp, J. L.; Rittschof, D.; Wahl, K. J., Characterization of the Adhesive Plaque of the Barnacle *Balanus Amphitrite*: Amyloid-Like Nanofibrils Are a Major Component. *Langmuir* **2010**, *26*, 6549-6556.

14. Chapman, M. R.; Robinson, L. S.; Pinkner, J. S.; Roth, R.; Heuser, J.; Hammar, M.; Normark, S.; Hultgren, S. J., Role of Escherichia Coli Curli Operons in Directing Amyloid Fiber Formation. *Science* **2002**, *295*, 851-855.
15. Maji, S. K.; Perrin, M. H.; Sawaya, M. R.; Jessberger, S.; Vadodaria, K.; Rissman, R. A.; Singru, P. S.; Nilsson, K. P. R.; Simon, R.; Schubert, D., *et al.*, Functional Amyloids as Natural Storage of Peptide Hormones in Pituitary Secretory Granules. *Science* **2009**, *325*, 328-332.
16. Iconomidou, V. A.; Vriend, G.; Hamodrakas, S. J., Amyloids Protect the Silkworm Oocyte and Embryo. *FEBS Lett.* **2000**, *479*, 141-145.
17. Knowles, T. P.; Fitzpatrick, A. W.; Meehan, S.; Mott, H. R.; Vendruscolo, M.; Dobson, C. M.; Welland, M. E., Role of Intermolecular Forces in Defining Material Properties of Protein Nanofibrils. *Science* **2007**, *318*, 1900-1903.
18. Podrabsky, J. E.; Carpenter, J. F.; Hand, S. C., Survival of Water Stress in Annual Fish Embryos: Dehydration Avoidance and Egg Envelope Amyloid Fibers. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **2001**, *280*, R123-R131.
19. Haynes, M.; Garrett, R.; Gratzer, W., Structure of Nucleic Acid-Poly Base Complexes. *Biochemistry* **1970**, *9*, 4410-4416.
20. Needleman, D. J.; Ojeda-Lopez, M. A.; Raviv, U.; Miller, H. P.; Wilson, L.; Safinya, C. R., Higher-Order Assembly of Microtubules by Counterions: From Hexagonal Bundles to Living Necklaces. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 16099-16103.
21. Tang, J. X.; Janmey, P. A., The Polyelectrolyte Nature of F-Actin and the Mechanism of Actin Bundle Formation. *J. Biol. Chem.* **1996**, *271*, 8556-8563.
22. Tang, J. X.; Wong, S.; Tran, P. T.; Janmey, P. A., Counterion Induced Bundle Formation of Rodlike Polyelectrolytes. *Berichte der Bunsengesellschaft für physikalische Chemie* **1996**, *100*, 796-806.
23. Wong, G. C. L.; Lin, A.; Tang, J. X.; Li, Y.; Janmey, P. A.; Safinya, C. R., Lamellar Phase of Stacked Two-Dimensional Rafts of Actin Filaments. *Phys. Rev. Lett.* **2003**, *91*.
24. Oosawa, F., Interaction between Parallel Rodlike Macroions. *Biopolymers* **1968**, *6*, 1633-1647.
25. Ha, B. Y.; Liu, A. J., Counterion-Mediated Attraction between Two Like-Charged Rods. *Phys. Rev. Lett.* **1997**, *79*, 1289-1292.
26. Ha, B. Y.; Liu, A. J., Effect of Non-Pairwise-Additive Interactions on Bundles of Rodlike Polyelectrolytes. *Phys. Rev. Lett.* **1998**, *81*, 1011-1014.

27. Podgornik, R.; Parsegian, V. A., Charge-Fluctuation Forces between Rodlike Polyelectrolytes: Pairwise Summability Reexamined. *Phys. Rev. Lett.* **1998**, *80*, 1560-1563.
 28. Gronbech-Jensen, N.; Mashl, R. J.; Bruinsma, R. F.; Gelbart, W. M., Counterion-Induced Attraction between Rigid Polyelectrolytes. *Phys. Rev. Lett.* **1997**, *78*, 2477-2480.
 29. Kornyshev, A. A.; Leikin, S., Electrostatic Zipper Motif for DNA Aggregation. *Phys. Rev. Lett.* **1999**, *82*, 4138-4141.
 30. Shklovskii, B. I., Wigner Crystal Model of Counterion Induced Bundle Formation of Rodlike Polyelectrolytes. *Phys. Rev. Lett.* **1999**, *82*, 3268-3271.
 31. Netz, R. R., Electrostatics of Counter-Ions at and between Planar Charged Walls: From Poisson-Boltzmann to the Strong-Coupling Theory. *European Physical Journal E* **2001**, *5*, 557-574.
 32. Angelini, T. E.; Liang, H.; Wriggers, W.; Wong, G. C. L., Like-Charge Attraction between Polyelectrolytes Induced by Counterion Charge Density Waves. *Proceedings of the National Academy of Sciences* **2003**, *100*, 8634-8637.
 33. Fauerbach, J. A.; Yushchenko, D. A.; Shahmoradian, S. H.; Chiu, W.; Jovin, T. M.; Jares-Erijman, E. A., Supramolecular Non-Amyloid Intermediates in the Early Stages of Alpha-Synuclein Aggregation. *Biophys. J.* **2012**, *102*, 1127-1136.
 34. Rooijen, B. D. v. Structural and Functional Insights into Interactions of Oligomeric Alpha-Synuclein with Lipid Membranes. PhD Thesis, University of Twente, Enschede, 2009.
 35. Binolfi, A.; Rasia, R. M.; Bertoncini, C. W.; Ceolin, M.; Zweckstetter, M.; Griesinger, C.; Jovin, T. M.; Fernandez, C. O., Interaction of Alpha-Synuclein with Divalent Metal Ions Reveals Key Differences: A Link between Structure, Binding Specificity and Fibrillation Enhancement. *J. Am. Chem. Soc.* **2006**, *128*, 9893-9901.
 36. Nielsen, M. S.; Vorum, H.; Lindersson, E.; Jensen, P. H., Ca²⁺ Binding to Alpha-Synuclein Regulates Ligand Binding and Oligomerization. *J. Biol. Chem.* **2001**, *276*, 22680-22684.
 37. Wright, J. A.; Brown, D. R., Alpha-Synuclein and Its Role in Metal Binding: Relevance to Parkinson's Disease. *J. Neurosci. Res.* **2008**, *86*, 496-503.
 38. Hoyer, W.; Cherny, D.; Subramaniam, V.; Jovin, T. M., Impact of the Acidic C-Terminal Region Comprising Amino Acids 109-140 on Alpha-Synuclein Aggregation in Vitro. *Biochemistry* **2004**, *43*, 16233-16242.
 39. Groenewold, J.; Kegel, W. K., Anomalously Large Equilibrium Clusters of Colloids. *J. Phys. Chem. B* **2001**, *105*, 11702-11709.
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40. Sciortino, F.; Mossa, S.; Zaccarelli, E.; Tartaglia, P., Equilibrium Cluster Phases and Low-Density Arrested Disordered States: The Role of Short-Range Attraction and Long-Range Repulsion. *Phys. Rev. Lett.* **2004**, *93*, 0557011-0557014.
41. Stradner, A.; Sedgwick, H.; Cardinaux, F.; Poon, W. C. K.; Egelhaaf, S. U.; Schurtenberger, P., Equilibrium Cluster Formation in Concentrated Protein Solutions and Colloids. *Nature* **2004**, *432*, 492-495.
42. Giasson, B. I.; Murray, I. V. J.; Trojanowski, J. Q.; Lee, V. M. Y., A Hydrophobic Stretch of 12 Amino Acid Residues in the Middle of Alpha-Synuclein Is Essential for Filament Assembly. *J. Biol. Chem.* **2001**, *276*, 2380-2386.
43. Vilar, M.; Chou, H. T.; Luhrs, T.; Maji, S. K.; Riek-Loher, D.; Verel, R.; Manning, G.; Stahlberg, H.; Riek, R., The Fold of Alpha-Synuclein Fibrils. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 8637-8642.
44. Trexler, A. J.; Rhoades, E., Single Molecule Characterization of Alpha-Synuclein in Aggregation-Prone States. *Biophys. J.* **2010**, *99*, 3048-3055.
45. Iwai, A.; Masliah, E.; Yoshimoto, M.; Ge, N. F.; Flanagan, L.; Desilva, H. A. R.; Kittel, A.; Saitoh, T., The Precursor Protein of Non-a-Beta Component of Alzheimers-Disease Amyloid Is a Presynaptic Protein of the Central-Nervous-System. *Neuron* **1995**, *14*, 467-475.

CHAPTER 3

ELECTROSTATIC INTERACTIONS CONTROL APPARENT ALPHA-SYNUCLEIN AGGREGATION KINETICS AND THE MORPHOLOGY OF ALPHA-SYNUCLEIN SUPRAFIBRILLAR ASSEMBLIES

Abstract

The protein α S forms suprafibrillar aggregates (SFAs) with well-defined morphologies in a range of physico-chemical conditions. In this study, we expand the previously explored range of experimental conditions and demonstrate that the higher order fibrillar assemblies can attain a wide range of different morphologies. Concomitant with the change in morphology the aggregation kinetics of α S changes in the presence of mono- and divalent counterions. Our findings imply sensitivity of the aggregation process towards the valence of ions in a different context than simple charge screening. At higher divalent counterion concentrations, a retardation in the kinetics is observed. We couple this finding to the formation of SFAs. The incorporation of α S fibrils into higher order structures probably affects processes such as fibril fragmentation, secondary nucleation and monomer recruitment which ultimately slow down the apparent aggregation kinetics. These effects prove decisive for the size of the SFAs formed in the presence of CaCl_2 .

3.1 Introduction

The aggregation of proteins is a physico-chemical phenomenon that is of great importance for many scientific disciplines such as chemistry, bionanotechnology and biology. Biology is perhaps the most prominent one since protein aggregation is a common denominator of several diseases such as Parkinson's disease, type II Diabetes mellitus and Amyloid Light-chain amyloidosis to name but a few.¹⁻² Despite being symptomatically and physiologically different these diseases share an important pathological feature - the self-assembly of a disease-specific protein into amyloid fibrils. The accumulation of these insoluble amyloid fibrils in tissue gives rise to abnormal proteinaceous deposits. Such deposits are the pathological feature used to diagnose the protein aggregation disorders. The name of the amyloid deposits varies with the disease, their morphology, and formation location. In the case of Parkinson's disease these structures are called Lewy bodies (LB) or Lewy neurites (LN) and their main constituent is fibrillar α -synuclein.³ Neither the role of these structures in the development of the disease nor the mechanisms governing their formation is known. In amyloid diseases research is currently centered around the early stages of the protein aggregation process. The significance of the more mesoscopic suprafibrillar aggregates is much less well investigated. This may be due to the fact that, in protein aggregation diseases, these aggregates have been considered inert or even protective deposits that result from the activity of the cell's defence system against misfolded proteins.⁴ Even though this scenario sounds plausible, it is not uncontroversial. Although α S is the major component of Lewy bodies, these inclusion bodies are heterogeneous in composition. The presence of many other proteins in Lewy bodies questions their inertness. Furthermore, amyloid deposits originate in many different locations. In Parkinson's disease they are encountered extra- and intra-cellularly. In other diseases, amyloid deposits appear as plaques in the extracellular space and in some cases even as intranuclear inclusions.⁵⁻⁷ These findings cannot be rationalized – at least not solely – by the active retrograde transport provided by the cell. Moreover, there is evidence that amyloidogenic proteins can form higher order fibrillar structures in the test tube once the appropriate conditions are provided. This renders a physico-chemical fibril deposit formation process likely.⁸⁻¹⁰

An interesting feature of the amyloid deposits formed *in vivo* is the span of observed deposit morphologies. Parkinson's disease alone is accompanied by the formation of multiple species of LB. The classical morphology of LBs is spherical with a dense core surrounded by a fibrillary halo.³ However, depending on the location of formation in the brain these inclusions also appear without the halo.¹¹⁻¹² The α S deposits that form outside of the cells have more amorphous appearance in comparison with those formed intracellularly.¹³ Recent studies in which the formation of intracellular inclusions was induced in a cell model system also revealed proteinaceous deposits with a wide spectrum of morphologies.¹⁴ Interestingly, suprafibrillar α S aggregates (SFAs) formed *in vitro* can also adopt different shapes.⁸ Depending on the salt concentration SFAs have either sheet-like or cylindrical morphologies. These different morphologies suggest that the formation process is very sensitive to the solvent conditions. The analogical polymorphic assembly into superstructures of the SFAs produced *in vitro* and their *in vivo* counterparts implies that the formation process *in vivo* may also be sensitive to the environmental conditions. Drawing this analogy may be valid as it is well known that physico-chemical conditions such as pH and ion species balance can differ within different parts of the organism, in the cytosol and in the extracellular matrix, and even within different compartments of the cell.

The self-assembly of amyloid fibrils into many morphologically different SFAs is also very exciting in the context of the increasing interest in the engineering of amyloid materials. Amyloid-based assemblies have been observed to be suitable materials for the synthesis of network scaffolds and microgel drug delivery vehicles.¹⁵⁻¹⁸ However, the flexibility of using such materials is somewhat hindered by the fact that they usually evolve into macroscopic gels. This problem was overcome in a smart approach using microfluidics which yielded well defined amyloid microgel particles.¹⁸ However, a more direct control over the assembly of amyloids into either macroscopic gels or finite particles with a well-defined morphology would increase the versatility of amyloid materials. To be able to design amyloid materials, a good understanding of the hierarchical self-assembly process is imperative.

In the past decades there has been much interest in studying the aggregation kinetics of amyloidogenic proteins. Kinetic data proved to be rich sources of information that gave

rise to a better mechanistic and molecular understanding of the aggregation process. The realization that amyloid fibrils self-assemble into higher order structures may have implication for their aggregation. Often, kinetics models for the aggregation of such systems completely neglect the formation of higher order structure and the possible consequences thereof. This approach is justified when the aggregation conditions do not overlap with the conditions required for the formation of higher order structures. When the formation of SFAs affects the overall aggregation kinetics their contribution and the kinetics of fibril formation have to be deconvolved to properly interpret the experimental results.

Here we map the morphological diversity of α S SFAs by systematically modulating the electrostatic interactions which play an essential role in the formation of these higher order amyloid structures. To change electrostatic interactions we use two complementary approaches and vary either the pH or the ionic strength. Additionally we use two α S variants, the WT protein and the 1-108 fragment of the sequence. The WT protein and the truncation mutant differ significantly in terms of total amount of negative charges in the sequence. The range of ionic strengths used in this study is significantly extended compared to what was previously reported, resulting in a large spectrum of morphologies. To assess if the sensitivity of the SFA self-assembly process towards the valence of the used counterions extends beyond simple electrostatic screening, the α S WT aggregation kinetics was systematically screened as a function of counterion species and ionic strength. The influence of the formation of SFAs on the dynamics of the aggregation process was determined by obtaining kinetic data.

3.2 Materials and methods

3.2.1 Formation of suprafibrillar aggregates.

Salt morphology map. 100 μ M α S was incubated in 10 mM TRIS (Sigma-Aldrich, USA), pH 7.4, 37 °C and different concentrations of NaCl (Sigma-Aldrich, USA) and CaCl_2 (I) were included. The aggregations were performed in 96 wells plate (Nunc, Thermo Fisher Scientific, USA) while shaking at 900 RPM for a week. Phase contrast images were obtained

with a TE2000 microscope (Nikon, Japan) in transmission mode using objectives, PlanFluor 20x Ph1 DLL (Nikon, Japan objective (Nikon, Japan), PlanFluor 40x Ph2 DLL.

pH morphology map. μ M α S was incubated in 10 mM TRIS, 37 °C at different pH levels. The pH was adjusted by the addition of citric acid (Sigma-Aldrich, USA) or ammonia (Sigma-Aldrich, USA). The aggregations were performed in 96 wells plate (Nunc, Thermo Scientific, USA) while shaking at 900 RPM. Images were obtained with a Nikon TE 2000 microscope in bright field mode using PlanFluor 20x Ph1 DLL (Nikon, Japan objective (Nikon, Japan) and PlanFluor 40x Ph2 DLL objectives.

3.2.2 Kinetic studies.

Aggregations. 100 μ M α S was incubated in 10 mM TRIS (Sigma-Aldrich, USA), 5 μ M Thioflavin T (Fluka, Sigma-Aldrich, UK.), pH 7.4, 37 °C and at different concentrations of NaCl (Sigma-Aldrich, USA) and CaCl_2 (Sigma-Aldrich, USA). The aggregations were performed in 384 wells plate (Nunc, Thermo Scientific) while shaking at 500 RPM. The increase of Thioflavin T fluorescence was monitored with a plate reader (Infinite 200 Pro, Tecan Ltd.). The dye was excited at 446 nm and the fluorescence signal was collected at 485 nm every 15 minutes. Slit sizes for the excitation and emission light were 5 and 20 nm respectively. The seeded aggregations were performed under identical conditions but in the presence of 5 mol% (of 100 μ M total α S concentration) of seeds.

Seeds. A solution of fibrils was prepared by incubating 100 μ M of α S in 10 mM Tris (Sigma), 2 mM NaCl (Sigma), pH=7.4, 37 °C and shaking at 900 RPM. Once the aggregation was completed, the fibril solution was sonicated (Branson, Sonifier 250) on ice. The solution was sonicated at the lowest power for 5 seconds and left at rest for another 55 second. The cycle was repeated five times. Subsequently, the sonicated samples were tested for seeding efficiency by incubation with α S monomers (data not shown).

Analysis of aggregation kinetics. The normalized sigmoidal aggregation kinetics curves were fitted using the logistic equation:

$$I = \frac{1}{1 + e^{-(x-x_0)/k}} \quad 3.1$$

where x is time, x_0 is the aggregation halftime and k is the apparent rate constant. The lag phase is approximated by $x_0 - 2/k$. The three curves for each experimental condition were globally fitted with x and x_0 being free fit parameters.

3.2.3 Atomic force microscopy.

Aliquots from the early stages of an aggregating sample (6-8 hours after start) in 10 mM TRIS, 2.5 mM CaCl_2 , pH7.4, at 37°C and shaking, were placed on mica surfaces. The samples were left at rest for 10 min in ambient conditions. After 10 min the samples were washed with demineralized water and dried with nitrogen. Phase images were obtained using a Multimode 8 AFM (Bruker, USA) in tapping mode with a Si_3N_4 tip (Bruker AFM probes, USA) with expected resonance frequency 85-155 kHz and spring constant 0.5 Nm^{-1} .

3.2.4 Labelling of αS with fluorescent dyes.

To label αS , mutants with cysteine point mutations at positions 18, 25, 35, 41, 56 and 140 were used. The cysteines at positions 18, 25, 35, 41, 56 were labelled with Alexa Fluor 568 maleimide (Lifesciences, USA) and at position 140 with Alexa Fluor 488 maleimide (Lifesciences). First, the αS cysteine mutant was reduced with 2 mM DTT. After an hour, the DTT was removed with a desalting column (Zeba, Thermo Fisher Scientific, and USA). The sample was eluted by spinning it down at 1000 G for 2 min. A 10 fold molar excess of the Alexa Fluor maleimide dye was added to the sample and left to react for 2 hours in dark and shaking. The unreacted dye was removed from the sample with a desalting column (Zeba, Thermofisher Scientific) by spinning it down at 1000 G for 2 min. The procedure was repeated 3 times. The labelling efficiency was estimated by measuring the absorbance (UV 2401 PC, Shimadzu) at 276 nm (αS) and at 575 nm (Alexa Fluor 568) or at 493 nm (Alexa Fluor 488). Labelling efficiency was $\geq 90\%$ for all the labelled mutants.

3.2.5 Proteinase K digestion assay.

Fibrils were grown by incubating 100 μM αS with 10 mM Tris (Sigma), 10 mM NaCl (Sigma), 5 mM seeds, pH=7.4, 37 °C and in quiescent conditions for 3 weeks. The wild type αS was mixed with (1 mol% from the total αS concentration) αS 140C-A1488 and (2 mol%)

α S XC-A1568 (X = 18, 25, 35, 41 or 56). After the aggregation was complete, samples were diluted 10 times and incubated with 0.5 mg/ml proteinase K (Thermo Fisher Scientific, USA) in a microscopy chamber and monitored in TIRF mode on a Nikon Ti-E setup coupled with an Argon laser (35-IMA-040, Melles Griot, USA). Images were acquired with a CFI Apo TIRF 100x objective (Nikon, Japan) and iXon 3 DU-897 EMCCD camera (Andor, UK) using the 488 nm line of the laser for the excitation of the Alexa Fluor 488 and the 515 nm line for the excitation of the Alexa fluor 568. For the Alexa Fluor 488 signal a filter cube with 480 nm excitation filter and 10 nm bandpass, 488 nm dichroic mirror and 520 nm emission filter with 30 nm bandpass. For the Alexa Fluor 568, a filter cube with 514 nm excitation filter and 10 nm bandpass, 514 nm dichroic and 525 long pass emission filter.

3.3 Results and discussion

3.3.1 Morphology of SFAs as a function of salt concentration

To evaluate the effect of electrostatic interactions (charge) on the morphology of suprafibrillar aggregates (SFAs) we first modulate the interfibrillar electrostatic interactions by changing the salt concentration to effectively alter charge screening. We do that for two alpha-synuclein (α S) variants: the wildtype protein (WT) and a truncated version (α S 1-108) lacking the last 32 amino acid residues. With this truncation a large part of the negatively charged C-terminal residues are removed from the protein sequence. Focusing on the WT protein we notice that the first fibrillar sheet-like aggregates emerge at 300 μ M CaCl_2 . This concentration marks the onset of salt conditions that promote fibril clustering (Figure 3.1). This transition point is in good agreement with previous findings (chapter 2). A comparison of the presented data with the phase behaviour of α S in the presence of NaCl (chapter 2) shows that the transition to fibril clustering is not a simple consequence of a more efficient charge screening provided by the divalent calcium counterions. Translating the transition points to ionic strengths shows that in the presence of NaCl the first fibril clusters appear at 10 mM while for CaCl_2 clustering starts at $\approx$ 1 mM ionic strength. This finding implies that in addition to the interactions that cause fibril clustering in monovalent salts, there must be other sources of interactions between fibrils present when multivalent ions are used to induce α S aggregation.

Besides the salt specific effect on the onset of fibril clustering, electrostatic interaction also have a profound effect on the shape of the SFAs. Increasing the concentration of CaCl_2 leads to a transition in the morphology of the suprafibrillar structures. While a sheet-like morphology is observed at low CaCl_2 concentrations the SFAs obtain a more cylindrical geometry at higher salt concentrations. The shape-shifting behaviour continues with the increase in salt concentration. Besides the morphology, the overall size of the aggregates is strongly affected by the changing conditions. SFAs become smaller and smaller with increasing ionic strength. At very high salt concentrations the fibrils fail to assemble into well-defined structures and aggregate in amorphous fibrillar flocks. This mode of assembly is reminiscent of macroscopic phase separation.

To obtain more insight into the effect of electrostatic interactions on the morphology of the SFAs, the role of the heavily negatively charged C-terminal region was investigated by additionally mapping the phase behavior and kinetics of the αS 1-108 deletion mutant. From the aggregation kinetics of αS 1-108 it is evident that the absence of the highly charged C-terminal residues has an enormous effect on the aggregation behaviour of the protein. The lag phase is significantly shorter and aggregation rate much higher for the truncated version of the protein compared to its wild type counterpart at the same physico-chemical conditions.¹⁹ The electrostatic interactions that govern SFA formation also act at the monomeric level and therefore affect the aggregation of the monomers into amyloid fibrils. Because the electrostatic repulsion between fibrils is significantly reduced in αS 1-108 monomers and fibrils, the protein readily aggregates and the fibrils agglomerate into higher order structures even in the absence of salt (Figure 3.3). The shorter C-terminal region most likely not only affects electrostatic interactions, there may be an additional effect that facilitates the clustering of fibrils into SFAs. Besides omitting several charged amino acid residues, the truncation probably increases the exposure of the core of the fibril which consists predominantly of hydrophobic amino acid residues (Chapter 5).^{8, 20-21} Thus, with truncation of the C-terminal region we may not only decrease inter monomer and fibril repulsion but also increase the attraction between fibrils. However although the charges on the protein molecule are significantly reduced, the morphology of the resulting SFAs is still sensitive to changes CaCl_2 concentration (Figure 3.1). It is surprising that the small

Figure 3.1 Salt-morphology map of the WT and α S 1-108 SFAs formed at different CaCl_2 concentrations. The bottom part of the graph corresponds to the morphologies as function of salt concentration observed for the wild type alpha synuclein (α S WT). Below 100 μM CaCl_2 , the aggregation was either very slow or did not occur at the experimental time scale. Aggregates above 300 mM CaCl_2 , did not show defined morphology but were often positive for Thioflavin T fluorescence signal, indicating cross-beta sheet content. The top part of the graph shows morphologies observed for the truncation mutant α S 1-108. Because it lacks a large part of the C-terminal region (negative charge carrier) the mutant aggregated throughout the whole range of used salt concentrations. Yet, the morphology is influenced by the ionic strength. The boundaries beyond which SFAs do not form are with limited accuracy because of the low 'salt concentration resolution' set by the number of salt concentrations used.

remainder of the C-terminus still imposes such a dramatic impact on the final SFA morphology. This finding can possibly be interpreted as an indication that the other charge carrier in the protein sequence, the N-terminal region, also influences the final SFA morphology. To verify this claim we need to consider the architecture of the fibril. It has

been established that the C-terminal region shields the hydrophobic core of the fibril and the whole stretch of the last 45 amino acids is exposed to the solvent.²¹⁻²² Reports also show that N-terminal region – or at least part of it – is solvent exposed.²² On the other hand the N-terminal region has been reported to contribute strands to the cross β -sheet backbone of the fibril. The number of amino acids in the N-terminal region that contribute to the fibril backbone varies within reports and in some cases it is suggested that the whole N-terminal region is incorporated in the core.^{21, 23-25} It is therefore not clear which part of the N-terminus is located outside the core and how much of it remains inside. This makes it difficult to judge if the fraction of the first 60 amino acids that protrudes from the core (if it does at all) is sufficient to affect interfibril interaction. To resolve which part of the N-terminal region is solvent exposed we have used a proteinase K digestion assay on fibrils. The fibrils were fluorescently labelled using α S variants containing a cysteine mutation at different positions along the N-terminal region. The β -sheet backbone of the fibril is resistant to proteinase activity and shields all the amino acids incorporated in the core from cleavage. By introducing fluorescent labels at engineered cysteine residues and tracing their susceptibility to proteinase digestion we were able to approximate which part of the N-terminus is solvent exposed (Figure 3.2). The N-terminus seems to be vulnerable to proteinase attack up to residue 25 Gly and resistant after residue 35 Glu. This implies that at least 25 residues are radiating out of the core and we therefore conclude that the N-terminus probably plays a role in modulating the interfibrillar interaction in the case of α S 1-108 fibrils at low ionic strengths. Because of the amphoteric nature of the N-terminal region, this part of the sequence might provide inter-fibrillar interactions similar to those observed between neurofilaments where polyelectrolyte arms mediate the attractive interaction among the filaments.²⁶⁻²⁷ Such a scenario would provide a rationale why we observe significant changes in morphology of the α S 1-108 SFAs with increasing salt, despite the absence of most of the negatively charged C-terminal region. At low ionic strengths such type of attraction will act complementary to the hydrophobic interaction mentioned earlier. Increasing the ionic strength will gradually screen the electrostatic interactions and eventually hydrophobic forces will dominate the inter-fibril interaction.

Figure 3.2 Proteinase K digestion assay for fibrils doped with different α S cysteine mutants (α SXC-AIY) labelled with an Alexa fluorophore. Green coded images (488 nm) correspond to the signal coming from the α S140C-AI488. Yellow color codes for the signal from the mutant α S labelled at different positions in the N-terminal region (α SXC-AI568) with Alexa Fluor 568 (568 nm). Scale bar is 5 μ m. (Inset) Thioflavin T channel showing that fibrils are still intact despite the cleavage of segments from the N- and C-terminal regions. Positions 18 and 25 are susceptible to cleavage indicating that at least 25 amino acids for the N-terminus are solvent exposed and thus, protruding out of the fibril core. The rest of the N-terminal residues seem to be buried inside the core and protected from enzymatic digestion.

3.3.2 Morphology of SFAs as a function of pH

The importance of the electrostatic interactions in the formation of the SFAs was additionally investigated by modulating the charges on the protein molecule by changing the pH. In the absence of additional salt, the α SWT SFAs form only in pH regimes in the vicinity of the isoelectric point of the protein molecule (Figure 3.3). At very low pH, almost all of the side chain carboxylic groups of Glu residues are protonated and the protein acquires a significant positive net charge. These positively charged residues are located exclusively in the first 60 amino acids of the sequence. With the charge accumulation it becomes a serious obstacle for the N-terminal regions (the part that participates in the cross β -sheets) to pack inside the core of the fibril and fibril formation is thus prevented. Once the pH is sufficiently increased the excess positive charge is counterbalanced by the newly generated negatively charges. The α S molecule attains a lower net positive charge which at some point allows it to aggregate into amyloid fibrils and subsequently form SFAs (Figure 3.3). In a similar fashion as was observed with increasing salt concentration, the morphology of the SFAs changes with increasing pH. Interestingly, the observed transformation is even more dramatic with the morphology going through elongated cup shapes, disc-like aggregates and cups with onion-like layering (Figure 3.3). This abrupt shape shifting with changing pH emphasizes the prominent role of the electrostatic interactions in the self-assembly process. At higher pH values the C-terminus becomes charged and the α S molecule starts attaining a net negative charge which at some point hinders the formation of SFAs although individual fibrils were able to form. At even higher pH levels more side chain carboxylic and amino groups are deprotonated which increases the net negative charge to an extent that even renders the α S monomers stable against aggregation into fibrils (at least on the time scale of the experiments).

The morphology of the α S 1-108 SFAs is also sensitive to the pH. Similar to its WT counterpart, the α S 1-108 fails to aggregate at low pH because of the excess positive charge on the N-terminal region of the molecule. Compared to WT, the first fibrils and SFAs with the truncated protein appear at relatively high pH. This is attributed to the lacking C-terminal region which otherwise provides the negative charges balancing the net charge of the molecule. The morphology of the 1-108 SFAs again changes with increasing pH and again

Figure 3.3 pH-morphology map of WT and α S 1-108 SFAs formed at different pH levels. (Bottom part) α S WT forms aggregates in a finite pH range within which the morphology of the SFAs is sensitive to the protein net charge. At low and high pH, the net positive and negative charge respectively, is very high and prevents either the formation of SFAs or even of fibrils. (Top part) pH morphology map for α S 1-108. The formation of SFAs and fibrils is only suppressed in the low pH range as the charge balance is shifted because the mutant lacks a large part of the negatively charged C-terminal region. The N-terminal region contains numerous amine groups. With increasing pH, carboxylic groups in the N-terminal region and in the residual part of the C-terminal region start to deprotonate and balance the positive charges. Above a threshold pH, fibrils and SFA aggregates with different morphology start to form. The purple, green, red and blue curves represent the calculated net charge as function of pH for the α S WT (—), α S 1-108 (—), N-terminus (—) and C-terminus (—) respectively. The boundaries beyond which SFAs do not form are with limited accuracy because of the low ‘pH resolution’ set by the number of pH conditions probed.

in contrast to the WT the SFAs keep on forming throughout the pH range used simply because the net charge of the molecule maintains moderate values.

3.3.3 SFAs and their influence on aggregation kinetics

The plethora of SFA morphologies that could be generated unambiguously shows that electrostatics play a crucial role in the formation of higher order fibrillar structures. The electrostatic interactions probably play a role at multiple levels in the self-assembly. The charge distribution on the fibril's surface most probably steers the packing and arrangement of the fibrils inside the higher order structure and by that it inherently affects the overall morphology of the SFAs. Besides SFA morphology, the kinetics of the aggregation of monomers into fibrils is also known to be extremely sensitive to the physiochemical conditions and specifically to the presence of ions. This indicates that also at this level electrostatics interactions are important. The convolution of multiple electrostatic effects probably modulates the observed self-assembly into a wide range of different morphologies. To gain further understanding of the complex role of electrostatics in α S aggregation and SFA formation we focus on the salt dependent SFA morphology of the WT protein. From the difference in the onset concentration of SFA formation in mono- and divalent salt we already concluded that multivalent counterions might contribute more to the self-assembly process (with the WT protein) than simple charge screening. This effect is also visible in the sensitivity of the aggregation kinetics to the valence of the counter ions. At comparable ionic strengths the aggregation kinetics significantly differs between counterions of different valence (Figure 3.4 A, B). This is especially apparent at low ionic strengths where the aggregation kinetics speeds up much faster as a function of ionic strength in the presence of Ca^{2+} . As a result all kinetic curves describing the aggregation of α S in the presence of Ca^{2+} shift faster to shorter time scales as compared to aggregations at the same ionic strength but in the presence of monovalent Na^+ (Figure 3.4 A,B). This indicates that the first signatures of amyloid formation, detected in a ThT assay, appear at much earlier times (shorter lag phase) in the presence of Ca^{2+} . To quantify the difference in aggregation kinetics two characteristic parameters, the apparent rate constant k^i where i denotes the type of counterion and aggregation half-time x_o^i were determined. At low ionic

Figure 3.4 Aggregation kinetics in the presence of NaCl and CaCl_2 . (A) Thioflavin T aggregation curves for NaCl and (B) CaCl_2 . In both A and B circles are used for the conditions at which kinetics speed up and the crosses are used for the conditions at which the kinetics start to slow down with increasing ionic strength. (C) The apparent rate constant of aggregation k and aggregation half time x_0 obtained from the fits for the aggregation curves shown in A and B. Blue lines represent the Debye length (κ^{-1}) as a function of ionic strength to guide the eye. Squares (■) and crosses (×) depict parameters obtained from aggregations performed in the presence of CaCl_2 and NaCl respectively. Each aggregation is performed in a triplicate and the kinetic parameters are obtained from global fits to these triplicates.

strengths both the rate constant and half-time for Ca^{2+} induced aggregations significantly differ in value compared to the values characterizing the Na^+ facilitated aggregation. The $k^{\text{Ca}^{2+}}$ is much higher than $k^{\text{Ca}^{2+}}$ with the maximal difference approaching an order of magnitude at 7.5 mM ionic strength. The same is observed for the $x_0^{\text{Ca}^{2+}}$ and $x_0^{\text{Na}^+}$ where the former is almost an order of magnitude lower. We therefore conclude that the difference between mono- and divalent cations is visible at all length scales. An extra attractive interaction between monomer in solution and in the fibril possibly accounts for this observation. At first, the mechanism of ion bridging seems to be a good candidate for explaining the experimental results. Bridging of divalent ions (especially calcium) by ionized carboxylic groups plays an important role in interactions of biopolymers. A classic example is the case of charged polysaccharides where divalent cations (especially Ca^{2+}) bridge between the ionized carboxylic groups and effectively cross-link neighboring polysaccharide chains.²⁸⁻³⁰ Intermolecular interactions mediated through bridging multivalent ions have also been observed in proteins (Chapter 6).³¹⁻³⁴ Another possible mechanism that could provide an extra source of attraction is counterion condensation and subsequent intramolecular movement correlations of the condensed multivalent counter ions.³⁵⁻³⁷ As already mentioned the C-terminal region of αS is highly charged and once monomers are incorporated in a fibril the charge densities at the surface of the fibril will become even larger. Thus, the prerequisite for ion condensation to take place is met. Since both proposed mechanisms operate on the inter-monomer and the inter-fibril level the kinetic data - which reflects interactions between monomers – can be used to infer information on interactions between fibrils. However it is difficult to pinpoint which of the two mechanisms is responsible for the observed experimental outcomes. Considering the length scales associated with the two processes may help to tip the balance in favour of one of them. In the case of ion bridges the interactions take place on the Ångstrom scale. With pectin for example, the distance between two adjacent polysaccharide chains that are cross-linked with ions is 5-6 Å.³⁸ On the other hand, the onset of attractive interactions between like charged rods generated by ion correlations is expected at around 2.8 nm in the case of divalent ions.³⁵ When we compare these two length scales to the Debye length (κ^{-1}) of 3.6

nm corresponding to the ionic strength of the peak of $k^{Ca^{2+}}$ the more long ranged mechanism appears a more probable candidate to explain the experimental findings.

Another interesting feature in the aggregation kinetics is the non-monotonic character of the changes in k^i and x_o^i . This is especially obvious for the aggregation series in the presence of $CaCl_2$. Initially the apparent rate constant $k^{Ca^{2+}}$ seems to grow with the increasing ionic strength but after peaking at 7.5 mM it decreases (Figure 3.4). To some extent a non-monotonic behaviour is also observed for the half time $x_o^{Ca^{2+}}$, though the changes at higher ionic strength are not as dramatic. Interestingly, the NaCl series fail to replicate the non-monotonic behaviour of k^i or at least it is much less pronounced for the ionic strength range that was probed. It is unlikely that non-monotonicity is a consequence of a salting-out effect (Hofmeister series) as this usually takes place at much higher salt concentrations. Moreover, given the positions of Ca^{2+} and Na^+ in the cationic chaotropic series NaCl should be more effective in salting out (aggregating) the protein than $CaCl_2$ which is opposite to what is seen in the experiments. Charge inversion is another process that should be considered to account for the changes in the kinetics in the presence of divalent ions. Charge inversion is a somewhat counterintuitive phenomenon in which at the right conditions highly charged species can bind an excess of counterions leading to the sign change of its net charge. It has been theoretically predicted in the early 20th century and was later experimentally supported by showing that colloids can invert their charge in solution containing excess counterions.³⁹⁻⁴¹ Charge inversion has also been observed for highly charged biopolyelectrolytes such as DNA and proteins.⁴¹⁻⁴³ Even though the theoretical treatment of the charge inversion problem permits the phenomenon to occur in the presence of monovalent ions (given high enough salt concentrations) it has been experimentally shown that changes in the sign of the net charge of proteins usually occurs in the presence of multivalent ions and monovalent ions do not suffice⁴³⁻⁴⁴ Thus, charge inversion may account for the non-monotonic change of the rate constant $k^{Ca^{2+}}$ and the different behaviour of k^{Na^+} with changing ionic strength. However, if charge inversion is the sole mechanism governing the aggregation kinetics then a similar non-monotonicity should be observed for $x_o^{Ca^{2+}}$. The non-monotonic behaviour of the half time is however much

more subtle and surprisingly also observed at higher ionic strength in the aggregation series in the presence of NaCl which shouldn't be the case. Also, the phase behaviour of the fibrils does not completely fit in the charge inversion scenario. We have shown earlier that above a threshold concentration of ≈ 2 mM CaCl_2 αS organizes into SFAs. The formation of SFAs at this concentration does not agree with the increase in electrostatic repulsion that accompanies charge inversion at elevated salt levels. For some proteins, the overcharging resulting from charge inversion was so large that it resulted in re-entrant behaviour of the aggregating system and re-solubilisation of the formed proteinaceous aggregates.⁴²

Although the formation of SFAs does not agree with the overcharging hypothesis, SFA formation itself perhaps accounts for the observed kinetic behavior. It would be rather surprising if the appearance of SFAs did not affect the aggregation kinetics. In our analysis we have significantly simplified the description of the aggregation process and reduced it to two parameters, k^i and x^i_o . In reality, the aggregation process is much more complex. The apparent rate constant k^i used here is basically a convolution of several sub-processes that occur simultaneously: i) primary nucleation; ii) addition of monomers to fibrils; iii) secondary nucleation; iv) fibril breakage⁴⁵⁻⁴⁶, where each of these sub-processes is characterized by its own rate constant and contributes to the apparent aggregation kinetics. Looking at the SFAs in this perspective at least 3 of the above mentioned processes are expected to be affected. In the SFAs the breaking of fibrils will for example, be significantly suppressed as they are reinforced by the surrounding matrix of the adjacent fibrils. Additionally the numerous interfibrillar contacts probably decrease the accessible fibril surface area at which secondary nucleation effects can take place. SFA formation thereby also effectively suppresses secondary nucleation. Not only less surface is accessible in SFAs. The growing ends of many fibrils will eventually become buried inside the aggregate. To elongate these fibrils monomeric αS will have to diffuse into the SFA which will probably result in a retardation of the addition rate. All the above would inevitably lead to an overall slowdown of the aggregation kinetics. Interestingly the starting point of the kinetics slowdown in our experiments seem to approximately coincide with the ionic strength at which we register the first occurrence of SFAs with cylindrical morphology (Figure 3.1). This holds for both the NaCl and CaCl_2 series. The idea that retardation in kinetics is caused by

the formation of SFAs is further supported by the kinetic data generated from aggregations seeded by preformed fibrils at comparable salt conditions. It is clearly visible that the seeding efficiency of the preformed fibril is significantly diminished with increasing ionic strength (Figure 3.5). The potency of the seeds seems unperturbed at low ionic strengths and gradually starts to deteriorate with increasing salt concentration. At high enough ionic strength the seeding is completely inhibited and the aggregation starts the conventional way with primary nucleation events. The full seeding suppression is nicely portrayed by the complete overlap between the seeded and non-seeded aggregation curves (Figure 3.6). The suppression of seeding is induced at elevated levels of both types of counterions, Na^+ and Ca^{2+} . This indicates that charge inversion is indeed an unlikely reason (or the least not the main one) for the retardation in the aggregation kinetics.

The formation of SFAs and aggregation kinetics are tightly connected. The appearance of SFAs results in aggregation kinetics that is significantly different from the kinetics observed in the absence of SFAs. However, the aggregation kinetics may also feed back into the morphology of the SFAs. The morphology of the α S WT SFAs formed in the presence of Ca^{2+} significantly changes with increasing ionic strength. Besides the shape, the overall size of the resulting SFAs seems to alter (Figure 3.1, Figure 3.6). With increasing CaCl_2 concentration the SFAs size distribution shifts towards smaller length scales. The changes in $x_o^{\text{Ca}^{2+}}$ seem to correlate with the size of the aggregates. With increasing ionic strength $x_o^{\text{Ca}^{2+}}$ decreases. It apparently becomes easier to nucleate the growth of new fibrils. Intuitively, decrease in size of the SFAs with increasing CaCl_2 concentration is thus attributed to the formation of more growth nuclei and limited amount of available material. However, previous experiments have shown that the size of SFAs is not simply limited by depletion of monomers and fibrils from solution. Instead, SFA size is electrostatically contained, at some point the formation of additional cohesive contacts between fibrils cannot energetically compensate for the entropy loss originating from the confinement of counter-ions in the vicinity of the fibril surface.⁴⁶

The relation between the size of the aggregates and $k^{\text{Ca}^{2+}}$ is less clear. Whereas $k^{\text{Ca}^{2+}}$ goes through a maximum the size of the aggregates continuously decreases with

Figure 3.5 Aggregation kinetics of seeded and non-seeded solutions of α S monomers in presence of mono- and divalent counterions. Seeded aggregations are depicted in red and the non-seeded are in black. For all aggregations the concentration of α S was 100 μ M. In the presence of NaCl (left column) and CaCl_2 (right column) a systematic decrease in the seeding efficiency with increasing ionic strength is observed. The retardation in kinetics is indicative for fibril (seed) clustering which interferes with the seeding efficiency. Experiments are performed and shown in triplicates for each condition.

Figure 3.6 Size distribution of SFAs and dependence on aggregation kinetics. (A) Size distributions of the SFAs at different CaCl_2 concentrations. The counts in the histogram are normalized to the total number of SFAs for each condition. (B) Apparent rate constant as a function of the lag phase (LF) for different salt levels. The lag phase was determined from logistic fits to the data presented in Figure 3.4 and is estimated using $x_0 - 2/k$.

increasing CaCl_2 concentration. As discussed above the slowdown in k with CaCl_2 concentration follows from enhanced fibril clustering and the resulting decrease in secondary nucleation and monomer addition rate in the clusters. Since we do not observe a clear correlation and excluded kinetic arguments for the limited size of the aggregates the observed kinetics cannot be directly responsible for the observed size dependence. However, at higher ionic strength the SFAs may become more densely packed with fibrils. With this in mind we therefore argue that the decrease in size with increase in ionic strength results from denser packing of fibrils in the aggregates formed at higher CaCl_2 concentrations.

3.4 Conclusion

In summary αS aggregation and SFAs formation are very sensitive to electrostatic interactions. Electrostatic interactions shape SFAs resulting in a plethora of different morphologies. These morphologies depend on the charge on the protein and salt conditions. In the presence of multivalent counterions charge screening alone fails to describe the experimental observations which implies that additional electrostatic interactions play a role. The onset of SFAs formation in the presence of Ca^{2+} at very low ionic strengths suggest that ion bridging or inter-fibrillar (maybe also inter-monomeric) correlated movement of condensed counterions are the source of additional attraction. The numerous types of interactions that contribute to the assembly of fibrillar structures not only opens up the possibility that αS amyloids can be used as a multi-stimuli responsive materials but also suggest that they may also be partially responsible for the sensitivity of the SFA formation process to the solvent conditions and through that for the polymorphism of the *in vitro* and perhaps *in vivo* formed SFAs such as LB.

The formation of SFAs also affects the aggregation kinetics. The inhibitory effect of increasing ionic strength on the seeding efficiency of preformed fibrils implies that the formation of SFAs, rather than charge inversion, is responsible for the observed non-monotonic behaviour of the aggregation kinetics. The appearance of SFAs significantly alters the aggregation dynamics as in such higher order structures rate-determining processes such as secondary nucleation, fibril fragmentation and monomer addition to

active fibril ends are probably suppressed. This finding makes room for some interesting considerations. If fibrils in SFAs are not efficient in sequestering free monomers, how protective can the formation of LB and other amyloid deposits be in protein aggregation diseases? If amyloid fibrils fail to recruit α S monomers fast enough they cannot prevent primary nucleation events and thus the formation of potentially toxic oligomer and fibril species in solution. This observation is also a warning when it comes to the interpretation of kinetic data obtained under conditions where SFAs form.

References

1. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., Alpha-Synuclein in Lewy Bodies. *Nature* **1997**, *388*, 839-840.
2. Westermark, P.; Wernstedt, C.; Wilander, E.; Sletten, K., A Novel Peptide in the Calcitonin Gene Related Peptide Family as an Amyloid Fibril Protein in the Endocrine Pancreas. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 827-831.
3. Spillantini, M. G.; Crowther, R. A.; Jakes, R.; Hasegawa, M.; Goedert, M., Alpha-Synuclein in Filamentous Inclusions of Lewy Bodies from Parkinson's Disease and Dementia with Lewy Bodies. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 6469-6473.
4. Kopito, R. R., Aggresomes, Inclusion Bodies and Protein Aggregation. *Trends Cell Biol* **2000**, *10*, 524-530.
5. Meyer-Luehmann, M.; Stalder, M.; Herzig, M. C.; Kaeser, S. A.; Kohler, E.; Pfeifer, M.; Boncristiano, S.; Mathews, P. M.; Mercken, M.; Abramowski, D., *et al.*, Extracellular Amyloid Formation and Associated Pathology in Neural Grafts. *Nat. Neurosci.* **2003**, *6*, 370-377.
6. LaFerla, F. M.; Green, K. N.; Oddo, S., Intracellular Amyloid-Beta in Alzheimer's Disease. *Nat. Rev. Neurosci.* **2007**, *8*, 499-509.
7. Fernandez-Nogales, M.; Cabrera, J. R.; Santos-Galindo, M.; Hoozemans, J. J. M.; Ferrer, I.; Rozemuller, A. J. M.; Hernandez, F.; Avila, J.; Lucas, J. J., Huntington's Disease Is a Four-Repeat Tauopathy with Tau Nuclear Rods. *Nat. Med. (N. Y., NY, U. S.)* **2014**, *20*, 881-885.
8. Semerdzhiev, S. A.; Dekker, D. R.; Subramaniam, V.; Claessens, M. M., Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. *ACS Nano* **2014**, *8*, 5543-5551.
9. Krebs, M. R. H.; MacPhee, C. E.; Miller, A. F.; Dunlop, L. E.; Dobson, C. M.; Donald, A. M., The Formation of Spherulites by Amyloid Fibrils of Bovine Insulin. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14420-14424.
10. Krebs, M. R. H.; Devlin, G. L.; Donald, A. M., Protein Particulates: Another Generic Form of Protein Aggregation? *Biophys. J.* **2007**, *92*, 1336-1342.
11. Kosaka, K.; Oyanagi, S.; Matsushita, M.; Hori, A.; Iwase, S., Presenile Dementia with Alzheimer-Body, Pick-Body and Lewy-Body Changes. *Acta Neuropathol* **1976**, *36*, 221-233.

12. Okazaki, H.; Lipkin, L. E.; Aronson, S. M., Diffuse Intracytoplasmic Ganglionic Inclusions (Lewy Type) Associated with Progressive Dementia and Quadriparesis in Flexion. *Journal Of Neuropathology and Experimental Neurology* **1961**, 20, 237-&.
 13. Iseki, E.; Marui, W.; Akiyama, H.; Ueda, K.; Kosaka, K., Degeneration Process of Lewy Bodies in the Brains of Patients with Dementia with Lewy Bodies Using Alpha-Synuclein-Immunohistochemistry. *Neurosci. Lett.* **2000**, 286, 69-73.
 14. Raiss, C. C.; Braun, T. S.; Konings, I. B. M.; Grabmayr, H.; Hassink, G. C.; Sidhu, A.; le Feber, J.; Bausch, A. R.; Jansen, C.; Subramaniam, V., *et al.*, Functionally Different Alpha-Synuclein Inclusions Yield Insight into Parkinson's Disease Pathology. *Sci Rep-Uk* **2016**, 6.
 15. Humblet-Hua, K. N. P.; Scheltens, G.; van der Linden, E.; Sagis, L. M. C., Encapsulation Systems Based on Ovalbumin Fibrils and High Methoxyl Pectin. *Food Hydrocolloids* **2011**, 25, 307-314.
 16. Maji, S. K.; Schubert, D.; Rivier, C.; Lee, S.; Rivier, J. E.; Riek, R., Amyloid as a Depot for the Formulation of Long-Acting Drugs. *PLoS Biol.* **2008**, 6, 240-252.
 17. Zhang, S. G., Fabrication of Novel Biomaterials through Molecular Self-Assembly. *Nat. Biotechnol.* **2003**, 21, 1171-1178.
 18. Shimanovich, U.; Efimov, I.; Mason, T. O.; Flagmeier, P.; Buell, A. K.; Gedanken, A.; Linse, S.; Akerfeldt, K. S.; Dobson, C. M.; Weitz, D. A., *et al.*, Protein Microgels from Amyloid Fibril Networks. *ACS Nano* **2015**, 9, 43-51.
 19. Hoyer, W.; Cherny, D.; Subramaniam, V.; Jovin, T. M., Impact of the Acidic C-Terminal Region Comprising Amino Acids 109-140 on Alpha-Synuclein Aggregation in Vitro. *Biochemistry* **2004**, 43, 16233-16242.
 20. Giasson, B. I.; Murray, I. V. J.; Trojanowski, J. Q.; Lee, V. M. Y., A Hydrophobic Stretch of 12 Amino Acid Residues in the Middle of Alpha-Synuclein Is Essential for Filament Assembly. *J. Biol. Chem.* **2001**, 276, 2380-2386.
 21. Vilar, M.; Chou, H. T.; Luhrs, T.; Maji, S. K.; Riek-Loher, D.; Verel, R.; Manning, G.; Stahlberg, H.; Riek, R., The Fold of Alpha-Synuclein Fibrils. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, 105, 8637-8642.
 22. Miake, H.; Mizusawa, H.; Iwatsubo, T.; Hasegawa, M., Biochemical Characterization of the Core Structure of Alpha-Synuclein Filaments. *J. Biol. Chem.* **2002**, 277, 19213-19219.
 23. Chen, M.; Margittai, M.; Chen, J.; Langen, R., Investigation of Alpha-Synuclein Fibril Structure by Site-Directed Spin Labeling. *J. Biol. Chem.* **2007**, 282, 24970-24979.
-

24. Heise, H.; Hoyer, W.; Becker, S.; Andronesi, O. C.; Riedel, D.; Baldus, M., Molecular-Level Secondary Structure, Polymorphism, and Dynamics of Full-Length Alpha-Synuclein Fibrils Studied by Solid-State Nmr. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 15871-15876.
 25. Gath, J.; Bousset, L.; Habenstein, B.; Melki, R.; Meier, B. H.; Bockmann, A., Yet Another Polymorph of Alpha-Synuclein: Solid-State Sequential Assignments. *Biomol Nmr Assign* **2014**, *8*, 395-404.
 26. Deek, J.; Chung, P. J.; Kayser, J.; Bausch, A. R.; Safinya, C. R., Neurofilament Sidearms Modulate Parallel and Crossed-Filament Orientations Inducing Nematic to Isotropic and Re-Entrant Birefringent Hydrogels. *Nat Commun* **2013**, *4*.
 27. Hudalla, G. A.; Sun, T.; Gasiorowski, J. Z.; Han, H. F.; Tian, Y. F.; Chong, A. S.; Collier, J. H., Gradated Assembly of Multiple Proteins into Supramolecular Nanomaterials. *Nat. Mater.* **2014**, *13*, 829-836.
 28. Morris, E. R.; Rees, D. A.; Thom, D.; Boyd, J., Chiroptical and Stoichiometric Evidence of a Specific, Primary Dimerization Process in Alginate Gelation. *Carbohydr. Res.* **1978**, *66*, 145-154.
 29. Ravanat, G.; Rinaudo, M., Investigation on Oligogalacturonic and Polygalacturonic Acids by Potentiometry and Circular-Dichroism. *Biopolymers* **1980**, *19*, 2209-2222.
 30. Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. J. C.; Thom, D., Biological Interactions between Polysaccharides and Divalent Cations - Egg-Box Model. *FEBS Lett.* **1973**, *32*, 195-198.
 31. Zhang, F. J.; Zocher, G.; Sauter, A.; Stehle, T.; Schreiber, F., Novel Approach to Controlled Protein Crystallization through Ligandation of Yttrium Cations. *J. Appl. Crystallogr.* **2011**, *44*, 755-762.
 32. Lin, Y. C.; Broedersz, C. P.; Rowat, A. C.; Wedig, T.; Herrmann, H.; MacKintosh, F. C.; Weitz, D. A., Divalent Cations Crosslink Vimentin Intermediate Filament Tail Domains to Regulate Network Mechanics. *J. Mol. Biol.* **2010**, *399*, 637-644.
 33. Wong, G. C. L.; Lin, A.; Tang, J. X.; Li, Y.; Janmey, P. A.; Safinya, C. R., Lamellar Phase of Stacked Two-Dimensional Rafts of Actin Filaments. *Phys. Rev. Lett.* **2003**, *91*.
 34. Yao, N. Y.; Broedersz, C. P.; Lin, Y. C.; Kasza, K. E.; MacKintosh, F. C.; Weitz, D. A., Elasticity in Ionically Cross-Linked Neurofilament Networks. *Biophys. J.* **2010**, *98*, 2147-2153.
 35. Oosawa, F., Interaction between Parallel Rodlike Macroions. *Biopolymers* **1968**, *6*, 1633-1647.
-

36. Ha, B. Y.; Liu, A. J., Counterion-Mediated Attraction between Two Like-Charged Rods. *Phys. Rev. Lett.* **1997**, *79*, 1289-1292.
37. Gronbech-Jensen, N.; Mashl, R. J.; Bruinsma, R. F.; Gelbart, W. M., Counterion-Induced Attraction between Rigid Polyelectrolytes. *Phys. Rev. Lett.* **1997**, *78*, 2477-2480.
38. Braccini, I.; Perez, S., Molecular Basis of Ca^{2+} -Induced Gelation in Alginates and Pectins: The Egg-Box Model Revisited. *Biomacromolecules* **2001**, *2*, 1089-1096.
39. Bungenberg de Jong, M. G., Colloid Science, 2. Reversible Systems, Kruyt, G.R. In New York: Elsevier: 1949; pp 333-432.
40. Grosberg, A. Y.; Nguyen, T. T.; Shklovskii, B. I., Colloquium: The Physics of Charge Inversion in Chemical and Biological Systems. *Rev Mod Phys* **2002**, *74*, 329-345.
41. Vanmegen, W.; Snook, I., The Grand Canonical Ensemble Monte-Carlo Method Applied to the Electrical Double-Layer. *J. Chem. Phys.* **1980**, *73*, 4656-4662.
42. Zhang, F.; Skoda, M. W. A.; Jacobs, R. M. J.; Zorn, S.; Martin, R. A.; Martin, C. M.; Clark, G. F.; Weggler, S.; Hildebrandt, A.; Kohlbacher, O., *et al.*, Reentrant Condensation of Proteins in Solution Induced by Multivalent Counterions. *Phys. Rev. Lett.* **2008**, *101*.
43. Garcia-Gimenez, E.; Alcaraz, A.; Aguilera, V. M., Overcharging Below the Nanoscale: Multivalent Cations Reverse the Ion Selectivity of a Biological Channel. *Phys Rev E* **2010**, *81*.
44. Roosen-Runge, F.; Heck, B. S.; Zhang, F. J.; Kohlbacher, O.; Schreiber, F., Interplay of Ph and Binding of Multivalent Metal Ions: Charge Inversion and Reentrant Condensation in Protein Solutions. *J. Phys. Chem. B* **2013**, *117*, 5777-5787.
45. Shvadchak, V. V.; Claessens, M. M. A. E.; Subramaniam, V., Fibril Breaking Accelerates Alpha-Synuclein Fibrillization. *J. Phys. Chem. B* **2015**, *119*, 1912-1918.
46. Buell, A. K.; Galvagnion, C.; Gaspar, R.; Sparr, E.; Vendruscolo, M.; Knowles, T. P. J.; Linse, S.; Dobson, C. M., Solution Conditions Determine the Relative Importance of Nucleation and Growth Processes in Alpha-Synuclein Aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 7671-7676.

CHAPTER 4

ON THE ANISOTROPIC ARCHITECTURE OF SUPRAFIBRILLAR AGGREGATES

Abstract

Depending on the physico-chemical conditions, the neuronal protein alpha-synuclein can self-assemble into individual amyloid fibrils or suprafibrillar assemblies (SFAs) of well-defined morphologies. The major fraction of the SFAs volume consists of water, and can therefore be classified as hydrogel particles. The architecture of the fibril networks in these hydrogel particles is however not homogenous. Here we show that the fibrils are organized differently in the interior and exterior of the fibrillar assemblies. While the fibrils in the central part of the SFAs are randomly oriented, the fibrils located in the periphery show a preference to cross neighboring fibrils at high angles. This difference in fibril organization coincides with a significant difference in polarity of the fibril environment in the central and peripheral parts of the SFA. An explanation for the structural anisotropy of SFAs is proposed in the light of the observation that α S fibrils bind a substantial amount of counterions. We hypothesize that the change in the ionic environment caused by the appearance of more and more fibrils triggers the change in fibril organization.

4.1 Introduction

Under specific physico-chemical conditions, numerous proteins can self-assemble into filamentous aggregates called amyloid fibrils. In the case of the neuronal protein alpha-synuclein (α S), this phenomenon has been associated with the pathology observed in the brains of patients suffering from Parkinson's disease.¹⁻² With the onset of this neurodegenerative disorder, α S monomers aggregate into chemically and mechanically stable amyloid fibrils, which in turn accumulate in the tissue and give rise to morphologically distinct higher order assemblies such as Lewy bodies and Lewy neurites. The formation of amyloids and their accumulation in higher order structures also accompanies the pathology of many other diseases such as Alzheimer's disease, diabetes type II and Huntington's disease to name but a few.³⁻⁵ Besides the importance of amyloids in disease, numerous functional forms of amyloids have been discovered. The existence of functional amyloids, in combination with the chemical and mechanical characteristics of amyloid fibrils, has triggered much interest in using amyloid assemblies as building materials in biomedical and nanotechnological applications.⁶⁻¹²

Recent studies showed that α S can not only self-assemble into a solution of fibrils. It also spontaneously forms higher order suprafibrillar aggregates (SFAs) in the test tube. Depending on the solution conditions these suprafibrillar structures adopt different morphologies, as observed for their *in vivo* counterparts.¹³ Understanding the interactions governing the *in vitro* formation and architecture of these SFAs, might thus provide valuable insights into how such structures form *in vivo*. In addition, a better understanding of the organization into suprafibrillar structure may contribute to the knowledge required to manipulate the properties of new amyloid based materials.

Here we use several approaches to elucidate the internal structure of α S SFAs formed *in vitro* with a cylinder-like morphology. The high water content, in combination with their fibrillar architecture, classifies the SFAs as hydrogel particles. We show that α S amyloid fibrils are birefringent and exploit this property to obtain information about the fibril organization in the SFAs. Birefringence data indicates that the interior and outer parts of the SFA do not share the same fibril organization. Inside the SFAs, fibrils seem to be randomly oriented rendering the interior of the aggregate optically isotropic while at the

periphery, SFAs show clear signs of birefringence indicating a non-random organization of the fibrils in this part of the aggregates. AFM experiments indicate that fibrils at the surface have a preference to cross other fibrils at high angles probably because this minimizes the repulsive electrostatic interactions between adjacent fibrils. We rationalize the difference in fibril organization in the SFA interior and exterior in the context of previous findings that show that amyloid fibrils bind counterions. Finally, we deploy a solvatochromic dye with multiband emission to sense the polarity of the environment in the different parts of the SFA. This approach confirms the modular design of SFAs and shows different polarity in the interior and the peripheral parts. We consider this finding as a consequence of the structural anisotropy in the different parts of the aggregates.

4.2 Materials and methods

4.2.1 α S monomers.

Expression of the human α S wild-type and the 140C mutant (α S140C) with a single alanine to cysteine substitution at residue 140, was performed in *E. coli* B121 (DE3) using the pT7-7-based expression system. Details on the purification procedure for α S and α S140C are described elsewhere.¹⁴

4.2.2 α S gels.

Gels were prepared by incubating 2mM of α S with 1 mol% (of the total protein concentration) of α S seeds in 10 mM Tris, pH=7.4, 2 mM NaCl for 7 days at 37 °C at quiescent conditions. After 7 days, the sample was stored for 2 months at room temperature.

4.2.3 α S seeds.

100 μ M of α S was incubated in 10 mM Tris (Sigma), 2 mM NaCl (Sigma), pH=7.4, 37 °C and shaking at 900 RPM. Once the aggregation was completed, the fibril solution was sonicated (Branson, Sonifier 250) on ice. The solution was sonicated at the lowest power for 5 seconds and left at rest for another 55 second. The cycle was repeated five times. Subsequently, the sonicated samples were tested for seeding efficiency by incubation with fresh α S monomers (data not shown).

4.2.4 α S suprafibrillar aggregates.

100 μ M α S was incubated in 2 mM CaCl₂ (Sigma), 10 mM TRIS (Sigma), pH 7.4, 37 °C for 48 hours. The aggregation was performed in 96 wells plate (Nunc, Thermo Scientific) while shaking at 900 rpm (Heidolph Int., Titramax 100).

4.2.5 Polarized light microscopy (PLM).

A glass cell was prepared using a cover slip, glass spacers (1 mm) and a glass slide which were glued together with UV-curable glue. The sample was injected into the glass cell ($\approx$ 100 μ l) with a pipette and was subsequently sealed with vacuum grease. PLM images were acquired with a Leica D/M microscope under crossed polarizers using Leica 20x air objective and recorded with a Leica DFC450C camera.

4.2.6 Confocal Laser Scanning Microscopy (CLSM)

The cysteine point mutant α S140C was labelled with AlexaFluor 647 maleimide (ThermoFisher scientific, USA) in accordance with the instructions of the manufacturer. SFAs were formed (see material and methods, α S suprafibrillar aggregates) in the presence of 1 mol% of α S140C-AI647. After the SFAs were formed, Thioflavin T (ThT) (Sigma-Aldrich, USA) was added to reach a final concentration of 5 μ M. An aliquot was then pipetted into custom made microscopy chambers and imaged on a Nikon Eclipse Ti microscope in confocal laser scanning mode. The ThT and AlexaFluor 647 were excited using 402 nm (CUBE, Coherent Inc., USA) and 647 nm (2RU-VFL-P-300-647, MPB Communications Inc., Canada) lasers, respectively. The signal from the ThT dye was collected using a 450/50 nm bandpass emission filter and from the AlexaFluor 647 using a 700/75 nm bandpass filter.

4.2.7 AFM

A solution of the SFA was deposited on a mica surface and left at rest for 20 min for the aggregates to sediment and adhere to the substrate. The sample was then gently washed with deionized water (Milli-Q, Milipore Corp., USA) and dried with a nitrogen flow. Height and phase images were obtained using a Multimode 8 AFM (Bruker, USA) in tapping mode and a MSCT Si₃N₄ tip (Bruker AFM probes) with expected resonance frequency and spring constant of 85-155 kHz and 0.5 N/m respectively.

4.2.8 Fibril organization and cross-angle analysis.

The average orientation of the fibrils in the AFM Phase images was determined using the ImageJ plugin FibrilTool. The details how the plugin operates are described elsewhere.¹⁵ Briefly, the program deploys the concept of nematic tensor to identify the average direction of the fibrils and to what extent they are aligned. The tensor is computed on the basis of the pixels intensities in the selected region of interest (ROI). After the intensity gradient is estimated, a unit vector is defined which is a local tangent to the fibrils. The circular average orientation of the unit vector defines the director in the ROI and the circular variance of it determines the degree of ordering (anisotropy).

4.2.9 Solvatochromic dye imaging.

The cysteine point mutant α S140C was first incubated in excess of DTT for an hour. The DTT was removed using a desalting column (Zeba spin, Thermofisher Scientific, USA). α S140C was incubated with molar excess of Dimethylamino-3-hydroxyflavone (MFM-maleimide) for two hours at room temperature and in dark. The unreacted dye was removed with a desalting column (Zeba spin, Thermofisher Scientific, USA). SFAs were grown by incubating α S with 5 mol% of α S140C-MFM (100 μ M total protein concentration) in identical conditions as above (see MATERIALS AND METHODS, α S suprafibrillar aggregates). The two tautomeric forms of MFM were excited using a 402 nm (CUBE, Coherent Inc., USA) and 488 nm (488-100 CW CDRH, Coherent Inc., USA) laser. The fluorescence signal from the two forms of the dye was collected using 482/35 nm and 595/50 bandpass emission filters.

4.3 Results and discussion

4.3.1 Composition of cylindrical SFAs

The volume of suprafibrillar aggregates (SFAs) significantly reduces upon drying.¹³ The initially cylindrical SFAs collapse into fibrillar sheets as thin as several hundreds of nanometers while when swollen the SFAs have a diameter d of tens of micrometers. The extent of shrinkage suggests that the solvent (i.e. water) comprises a large fraction of the SFA's volume. With simple geometrical considerations one can easily obtain an estimate for

Figure 4.1 Geometrical configurations of a dried and swollen suprafibrillar aggregate used to estimate the water fraction.

the water fraction. In order to determine the volume of the hydrated and dried aggregate we assume that for both species the circumference P of the cross section remains the same when an initially cylindrical aggregate with diameter d is dried and collapses on a mica surface (Figure 4.1, Figure 2.3), or:

$$P = \pi d = 2(a + b) \quad 4.1$$

where a is the height and b is the width of the dried rectangular aggregate Figure 4.1. This approximation seems reasonable since there were no cracks or integrity breaches observed in the top layers of the aggregates that would suggest spreading and therefore mismatch between the circumferences of the dried and swollen structures. Using equation 4x we can calculate back the diameter of the swollen aggregate and subsequently its volume:

$$V_{swollen} = \frac{c(a+b)^2}{\pi} \quad 4.2$$

The volume fraction of the water then can be estimated:

$$\phi_{H_2O} = 1 - \frac{V_{collapsed}}{V_{swollen}} \quad 4.3$$

Using the equations above one arrives at values for $\phi_{H_2O} \approx 0.92$ confirming the high water content in the SFAs. This high water content suggest that the SFAs are particles with hydrogel-like architecture. It is surprising that despite the high water content these fibrillar

assemblies still maintain such a well-defined morphology. Considering their well-defined shape, fibrils within the aggregates may very well be organized in ordered arrays.

4.3.2 Optical properties of α S fibrils

To investigate the internal structure of the SFAs and probe for signs of long- or short-range order we first deploy polarized light microscopy (PLM). Proteinaceous materials which exhibit ordering at molecular level are often optically anisotropic and display birefringence. For example, filamentous protein structures such as collagen fibers and silk threads are strongly birefringent upon illumination with polarized light.¹⁶⁻¹⁷ The polarizability along the protein fibril backbone is different from the polarizability at right angles to the backbone. This effectively gives different refractive indices for the two directions along the fibril.¹⁸ Once the protein molecules are arranged in a non-isotropic manner they thus render the resulting material birefringent. The common feature of amyloid fibrils is the cross-beta sheet which organizes the amyloidogenic protein monomers (or parts of them) into a 1D semi-crystalline array. Thus, periodically arranged amyloid fibrils have all the above mentioned prerequisites for birefringence. Indeed, amyloid fibrils or structures thereof have been shown to display strong birefringence. Higher order fibrillar assemblies of different amyloidogenic proteins such as β -lactoglobulin, insulin and amyloid-

Figure 4.2 α S gels with liquid crystal-like domains. (Left) a 10x image of a 2 mM α S gel. (Right) Zoomed image of the same gel. Scale bars are 1000 and 100 μ m.

beta (42) clearly show double refraction under polarized light.¹⁹⁻²¹ At sufficiently high concentrations, suspensions of lysozyme fibrils form liquid crystalline phases which are visible as birefringent domains when studied with polarized light.²² To check if α S fibrils can organize into birefringent domains, we have prepared soft amyloid gels. To induce nematic ordering in these gels, they were prepared at relatively high protein concentration (2 mM) and low ionic strength (2 mM NaCl) to ensure that fibrils repel each other. At these conditions birefringent clusters could be identified within the gel (Figure 4.2) confirming that α S fibrils are optically anisotropic. The architectural similarity between the cross- β -sheet backbones of α S and insulin fibrils permits a direct comparison of their optical properties. In both insulin and α S fibrils, monomers stack on top of each other in the longitudinal direction along the fibrils while the backbone of the monomers runs at right angles to it. With such arrangement of the protein molecules the optical axis of the fibrils coincides with its longitudinal axis and the refractive index in the lateral and parallel

Figure 4.3 Optical properties of α S fibrils. The blue dotted line vectors represent the ordinary n_o and extra ordinary n_e optical axes of the fibril. P and A designate the polarizer and analyzer respectively. The blue continuous line vectors and r_e show the polarization of the ordinary and extra ordinary rays. r' is the resultant; the orientation of this vector shows the polarization at which maximum amount of light is transmitted by the fibril, and the length is proportional to the intensity. The red arrows show the polarization of the illumination light. (Left and Right) If the optical axis of the fibril stays at an angle that is a multiple of 90° with respect to the polarization axis of A (or of P), the light is completely extinguished $r' \perp A \rightarrow$ no transmitted light. (Middle) In contrast, if the angle is a multiple of 45° , maximal amount of light is transmitted $r' = r_o + r_e$.

direction should be different (Figure 4.3). Such a configuration would also be in line with the observed optical properties of materials consisting of aligned protein molecules.¹⁸

4.3.3 Architecture of the cylindrical SFAs

With this in mind, it is clear that the SFA should be birefringent if the orientation of the fibrils within the aggregates departs from a random distribution. Upon illuminating the SFAs with plane polarized light and placing them in the right orientation with respect to the crossed polarizers, the birefringent properties of the aggregates become visible (Figure 4.4). When viewed from the front the SFAs exhibit a Maltese cross like extinction pattern (Figure 4.4 D, E). A similar pattern is observed in spherulitic higher order structures of amyloidogenic proteins and other (bio)polymers.^{19-21, 23-31} In spherulites, the Maltese cross pattern is a direct consequence of its molecular architecture. Spherulites consist of fibrils radiating out from a common center. Because of the optical anisotropy in the fibrils and their sphero-symmetric arrangement in the spherulites, under crossed polarizers, the light is completely extinguished at right angles (relative to the polarization axes of the polarizer and analyzer) and attains maximum transmission at 45° (Figure 4.3). However, despite the similarity between the Maltese cross extinction pattern of SFAs and spherulites, there is also a marked difference. While for spherulites the Maltese cross seems to be spread throughout the whole cross-section, in SFAs a large portion of the aggregate remains dark; only the SFA periphery periodically lights up (figure 4.4 D, E). The inability of material from the SFA interior to rotate the polarization plane of the light upon transmission can be interpreted in several ways. SFAs with a hollow interior or with an interior composed of amorphously aggregated protein would account for the observed experimental result. The observation of amyloid superstructures with an amorphous core would not be unprecedented as it has previously been observed for bovine insulin spherulites.²⁰ However, the existence of hollow SFAs or SFAs with unstructured cores (no cross-beta sheet content) conflicts with other experimental observations. Sections of the SFA interior obtained with 3D confocal microscopy clearly show the presence of protein inside the SFAs. The bright Thioflavin T (ThT) fluorescence observed there indicates that these proteins have cross-beta sheet conformation, i.e. they are most probably present in fibrillar form (Figure 4.4 F). Thus,

Figure 4.4 PLM and CLSM images of SFAs. Side view PLM images of SFA rotated at A) 45°, B) 0° and C) 315° relative to the polarization axis of the analyzer. Front view PL micrographs of an SFA at D) 45° and E) 0°. F) Confocal z-stack slice from the middle section of an SFA formed within the presence of α S140C-A1647 (red) and stained with the amyloid specific dye Thioflavin T (green). Scale bars are 10 μ m.

the only explanation that reconciles the data from the confocal and polarized light microscopy is that the core of the aggregates is composed of fibrillar α S and either: i) all possible orientations of the fibrils have equal weight giving no net director and an optically isotropic material; or ii) all the fibrils are lying with their optical axis parallel to the long axis of the aggregate. This would cause the light to propagate parallel to the fibrils optical axes (front view Figure 4.4 D, E) and it therefore experiences no optical anisotropy. If ii) was true then the SFAs should be strongly birefringent when viewed from the side (Figure 4.4 A-C). Even though the SFAs show birefringence when viewed from the side it is weak compared to the birefringent periphery visible in the front view (Figure 4.4). Thus we conclude that the SFAs seem to contain a core with randomly oriented fibrils. Interestingly, the orientation of fibrils changes in the outer layers of the SFAs as is evident from the strongly birefringent periphery of the aggregate when viewed from the front (Figure 4.4 D, E). The characteristic

extinction pattern doesn't change upon rotation implying a cylindrical symmetric arrangement of the fibrils in the periphery. To elucidate the source of the strong birefringence originating from the SFAs periphery, we visualize the fibril arrangement in the outer layers of the aggregates by mapping the surface topology using atomic force microscopy (AFM). Subsequently, we analyze the fibril arrangement at the SFA surface in terms of average orientation. This average orientation gives an indication for the source of the observed birefringence. Surprisingly, the AFM micrographs do not show large scale order in the fibril arrangement (Figure 4.5 A). Interestingly, the local directors belonging to the different analyzed surface regions do show a similar orientation. The orientation of the directors often seem close to 45° relative to the longitudinal (central) axis of the SFAs (Figure 4.5 A). This finding seems to be in conflict with the PLM images. If the fibrils had an average orientation of 45° from the central axis of the aggregates, the maximum transmission of the polarized light would be expected when the central axis of the aggregates approaches 90° (or multiples of it) with respect to the analyzer which is exactly opposite to what is observed (Figure 4.4 A,B and C). A possible arrangement of the fibrils that would be in agreement with both the PLM and AFM data is a raft like structure (somewhat reminiscent to the one observed of f-actin) in which the fibrils cross each other at high angles. If the fibrils have a bimodal preference for a direction such that the optical axis runs either parallel with or perpendicular to the central axis (keeping the high angles at the cross-points) of the aggregate, then the SFAs would give maximum transmission of polarized light in positions similar to those observed in Figure 4.4 A and C. Collecting the distribution of angles at which fibrils cross each other on the surface of the SFAs results in a histogram that clearly shows a preference for high angles which is in line with the proposed arrangement of the fibrils (Figure 4.5 C,D). It should be noted that this arrangement is also in line with the extinction patterns that are observed when the SFAs are imaged from the front (Figure 4.4 D and E). If one assumes that the outer layers of the aggregates are composed of stacks of 2D fibrils rafts or meshes, then it is easy to explain the extinction pattern observed in front view PLM images of SFAs. There are two points that need to be considered to explain the birefringence observed in front views of α S SFAs. One is that to get birefringence the fibril's optical axis should lie in an orientation that is not parallel to the direction in which the polarized light

Figure 4.5 Fibril orientation at the surface of the aggregate. (A) AFM phase image from the surface of an SFA. Red circles are the selected areas for which the local director (red lines) was computed and $\bar{\alpha}$ is the average anisotropy. The dashed line represents the central axis of the SFA (a central axis of an SFA can be seen in Figure 4.3 A, B and C). (B) Representative AFM image with measured fibril cross-angles and (C) and the corresponding distribution of cross-angles. Scale bars are 500 nm.

propagates and the second is that for birefringence to occur the slow and fast axes of the fibril should be at an angle different from 0° , 90° , 180° or 270° with respect to the analyzer (Figure 4.2 B). Since, there will always be a large fraction of fibrils in the rafts that do not align in parallel with the central axis of the SFA, the first condition is essentially fulfilled for the whole periphery of the aggregate (2π). That does not hold true for the second condition. The position of the fibrils confined in the 2D mesh fails to comply with the second condition at the top, bottom and the sides of the SFA periphery (when looked from the front) leaving those areas dark (Figure 4.4 D, E) under the cross polarizers resulting in the Maltese cross like extinction pattern.

The non-random organization observed for α S fibrils confined at the surface of a cylindrical is not unusual for semi-flexible (bio)polymers. Cellulose fibrils wrap the plant cell walls in a helical manner.³²⁻³³ Similarly, collagen fibers twist around blood vessels following a helical trajectory and interestingly in some cases - including the thoracic aorta and annulus fibrosus - a cross-hatched order of the filaments is observed.³⁴⁻³⁷ It has been previously reported that strain may induced the alignment of semiflexible polymers.³⁸ Helical organization of semiflexible polymers confined at the surface of carbon nanotubes has been

also observed in simulations.³⁹⁻⁴⁰ Recent simulations with 2D confined Mikado networks on cylindrical substrates showed a spontaneous bimodal orientation of the cross-linked filaments imposed by the interplay between stretching and bending energies of the individual polymers.⁴¹ This bimodal orientation of fibrils confined on cylindrical surfaces is reminiscent of the arrangement of α S fibrils in the periphery of the SFAs. The mechanisms that are responsible for the orientation of fibrils confined on cylinders may therefore possibly also be used to rationalize fibril orientation in SFAs. This cross-hatch model however, predicts a bimodal distribution of orientations centered around $\pm \pi/4$ with respect to the central axis of the cylinder, which disagrees with the PLM images. Moreover, the radius of curvature of the SFA is rather large with respects to the fibril persistence length, which makes the energy associated with fibril bending relatively low. In addition, the inter-fibril cross-links are transient which allows the fibrils to readjust and control the build-up of stresses and minimize fibril stretching. Thus, the role of stretching and bending fibrils in imposing order in the periphery of SFAs is probably small. Instead inter-fibril electrostatic interactions may be the force driving the high angle fibril arrangement. α S fibrils are highly negatively charged, high cross-angles are therefore favoured over parallel fibril orientation since they minimize the electrostatic repulsion. Electrostatic interactions may thus be responsible for the observed distribution of the fibril cross-angles at the surface of the SFAs (Figure 4.5 D). A similar arrangement of filaments has been observed in supramolecular raft assemblies formed by the negatively charged biopolymer f-actin⁴². In the presence of divalent counterions, including Ca^{2+} , f-actin assembles into ion cross-linked lamellar rafts. In this system the actin filaments are arranged at high angles to diminish the electrostatic repulsion. Interestingly, for f-actin rafts the Ca^{2+} concentration at which the transition from an isotropic to a 'raft-like' phase takes place is very close to the Ca^{2+} concentration used to form the α S SFAs. Moreover, there is a growing amount of evidence that multivalent ions can provide an additional source of inter α S amyloid fibril attraction that complements hydrophobic interactions (Chapters 2 and 6). At the relatively low Ca^{2+} concentrations used to induce the formation of SFAs, the number of condensed Ca^{2+} might not be sufficient to neutralize the charge of the fibrils. As a result of the relatively high fibril large density fibril prefer high angle crossings over parallel arrangements. However, although Ca^{2+} does not

neutralize the fibril charge, its concentration is high enough to establish cross-links between α S fibrils at the intersection points. This explains the orientation of fibrils at the surface of the SFAs but it still remains unclear what triggers the change in the organization of the fibrils at the peripheral layers of the SFA compared to the interior. It is difficult to pin point what exactly causes this anisotropy in the SFA architecture with the given set of experiments, but a hypothesis can be proposed. To obtain a non-isotropic fibril organization some dynamic parameter that determines the self-assembly process should be involved. It has been proposed previously that the strength of the electrostatic interactions can determine the morphology of protein aggregates.⁴³ This offered a plausible explanation for the polymorphism that is experimentally observed among SFAs of different proteins. In particular it could account for the structural anisotropy of the insulin spherulite core and periphery. There it is argued that the accumulation of repulsive electrostatic interaction forces causes the developing aggregates to change their fractal dimension. The internal architecture of the spherulite changes in order to compensate for energy build-up. Perhaps a similar reasoning can be applied for the α S SFAs. The proposed mechanism would agree with previous findings showing that the growth of SFAs can be reinitiated or stopped when the ionic strength of the solution is increased or decreased respectively.¹³ An alternative or complementary mechanism that could be considered is based on the change in availability of counterions. Experiments show that due to their surface charge α S (and other proteins) amyloid fibrils bind a significant amount of multivalent counter ions (Chapter 6).⁹ Thus, in the course of their development, SFAs abduct a significant amount of counterions and decrease the bulk concentration of 'free' ions. This has two consequences: i) in time the charge screening deteriorates and the new layers of the SFA form in this changed environment; ii) the amount of condensed ions on the new fibril layers is lower which reduces the charge neutralization and the 'cross-linking' that the Ca^{2+} provides at the fibril intersection points. The decrease in counterion concentration in solution may thus result in a change in the structure of the SFA. This interpretation agrees with the theoretical predictions for the phase behaviour of semi-flexible polymers in the presence of cross-linkers. At high cross-linker concentration, a gel is expected to be the equilibrium phase while at sufficiently low concentrations, raft-like structures are predicted to form.⁴⁴ Thus

the conditions at the first stages of SFAs formation might favour a gel-like interior while at a later stage, at which the cross-linkers are scarcer, a raft-like architecture may be preferred. Changes in ionic environment might thus give rise to the modular architecture observed for fibrils in the SFAs.

4.3.4 Polarity map of cylindrical SFAs

The modular architecture of the SFAs is very interesting but at the same time very surprising. To further investigate the structure of the SFAs we apply a novel approach and use the properties of a solvatochromic dye to sense the physico-chemical environment within the different regions of the SFAs. 3-hydroxyflavone fluorophores can undergo an excited state intramolecular proton transfer (ESIPT) which generates a second tautomeric (T^*) band in the emission spectrum (Fig 4.6).⁴⁵⁻⁴⁶ The intensity ratio of the normal (N^*) and tautomeric band is highly sensitive to the local environment.⁴⁷⁻⁴⁸ Diethylamino-3-hydroxyflavone (MFE) is able to distinguish between protic and aprotic environments as well as between environments with different polarity. Because of these features this class of dyes permit the spectroscopic discrimination between different membrane types.^{46, 48-50} Also, MFE has been successfully used to probe differences in supramolecular organization of amyloid fibrils.⁵¹ A homologous ESIPT probe (MFC) has been used to monitor α S aggregation.⁵² Here we use the MFM dye (Dimethylamino-3-hydroxyflavone), a closely related homologue of MFE with almost identical solvatochromic properties to determine if the difference in fibril organization in SFAs also results in differences in physico-chemical properties of the fibril environment. First, we investigate if MFM is able to differentiate the aggregation states of α S. To that end we record the emission spectra of the dye- α S conjugate in monomeric form, incorporated in a fibril and in a SFA. A marked difference in the emission spectra can be observed (Figure 4.7). As anticipated, the fluorophore detects a decrease in polarity of its environment in the order monomers > fibrils > SFAs (Figure 4.7). Attached to monomeric α S, MFM exhibits a single red-shifted band corresponding to the emission of the H-bonded N^* form (H-N). The H-coupling to the carbonyl group inhibits the ESIPT and thus the formation of the T^* tautomeric form. In fibrils, the MFM spectrum exhibits a dual emission with a short and a long wavelength band corresponding to the N^*

Figure 4.6 Spectral properties of 3HC dyes. (A) Ground (below) and excited (above) states of 3-hydroxy-flavone (3HC) derivatives in lipid membranes. Upward arrows represent excitation, and downward arrows represent emission. The corresponding band maxima (in nm) are indicated. (B) Fluorescence spectrum of 4-(dimethylamino)-3-hydroxy-flavone in phosphatidylcholine large unilamellar vesicles and its deconvolution into the emission bands of the N*, H-N*, and T* forms. Figure is adapted from ref. 44.

and the ESIPT tautomer T* form. The appearance of the dual emission mode is indicative for the changing polarity of the environment that the monomer attached dye experiences once the α S monomer is incorporated in a fibril. That effect is even more enhanced once SFAs are formed. The change in environment manifests as an increase of the I_{N^*}/I_{T^*} ratio of

Figure 4.7 Emission spectra for aS140C-MFM in monomeric form and different aggregation states. The environment becomes more polar in the order monomer < fibril < SFAs. Intensities for the N* and T* bands were measured at 480 nm and 576 nm respectively.

Figure 4.8 CLSM images (red and green overlay) of suprafibrillar aggregates formed in the presence of α S140C-MFM. The T* band signal is collected in the red channel (A) and is coded in red color on the images (B-D) while the N* band signal is collected in the green channel (A) and is coded in the same color (B-D).

MFM in the SFAs compared to in fibrillar α S. The intensity ratio is a convenient parameter to probe the polarity of the environment and is sensitive enough to distinguish between a fibril in solution and one embedded in a SFA. It is thus possible that the dye is sensitive enough to detect differences in the physico-chemical environment inside the SFAs. To put that conjecture to the test, we have acquired confocal laser scanning microscopy (CLSM) images of SFAs that were formed in the presence of α S cysteine mutant that was coupled at position 140 with a MFM dye (α S140C-MFM) (Figure 4.8). From these images it can be observed that the outer layers of the SFAs are more polar than the inner parts. Interestingly, the polarity pattern outlines a modular design that is strongly reminiscent of the architecture deduced from the PLM images. Since, the interior and the periphery of the SFAs are comprised of fibrils, most likely their arrangement and/or packing (i.e. density) causes the different behaviour of the dye localized in the different parts of the aggregate.

However, the ratio between the T* and N* signal remains constant despite the increase in the intensity of both (Figure 4.9). That holds for the interior and the peripheral part of the SFAs as can be seen by the two populations in the contour plots (Figure 4.9) this excludes that variations in fibril density are responsible for the changes in polarity. Differences in fibril arrangement remain a potential explanation for the observed spectroscopic behaviour of the solvatochromic dye incorporated in different regions of the SFAs. Thus, we rationalize these observations in the context of our previous finding which already proposed a different organization of the fibrils in the periphery and interior of the aggregates. The size of contact area between fibrils is strongly dependent on their mutual orientation. Thus, if the angles are higher – as it is expected for the interior of the SFAs - the interfibril contact area inside the aggregates will be large compared to the contact area of fibrils in the SFA periphery. We hypothesize that at higher interfibril angles, more of the unstructured C-terminal regions on the fibril surface (and therefore dye molecules) become ‘trapped’ between the two interfacing hydrophobic surfaces of the fibril cores. As a result the dyes coupled to the C-termini experience more apolar environment. Since it is expected that number of trapped C-termini are higher inside the SFAs than at their periphery, we stipulate that this leads to a lower I_{N^*}/I_{T^*} for the signal originating from the interior of the SFAs. Additionally, the degree of collapse of the C-terminal region may differ in the different parts of the SFAs. As mentioned earlier, because of the recruitment of counterions by the fibrils, the outer parts of the aggregates effectively form differently at different ionic strengths. Thus, C-terminal

4.9 Distribution analysis for aS140C-MFM in SFAs. (Left) CLSM image of an SFA that is analyzed. (Middle) Total intensity vs Blue/Red ratio contour map. (Right) Red intensity vs Blue intensity contour map. Scale bar is 10 μm.

regions with higher degree of collapse (interior of SFAs) effectively shorten the average distance between the hydrophobic core of the fibril and the last residue of the protruding C-termini of the monomers. Such phenomenon could also contribute to a lower I_{N^*}/I_{T^*} for the signal emanating from the interior of the aggregates.

4.4 Conclusion

In summary, we have shown that the SFAs are comprised of more than 90 % water which classify them as hydrogel particles. We have also shown that α S amyloid fibrils are, similar to fibrils of other amyloidogenic proteins, birefringent. We have exploited this optical property to probe the architecture of SFAs and have visualized their modular build-up, emanating from the different organization of the fibrils in the interior and periphery of the SFAs. The fibrils located in the internal parts of the aggregates have a random distribution of orientations while the fibrils at the periphery seem to have a preference for high crossing angles. We rationalize these findings in the light of previous studies indicating that α S fibrils bind divalent ions. While at the early stages of the self-assembly phenomenon SFAs form in a counterion “rich” environment, with the progression of the aggregation process increasing amount of counterions become trapped by the newly formed fibrils. Thus, at later stages, SFA growth continues in a different (poorer) ionic environment. As a response, the fibril organization changes in the newly formed parts of the aggregates. A higher crossing angle between fibrils is established which minimizes the electrostatic repulsion and allows for the aggregates to grow further. We further investigated the structure of the SFAs using a novel approach based on the conjugation of a solvatochromic dye with α S monomers. The α S bound solvatochromic dye incorporated in the SFA visualizes the existence of regions with different polarity. The outline of these regions coincides remarkably well with SFA structural design derived from the PLM image. It shows that the interior and periphery of the SFAs are different. This allow us to hypothesize that the difference in polarity sensed by the MFM dye is imposed by the different organization of the fibrils in the interior and peripheral parts of the aggregates. In the interior, where the orientation of fibrils is isotropic and low interfibril cross-angels are more probable, a larger fraction of C-terminal regions may find themselves trapped between the cores of adjacent

fibrils at the crossing points. That renders the environment that the MFM dye – which is coupled to the C-terminus of the protein - experiences inside the aggregate more apolar than the environment in the outer layers of the SFA where higher inter-fibril angles are preferred.

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References

1. Spillantini, M. G.; Crowther, R. A.; Jakes, R.; Hasegawa, M.; Goedert, M., A-Synuclein in Filamentous Inclusions of Lewy Bodies from Parkinson's Disease and Dementia with Lewy Bodies. *Proceedings of the National Academy of Sciences* **1998**, *95*, 6469-6473.
2. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., Alpha-Synuclein in Lewy Bodies. *Nature* **1997**, *388*, 839-840.
3. Westermark, P.; Wernstedt, C.; Wilander, E.; Sletten, K., A Novel Peptide in the Calcitonin Gene Related Peptide Family as an Amyloid Fibril Protein in the Endocrine Pancreas. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 827-831.
4. McGowan, D. P.; van Roon-Mom, W.; Holloway, H.; Bates, G. P.; Mangiarini, L.; Cooper, G. J. S.; Faull, R. L. M.; Snell, R. G., Amyloid-Like Inclusions in Huntington's Disease. *Neuroscience* **2000**, *100*, 677-680.
5. Selkoe, D. J., The Molecular Pathology of Alzheimers-Disease. *Neuron* **1991**, *6*, 487-498.
6. Iconomidou, V. A.; Vriend, G.; Hamodrakas, S. J., Amyloids Protect the Silkmoth Oocyte and Embryo. *FEBS Lett.* **2000**, *479*, 141-145.
7. Barlow, D. E.; Dickinson, G. H.; Orihuela, B.; Kulp, J. L.; Rittschof, D.; Wahl, K. J., Characterization of the Adhesive Plaque of the Barnacle Balanus Amphitrite: Amyloid-Like Nanofibrils Are a Major Component. *Langmuir* **2010**, *26*, 6549-6556.
8. Maji, S. K.; Perrin, M. H.; Sawaya, M. R.; Jessberger, S.; Vadodaria, K.; Rissman, R. A.; Singru, P. S.; Nilsson, K. P. R.; Simon, R.; Schubert, D., *et al.*, Functional Amyloids as Natural Storage of Peptide Hormones in Pituitary Secretory Granules. *Science* **2009**, *325*, 328-332.
9. Bolisetty, S.; Mezzenga, R., Amyloid-Carbon Hybrid Membranes for Universal Water Purification. *Nat. Nanotechnol.* **2016**.
10. Knowles, T. P. J.; Oppenheim, T. W.; Buell, A. K.; Chirgadze, D. Y.; Welland, M. E., Nanostructured Films from Hierarchical Self-Assembly of Amyloidogenic Proteins. *Nat. Nanotechnol.* **2010**, *5*, 204-207.
11. Yolamanova, M.; Meier, C.; Shaytan, A. K.; Vas, V.; Bertoncini, C. W.; Arnold, F.; Zirafi, O.; Usmani, S. M.; Muller, J. A.; Sauter, D., *et al.*, Peptide Nanofibrils Boost Retroviral Gene Transfer and Provide a Rapid Means for Concentrating Viruses. *Nat. Nanotechnol.* **2013**, *8*, 130-136.

12. Knowles, T. P. J.; Buehler, M. J., Nanomechanics of Functional and Pathological Amyloid Materials. *Nat. Nanotechnol.* **2011**, *6*, 469-479.
 13. Semerdzhiev, S. A.; Dekker, D. R.; Subramaniam, V.; Claessens, M. M., Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. *ACS Nano* **2014**, *8*, 5543-5551.
 14. Rooijen, B. D. v. Structural and Functional Insights into Interactions of Oligomeric Alpha-Synuclein with Lipid Membranes. PhD Thesis, University of Twente, Enschede, 2009.
 15. Boudaoud, A.; Burian, A.; Borowska-Wykret, D.; Uyttewaal, M.; Wrzalik, R.; Kwiatkowska, D.; Hamant, O., Fibriltool, an Imagej Plug-in to Quantify Fibrillar Structures in Raw Microscopy Images. *Nat. Protoc.* **2014**, *9*, 457-463.
 16. Gosline, J. M.; Demont, M. E.; Denny, M. W., The Structure and Properties of Spider Silk. *Endeavour* **1986**, *10*, 37-43.
 17. Hulmes, D. J. S., Building Collagen Molecules, Fibrils, and Suprafibrillar Structures. *J. Struct. Biol.* **2002**, *137*, 2-10.
 18. Senti, F. R.; Copley, M. J.; Nutting, G. C., Fibrous from Globular Proteins. *J. Phys. Chem.* **1945**, *49*, 192-211.
 19. Donald, A. M., Aggregation in Beta-Lactoglobulin. *Soft Matter* **2008**, *4*, 1147-1150.
 20. Krebs, M. R. H.; MacPhee, C. E.; Miller, A. F.; Dunlop, L. E.; Dobson, C. M.; Donald, A. M., The Formation of Spherulites by Amyloid Fibrils of Bovine Insulin. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14420-14424.
 21. Exley, C.; House, E.; Collingwood, J. F.; Davidson, M. R.; Cannon, D.; Donald, A. M., Spherulites of Amyloid-Beta(42) in Vitro and in Alzheimer's Disease. *J. Alzheimers Dis* **2010**, *20*, 1159-1165.
 22. Corrigan, A. M.; Muller, C.; Krebs, M. R. H., The Formation of Nematic Liquid Crystal Phases by Hen Lysozyme Amyloid Fibrils. *J. Am. Chem. Soc.* **2006**, *128*, 14740-14741.
 23. Coleman, J. E.; Allan, B. J.; Vallee, B. L., Protein Spherulites. *Science* **1960**, *131*, 350-352.
 24. Tanaka, S.; Yamamoto, M.; Ito, K.; Hayakawa, R.; Ataka, M., Relation between the Phase Separation and the Crystallization in Protein Solutions. *Phys Rev E* **1997**, *56*, R67-R69.
 25. Rill, R. L., Liquid-Crystalline Phases in Concentrated Aqueous-Solutions of Na⁺ DNA. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 342-346.
-

26. Olson, R. A., Microtubular Spherulites - Development and Growth in Solutions of Bacteriochlorophyll Protein. *Science* **1970**, *169*, 81-&.
27. Ring, S. G.; Miles, M. J.; Morris, V. J.; Turner, R.; Colonna, P., Spherulitic Crystallization of Short Chain Amylose. *Int. J. Biol. Macromol.* **1987**, *9*, 158-160.
28. Murray, S. B.; Neville, A. C., The Role of the Electrostatic Coat in the Formation of Cholesteric Liquid Crystal Spherulites from Alpha-Chitin. *Int. J. Biol. Macromol.* **1997**, *20*, 123-130.
29. Murray, S. B.; Neville, A. C., The Role of Ph, Temperature and Nucleation in the Formation of Cholesteric Liquid Crystal Spherulites from Chitin and Chitosan. *Int. J. Biol. Macromol.* **1998**, *22*, 137-144.
30. Kobayashi, S.; Hobson, L. J.; Sakamoto, J.; Kimura, S.; Sugiyama, J.; Imai, T.; Itoh, T., Formation and Structure of Artificial Cellulose Spherulites Via Enzymatic Polymerization (Vol 1, Pg 169, 2000). *Biomacromolecules* **2000**, *1*, 509-509.
31. Bassett, D. C., Polymer Spherulites: A Modern Assessment. *J Macromol Sci Phys* **2003**, *B42*, 227-256.
32. Klemm, D.; Heublein, B.; Fink, H. P.; Bohn, A., Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angew Chem Int Edit* **2005**, *44*, 3358-3393.
33. Donaldson, L., Microfibril Angle: Measurement, Variation and Relationships - a Review. *Iawa J* **2008**, *29*, 345-386.
34. Wagermaier, W.; Gupta, H. S.; Gourrier, A.; Burghammer, M.; Roschger, P.; Fratzl, P., Spiral Twisting of Fiber Orientation inside Bone Lamellae. *Biointerphases* **2006**, *1*, 1-5.
35. Giraudguille, M. M., Twisted Plywood Architecture of Collagen Fibrils in Human Compact-Bone Osteons. *Calcif. Tissue Int.* **1988**, *42*, 167-180.
36. Schriebl, A. J.; Reinisch, A. J.; Sankaran, S.; Pierce, D. M.; Holzapfel, G. A., Quantitative Assessment of Collagen Fibre Orientations from Two-Dimensional Images of Soft Biological Tissues. *J R Soc Interface* **2012**, *9*, 3081-3093.
37. Beckett, M. C.; Ralphs, J. R.; Caterson, B.; Hayes, A. J., The Transmembrane Heparan Sulphate Proteoglycan Syndecan-4 Is Involved in Establishment of the Lamellar Structure of the Annulus Fibrosus of the Intervertebral Disc. *Eur Cells Mater* **2015**, *30*, 69-88.
38. Vader, D.; Kabla, A.; Weitz, D.; Mahadevan, L., Strain-Induced Alignment in Collagen Gels. *PLoS One* **2009**, *4*.
39. Kusner, I.; Srebnik, S., Conformational Behavior of Semi-Flexible Polymers Confined to a Cylindrical Surface. *Chem. Phys. Lett.* **2006**, *430*, 84-88.

40. Zheng, M.; Jagota, A.; Semke, E. D.; Diner, B. A.; Mclean, R. S.; Lustig, S. R.; Richardson, R. E.; Tassi, N. G., DNA-Assisted Dispersion and Separation of Carbon Nanotubes. *Nat. Mater.* **2003**, 2, 338-342.
41. Vrusch, C.; Storm, C., Curvature-Induced Crosshatched Order in Two-Dimensional Semiflexible Polymer Networks. *Physical review. E, Statistical, nonlinear, and soft matter physics* **2015**, 92, 060602.
42. Wong, G. C. L.; Lin, A.; Tang, J. X.; Li, Y.; Janmey, P. A.; Safinya, C. R., Lamellar Phase of Stacked Two-Dimensional Rafts of Actin Filaments. *Phys. Rev. Lett.* **2003**, 91.
43. Fodera, V.; Zacccone, A.; Lattuada, M.; Donald, A. M., Electrostatics Controls the Formation of Amyloid Superstructures in Protein Aggregation. *Phys. Rev. Lett.* **2013**, 111.
44. Borukhov, I.; Bruinsma, R. F., Raft Instability of Biopolymer Gels. *Phys. Rev. Lett.* **2001**, 87.
45. Chou, P. T.; Martinez, M. L.; Clements, J. H., Reversal of Excitation Behavior of Proton-Transfer Vs Charge-Transfer by Dielectric Perturbation of Electronic Manifolds. *J. Phys. Chem.* **1993**, 97, 2618-2622.
46. Demchenko, A. P.; Mely, Y.; Duportail, G.; Klymchenko, A. S., Monitoring Biophysical Properties of Lipid Membranes by Environment-Sensitive Fluorescent Probes. *Biophys. J.* **2009**, 96, 3461-3470.
47. Klymchenko, A. S.; Demchenko, A. P., 3-Hydroxychromone Dyes Exhibiting Excited-State Intramolecular Proton Transfer in Water with Efficient Two-Band Fluorescence. *New J. Chem.* **2004**, 28, 687-692.
48. Klymchenko, A. S.; Mely, Y.; Demchenko, A. P.; Duportail, G., Simultaneous Probing of Hydration and Polarity of Lipid Bilayers with 3-Hydroxyflavone Fluorescent Dyes. *Bba-Biomembranes* **2004**, 1665, 6-19.
49. Oncul, S.; Klymchenko, A. S.; Kucherak, O. A.; Demchenko, A. P.; Martin, S.; Dontenwill, M.; Arntz, Y.; Didier, P.; Duportail, G.; Mely, Y., Liquid Ordered Phase in Cell Membranes Evidenced by a Hydration-Sensitive Probe: Effects of Cholesterol Depletion and Apoptosis. *Bba-Biomembranes* **2010**, 1798, 1436-1443.
50. Shynkar, V. V.; Klymchenko, A. S.; Kunzelmann, C.; Duportail, G.; Muller, C. D.; Demchenko, A. P.; Freyssinet, J. M.; Mely, Y., Fluorescent Biomembrane Probe for Ratiometric Detection of Apoptosis. *J. Am. Chem. Soc.* **2007**, 129, 2187-2193.
51. Celej, M. S.; Caarls, W.; Demchenko, A. P.; Jovin, T. M., A Triple-Emission Fluorescent Probe Reveals Distinctive Amyloid Fibrillar Polymorphism of Wild-Type

Alpha-Synuclein and Its Familial Parkinson's Disease Mutants. *Biochemistry* **2009**, *48*, 7465-7472.

52. Yushchenko, D. A.; Fauerbach, J. A.; Thirunavukkuarasu, S.; Jares-Erijman, E. A.; Jovin, T. M., Fluorescent Ratiometric Mfc Probe Sensitive to Early Stages of Alpha-Synuclein Aggregation. *J. Am. Chem. Soc.* **2010**, *132*, 7860-+.

CHAPTER 5

AGING AND THERMO-STIFFENING OF α -SYNUCLEIN AMYLOID NETWORKS

Abstract

Elucidating the nature of the interactions between amyloid fibrils is imperative for a proper understanding of how these structures accumulate and eventually become toxic in tissues in several protein aggregation diseases. Understanding the underlying interactions is also essential if we want to address the full potential of tunable amyloid based materials. Here we study the dynamics, the mechanical properties and the temperature response of aging and equilibrated α S amyloid networks. The equilibration of fibril networks requires a long period of time in quiescent condition, but is significantly faster at higher temperatures. Once equilibrated, the network stiffens drastically at elevated temperatures. This thermos-stiffening is reversible; the network relaxes to its original state upon the removal of the temperature stimulus. We attribute the thermo-stiffening to inter-fibril hydrophobic interactions that are enhanced at elevated temperatures and that mimic the effects of physical cross-links in the network. By incorporating an expression that accounts for the temperature dependence of the formation of these hydrophobic crosslinks, in a floppy mode-based scaling relation for the elastic response function G' of semiflexible networks, we obtain good agreement with the experimentally observed temperature dependence of G'' . In view of our findings, we stipulate the existence of hydrophobic patches in the surface architecture of the α S amyloid fibrils. We finally discuss our findings in the light of disease conditions and material properties.

5.1 Introduction

Amyloids are a fascinating class of 1D molecular assemblies formed by multiple monomeric protein units that interconnect via non-covalent interactions, among which intermolecular hydrogen bonding is the dominant one.¹⁻² These self-assembled structures have attracted much attention because of their biological relevance and interesting properties for new materials. Biologically, the appearance of amyloid fibrils is often associated with the onset of diseases such as Alzheimer's, Parkinson's.³ However, in many cases amyloids are functional and used by organisms to engineer materials for structural reinforcement and support, improved adhesion, biomolecular storage, cellular signal transduction and others.⁴⁻⁹

The self-assembly process that amyloidogenic proteins undergo is very complex.¹⁰ This becomes apparent in the rich palette of structures that amyloid materials can adopt and the multiple length scales these materials can bridge. Amyloidogenic proteins can organize into structures ranging from oligomers and fibrils populating the nanometer length scale to micron-sized particles with different morphologies and fibril organization.¹¹⁻¹³ The ability of amyloidogenic proteins to self-organize at different length scales implies that the interactions driving this self-assembly process are transmitted hierarchically. In a certain range of physico-chemical conditions amyloid fibrils can also organize into bulk materials, such as liquid crystalline phases and gels.¹⁴⁻¹⁶ Amyloid gels are particularly interesting class of materials. The bulk properties of such semi-flexible networks strongly depend on: i) the properties of the individual constituent fibrils; ii) the interactions between these fibrils. On one side, the tensile strength of amyloid fibrils is comparable to those of robust materials like silk and steel, which enables them to form stiff gels at low protein concentrations.¹⁷ On the other hand, interactions governing the multiscale self-assembly process are transmitted hierarchically from the inter-monomer to the inter-fibril level. Since these interactions are very sensitive to physico-chemical conditions, they can, in principle, be exploited to adjust the inter-fibril interactions in amyloid networks. The network properties can thus be fine-tuned and manipulated using physico-chemical triggers. This feature to control interactions by environmental parameters suggests that amyloid fibrils can be used to build multi-responsive materials.

Considering the above, it becomes clear that understanding the interactions that are responsible for this hierarchical self-organization is critical from both a biological and a material science perspective. Elucidating these interactions can aid us in identifying the role and formation mechanism of pathological fibrillar structures such as the α -synuclein (α S) rich inclusions known as Lewy bodies that are found in the brain of patients suffering from Parkinson's disease.³ Additionally, knowledge of how the interactions can be tuned is a first step toward building nanostructured amyloid based materials with tunable properties. Here, we use networks of α S fibrils as a model system to study the organization, dynamics and viscoelastic properties of this class of materials. We identify and exploit temperature as a physico-chemical stimulus to control the properties of the fibrillar network. By following the evolution of the fibrillar gel in time, we show that after the formation of the network, processes with slow dynamics remain active and gradually drive the system to its equilibrium state. These slow equilibration processes could be significantly sped up by a temperature increase. Also, the viscoelastic properties of the equilibrated α S network we find to be very sensitive to changes in temperature. We attribute this observation to the modulation of the inter-fibril hydrophobic interactions. Subsequently, we incorporate the effect of temperature in the strength of the hydrophobic contacts in established scaling relations and arrive at an expression describing the elastic response of the α S cross-linked semi-flexible polymer networks.

5.2 Materials and methods

5.2.1 α S gel preparation

Expression of the human α S wild type was performed in *E. coli* B121 (DE3) using the pT7-7 based expression system. Details on the purification procedure for α S are described elsewhere.⁵⁷ Gels were prepared in quiescent conditions and fibril growth was seeded of preformed 5 mol % α S seeds and total protein concentration in the sample of 300 μ M in 10 mM Tris, pH 7.4 and 10 mM NaCl (Sigma). The first 6 days samples were incubated at 37 °C and after that the gels were stored mature at room temperature. The gels for SAXS measurement were directly grown in quartz capillaries (Hilgenberg GmbH, L=80, OD=1.5,

Wall=0.01 mm) using the procedure described above. Samples for the TIRFM imaging were first stained with the amyloid specific fluorescent dye Thioflavin T (ThT) and sealed in custom made glass chambers.

5.2.2 α S seeds

A solution of fibrils was prepared by incubating 100 μ M of α S in 10 mM Tris (Sigma), 2 mM NaCl (Sigma), pH=7.4, 37 °C and shaking at 900 RPM. Once the aggregation was completed, the fibril solution was sonicated (Branson, Sonifier 250) on ice. The solution was sonicated at the lowest power for 5 seconds and left at rest for another 55 second. The cycle was repeated five times. Subsequently, the sonicated samples were tested for seeding efficiency by incubation with α S monomers (data not shown).

5.2.3 SAXS

Experiments were performed at the BM26 DUBBLE (Dutch-Belgian Beamline, ESRF, Grenoble, France). Two dimensional images were collected using Pilatus 1M photon counting detector. The sample to detector distance was 6.6 m. The wavelength for the incident X-ray was 0.1 nm and beam cross-section with dimensions 2.5 mm $\times$ 4.5 mm. The energy of the x-rays was 12 eV. The attained q range was 0.03-1.5 nm⁻¹. The ATSAS 2.6.0 software package was used for post-acquisition processing and analysis of the SAXS data.⁵⁸

5.2.4 Rheology

Rheology measurements were performed on Anton Paar MCR 301 rheometer using a plat-plate geometry d = 25 mm. Gel samples were carefully collected from the storage tubes using truncated pipette tips to minimize shearing and network disruption. The gel was then carefully deposited on the rheometer's stage. Measurements were conducted at gap size of 0.16 mm and the samples were covered with mineral oil to avoid evaporation during the temperature runs. For each temperature step the sample was first left to equilibrated until the $G_{0,T}$ (f = 0.5 Hz, γ = 0.5 %) attained a stationer value (usually within 1 hour). Subsequently, a frequency spectrum was recorder (γ = 1%). Stress relaxation test were conducted by first equilibrating the sample at a given temperature and then subjecting it to

a strain $\gamma = 3 \%$ which is within the linear viscoelastic response region of the sample. Subsequently the time relaxation modulus $G(t)$ was recorded.

5.2.5 TIRFM

The TIRFM imaging was performed using a Nikon Ti-E microscopy setup coupled with an Argon laser (35-IMA-040, Melles Griot, USA). Images were acquired with a CFI Apo TIRF 100x objective (Nikon, Japan) and iXon 3 DU-897 EMCCD camera (Andor, UK) using the 457 nm line of the laser for the excitation of the ThT dye. The used filter cubes contained a 455 nm excitation filter with 10 nm bandpass, a 458 nm long pass dichroic mirror and a 485 nm emission filter with 30 nm bandpass.

5.2.6 Determination of residual monomer concentration

Gel samples were centrifuged using a Sorvall WX 80 ultracentrifuge (Thermo Scientific, USA) and Fiberlite F50L-24 x 1.5 Fixed-Angle Rotor at 247 kG for 5 hours. Then the concentration of free α S monomers in the supernatant was determined using a UV-Vis spectrophotometer (UV 2401 PC, Shimadzu). Absorbance was measured at 276 nm and an extinction coefficient of $5600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used to calculate the protein concentration.

5.2.7 Persistence length analysis

An aliquot of a $300 \mu\text{M}$ α S gel, 10 mM NaCl and 10 mM Tris was diluted in the same buffer keeping the ionic strength constant. Thioflavin T (ThT) was subsequently added to a final concentration of $1 \mu\text{M}$ in order to stain the fibrils. A small volume of the diluted sample was sealed between a glass slide and a cover slip. The sample was left at rest for the fibrils to sediment on the cover slip and then imaged using Nikon Ti-E in TIRF mode (see section TIRFM). Images were analyzed using the MATLAB based package Easyworm.⁵⁹ Briefly, persistence length is determined via random resampling using bootstrap with replacement method. A bootstrap sample of n chains is randomly selected from the ensemble of all chains. For each bootstrap the average square of the end to end distance $\langle R^2 \rangle$ is binned at equal length intervals. Then all the data is fitted using the wormlike chain model:

$$\langle R^2 \rangle = 2l_p L \left[1 - \frac{l_p}{L} \left(1 - e^{-L/l_p} \right) \right] \quad 5.1$$

where L is the contour length.

5.3 Results and discussion

5.3.1 Network aging and equilibration.

The polymerization of α S into amyloid fibrils is a slow process. Within 7 days after the initiation of polymerization (see the Materials and Methods section), a solution of monomeric α -synuclein (α S) has typically evolved into amyloid fibril network. Visual inspection of samples of these networks, subjected to an inversion test, indicate that the solution has become a self-supporting gel (Figure 5.1). Total internal reflection microscopy (TIRF) images confirm the network nature of the sample (Figure 5.1). To examine the mechanical properties and probe the temporal evolution of the network, we systematically performed oscillatory rheology measurements on an aging sample. Rheologically, the

Figure 5.1 α S amyloid gel. (Left) Frequency sweeps for a 7 day (squares) and 37 days (circles) old sample. The storage modulus G' and the loss modulus G'' are designated with closed and open symbols respectively. (Middle) Self-supporting 300 μ M α S fibril network subjected to an inversion test. The gel was formed in 10 mM NaCl, 10 mM Tris and pH=7.4 (Right) TIRF image of α S network formed at the same conditions and stained with ThT (30 μ M). The scale bar is 5 μ m.

networks of semi-flexible amyloid fibrils behave as viscoelastic materials. The frequency sweep for an aged 37 days old network generates a relatively featureless spectrum and no cross-overs between the frequency (f) dependent storage modulus, $G'(f)$ and loss modulus $G''(f)$, were observed at the probed frequency range (Figure 5.1). The amyloid network properties are dominated by the storage modulus as is characteristic for viscoelastic solids.¹⁸ Interestingly, both G' and G'' seem to be weakly dependent on the frequency, a feature reminiscent of cross-linked materials. Since the α S amyloid networks are not chemically cross-linked, this observation implies significant inter-fibril interactions. Although 7 days were enough to form self-supporting gel, the viscoelastic properties of the

Figure 5.2 Spontaneous versus stimulated equilibration of a 300 μ M α S amyloid network. (Left) Time evolution of the G' (at 0.5 Hz) for two independently prepared aging α S networks (● and ●). The aging process is subdivided into three main stages: I) stiffening; II) relaxation and III) equilibrium. (Right) Response to temperature ramps of a young (●) and an equilibrated (●) α S amyloid network. The network was incubated for 2 hours at each temperature step.

amyloid fibril networks strongly depended on the age of the sample (Figure 5.2). The changes in the viscoelastic properties of the network during the aging process amounted up to an order of magnitude difference in the storage modulus at different point in time (the 7th and 37th day, for example) (Figure 5.2). The aging process can be tentatively divided

Figure 5.3 Absorbance spectra of the supernatant obtained after the ultracentrifugation of an 300 μM αS gel at different time points of the aging process. After 7 days, only have of the monomers have been required in fibrils and a detectable amount of free ThT has been is present in the solution. At 37 days the residual level of monomeric protein is below the threshold for an accurate determination and no free ThT is detected.

into three stages: I) the stiffening stage in which the G' continuously increases and reaches a maximum; II) a relaxation stage accompanied by a significant drop of the G' ; iii) a final equilibrium stage where the network relaxes to an energetic minimum (local or global) and G' attains a stationary value. The marked increase of the G' in the early stages was attributed to ongoing aggregation of αS , i.e., to the formation of new fibrils that reinforce the network. A slow equilibration and low aggregation rate would not be surprising, given that the amyloid gel was grown in the absence of mechanical agitation. Quiescent conditions are known to retard the aggregation kinetics as fibril breaking is suppressed and the formation of fibrils is thus governed by primary nucleation events and the addition of αS monomers to already existing fibrils.¹⁹ The high concentration of αS monomers found, after ultracentrifugation, in the supernatant of a 7 day old gel confirms that only half of the available monomers have aggregated into fibrils, whereas after 37 days the monomer

concentration is approaching values close to the critical concentration and the aggregation seems complete (Figure 5.3).¹⁹⁻²⁰ Even though this finding does explain the first part of the aging process, it does not provide a rationale for relaxation stage. Significant structural reorganization of the network, such as an increase of the mesh size, would explain the drop in the G' with time. At a fixed fibril concentration an increase of the mesh size can be achieved by fibril bundling or the formation of local fibril clusters. However, SAXS

Figure 5.4 SAXS measurements of a network at different temperatures. (Left) 1D SAXS curves of a 35 day old 300 μ M α S network. The sample was first measured at 20 $^{\circ}$ C and ($\times$) subsequently heated up to 80 $^{\circ}$ C ($\triangle$) and measured again. The final measurement was performed after the sample was cooled down to the starting temperature ($\circ$). (Right) Cross-sectional Guinier (CG) plots. The fibril thickness seems constant before, during and after the temperature treatment of an equilibrated α S network. Data sets are vertically shifted for better visualization.

measurements (Figure 5.4) do not provide evidence supporting reorganization. The cross-sectional Guinier analyses of SAXS curves obtained for mature networks (Figure 5.4) show values for the fibril thickness that are in good agreement with the height of α S fibrils obtained with AFM.²¹⁻²² This rules out fibril bundling as a possible cause of the decrease in G' with time.

Figure 5.5 Mechanical properties of αS fibrils. (Right) TIRF image of individual αS fibrils Scale bar is $5 \mu m$. (Middle) Traces for the set of analyzed fibrils (inset) with contour length vs mean square of the end to end distance (inset). (Right) High bending modes of fibrils embedded in a network (right images) that is indicative for the presence of stresses in the young αS network. Scales are $2 \mu m$.

The aging process, marked by slow relaxation dynamics, observed for αS networks is not unique. Colloidal gels and filamentous networks comprised of semi-flexible biopolymers have been shown to exhibit similar behavior which was interpreted in the context of soft glassy rheology.²³⁻²⁷ Aggregating systems usually explore very complex energy landscapes in the course of their evolution and can get kinetically trapped in deep local minima. The spontaneous migration of such a system to its equilibrium state usually involves slow activated dynamics. Internal stresses have been previously proposed as the source driving the spontaneous relaxation processes observed in soft colloidal gels.²⁵ The first direct experimental evidence for the buildup and subsequent relaxation of internal stresses was provided by actin/fascin networks.²⁴ In many aspects αS networks resemble actin networks. Both αS fibrils and actin filaments are semi-flexible (Figure 5.5) and once incorporated in a network, the inter-fibril or inter-filament interactions are mediated by transient physical cross-links. The transient nature of the bonds is essential for the ability of such systems to relax internal stresses by rearrangements of its constituents. The presence of pre-strained fibrils in an αS amyloid network is a good indication for the buildup of stresses during polymerization. Upon visual inspection of a young αS network we indeed observe local deformations such as high curvature bending modes in individual filaments that are indicative of stress buildup (Figure 5.5). This finding, in combination with the absence of

changes in network organization during aging, gives us the ground to argue that the origin of internal stresses and subsequent relaxation is identical for both F-actin and α S fibril networks.

If the network is indeed in a kinetically trapped state, heating up the system should speed up the aging process since most transient bonds are usually being opposed by thermal energy. Additionally, the elevated temperature will accelerate the α S aggregation and by that the stress buildup. The combined effects should significantly shorten the time that the system needs to reach its equilibrium state. Subjecting an already equilibrated network to a temperature treatment, starting from room temperature up to 50 °C, leads to an increase of the G' with T. In this treatment G' reaches a maximum value at the highest temperature (Figure 5.2). However, when the temperature is gradually lowered G' recovers to the initial value. The same temperature treatment invokes a markedly different response in a non-equilibrated network. As observed for the aged equilibrated network, the stiffness of the gel increases with temperature but at 50 °C, the G' of this network is much larger compared to its value of the equilibrated network at the same temperature. Interestingly, the storage modulus continues growing even in the cooling stage of the temperature treatment, implying ongoing formation of new filaments. Probably the nuclei that were generated during heating continue to grow even when the system is cooling down. A contribution from the formation of new growth centers during the cooling stage cannot be ruled out as the temperature of the system is still sufficiently high for more new nuclei to form. Another interesting observation is the very high maximum G' of ≈ 3200 Pa through which the storage modulus passes. This large increase cannot be attributed solely to the temperature, as the latter proved to cause a more modest increase of the G' in the case of the equilibrated network (Figure 5.2). The high value of G' indicates that a large part of the freshly formed fibrils are in a 'pre-strained' conformation. Additionally, the fibrils in the network seem to stick together. Such attractive interactions would retard the relaxation processes and allow the stress to build up macroscopically. Once the monomers are depleted from the solution the relaxation process catches up and G' decreases to values almost identical those of equilibrated networks after the temperature treatment. Note that

the relaxation process at high temperature is probably significantly sped up by the high levels of stress that were generated in the sample.

Figure 5.6 Thermal stiffening of α S amyloid networks. (Left) Frequency sweeps for an equilibrated network subjected to an extended temperature treatment (Right) The damping factor ($\tan\delta$) at 1 Hz as a function of temperature. The decreasing value of the damping factor shows an increase in the elastic portion of the mechanical response of the network.

5.3.2 Temperature response of equilibrated α S fibrillar networks

As mentioned above, the equilibrated network also exhibits a remarkable response to temperature changes. The gels not only remain stable at high temperatures, they even seem to get reinforced (Figure 5.2). To study this phenomenon in more detail we have expanded the range of temperatures to which the equilibrated network was subjected. In the range 15–85 °C, the gel significantly stiffens, which manifests in an order of magnitude increase in the G' (Figure 5.6). Even more interestingly, the stiffening seems to be reversible. The value of the storage modulus tightly follows the changes in the temperature even when the network is repeatedly subjected to temperature oscillations with different amplitudes (figure 5.7). This behavior is normally not observed in semi-flexible polymers, and strongly resembles rubber elasticity where the free energy cost of stretching out the stored contour length in the polymer becomes much larger at elevated temperatures. However, from TIRF images of α S fibrils, formed at the conditions used to prepare amyloid gels, appear to be rather stiff (Figure 5.5). An end-to-end distance *versus* contour length

analysis of the fibrils produces an estimate for the persistence length l_p of $\approx 85 \mu\text{m}$, which is much larger than the mesh size calculated for this network. It is therefore unlikely that the increased cost of reducing the fibril's conformational space contributes significantly to the observed increase in G' with temperature.

Figure 5.7 Reversibility and scaling of the temperature response. (Left) Reversible temperature stiffening of an equilibrated αS amyloid network that was repeatedly subjected to temperature oscillations. (Right) Scaling of the storage modulus with temperature. The circles represent the experimental data, while the red curve is the fit generated using scaling eq. 5.8.

An enormous experimental and theoretical effort has been invested the last decades to better understand the viscoelastic properties of networks comprised of semi-flexible polymers.²⁸ These efforts resulted in theoretical frameworks describing the scaling relations between quantities characterizing the properties of these networks.²⁸ Even though the need for a better understanding of cell mechanics has been an important driving force for progress in this field, the application scope of the developed framework has also significantly expanded. It has been successfully applied to describe wide range of biological and synthetic materials, all falling in the general class of semi-flexible polymer networks.²⁹⁻³⁵ In view of the featureless frequency sweeps, the αS fibril network seems to behave as a cross-linked network (Figure 5.1 and Figure 5.6). The preliminary observation that the fibril networks is probably crosslinked is further supported by creep-recovery experiments (Figure 5.8). Applying constant stress σ on the gel initially induces a time - dependent strain

Figure 5.8 Creep-recovery tests for an equilibrated 300 μM αS amyloid network. Squares designate the measured strain γ and the dashed block pulses represent the loading stages characterized by their duration and the amount of stress σ applied. Different shades of grey are used to discern the 3 subsequent creep-recovery test with increasing duration of the loading stage. The same sample was used for the three measurements. The estimate for the creep-compliance $J(t)$ is obtained from the slope of the dashed line.

response $\gamma(t)$ of the material. Shortly after the stress was applied, $J(t) = \partial\gamma(t)/\partial t$ reaches a steady state (creep). Interestingly, the network exhibits very low creep, a behavior reminiscent of highly viscous or (physically) cross-linked materials, implying significant inter-fibrillar interactions. Once the stress is removed the network shows very low levels of plastic deformation and recovers almost completely its original condition. The same behavior was observed even after extending the period during which the sample was subjected to stress (Figure 5.8). With this in mind, we adopted the scaling relation for cross-linked semi-flexible networks as a starting point to interpret the temperature behavior of the αS amyloid network. Because of the relatively high stiffness of αS fibrils, we deploy a scaling relation based on the floppy modes model:³⁶

$$G'_0 \sim \frac{\kappa \delta_{na}^2}{\xi^2 l_c^4} \quad 5.2$$

where G'_0 is the plateau modulus, κ is the bending modulus, ξ the average mesh size, l_c the average distance between cross – links and δ the strain. In contrast to our experimental findings, this scaling relation implies that heating up the system should not affect the stiffness of the network, at least if κ , ξ , l_c are independent of T . A possible interpretation of this discrepancy is that the bending rigidity κ of the polymers comprising the network increases with increasing T . Despite being somewhat counter intuitive at first glance, such a temperature effect on κ has been observed in synthetic semi-flexible polymer systems.³⁴ Driven by the enhanced hydrophobic interactions these synthetic polymers bundle into filaments at higher temperatures with significantly enhanced rigidity, and with that a vast increase of the storage modulus of the network. Even though hydrophobic interactions seem to play an important role at the intra- and interfibrillar level in α S networks, this mechanism cannot be used to explain the experimental observations.^{12, 37-38} As already mentioned, SAXS measurements do not provide evidence for significant structural changes in the network. The size of the fibril's cross-section remains more or less constant throughout the temperature ramps (Figure 5.4). The SAXS curves at the different temperature points remain identical indicating that there are no sizable changes in ξ either.

If stiffening of the individual fibrils is the reason for the increase in the G'_0 , then the bending rigidity of the fibrils should change drastically with T . Since $G'_0 \sim \kappa$, see eq. (5.2), the observed change in G' between 5-80 C° would imply 13 fold increase in κ . This estimate is based solely on changes in κ without taking into account changes in l_e . If the sensitivity of l_e and κ , through $l_c \sim l_e \sim l_p \sim (\kappa/kT)^{1/5}$, on the temperature is also taken into account, then the increase of κ with temperature would need to be even larger, see eq. (5.2). Such a drastic change in the mechanical properties of individual α S fibrils is unlikely, considering the generic architecture of amyloid fibrils. It is well known that the protein monomers in the cross β -sheets fibril backbone are held together by numerous intermolecular hydrogen bonds.^{2, 39} These hydrogen bonds are the main determinant for the fibril stiffness.⁴⁰ Also, the monomers fold in a way that assures maximum degree of internalization of apolar residues in the fibril's core, and by that the total apolar-polar interfacial area is minimized

(in aqueous solution). Keeping the above in mind, higher temperatures stimulate two counteracting effects: on one hand, the breaking of hydrogen bonds would reduce the rigidity of the fibrils and, on the other hand, the enhancement of the hydrophobic interaction would increase the stiffness. It is however unlikely that the inter-monomer hydrophobic interactions increase sufficiently to compensate for the lost hydrogen bonds and generate such a dramatic net increase of the rigidity. Moreover, the environment inside the fibril's core is rather apolar, suggesting that there is not much hydrophobic solvation taking place there.⁴¹ Therefore, a significant enhancement of the hydrophobic interactions inside the fibril core is not expected to be triggered by higher temperatures. Other molecular assemblies that are held together by hydrophobic interactions also do not show signs of abnormal stiffening induced by increase of temperature (in the temperature range comparable to the one used in this study). Lipid bilayers, for example, become more easy to deform with increasing temperature.⁴²⁻⁴³ The filamentous fd virus exhibits a non-monotonic change in the persistence length l_p with temperature. At higher temperature l_p decreases while initially an increase was registered at the lower temperature range.⁴⁴ This increase in l_p was however small, amounting to approximately 30% of the initial value.

With changes in κ being an unlikely culprit for the large temperature induced jump in G' , the only parameter left that could account for this phenomenon is the mean distance between cross-links l_p . At first, this appears as a counter-intuitive idea. Usually, thermal energy opposes the formation of physical crosslinks because it enhances bond breakage.⁴⁵ Cross-linked actin networks, for example, significantly soften upon heating. Higher temperatures facilitate the disruption of the cross-links by the unbinding events of the cross-linking proteins.⁴⁶ In general, all types of non-covalent interactions decrease in strength with temperature besides one; hydrophobic interactions.^{45, 47} We have previously shown that heating up a stable suspension of α S fibrils induces the formation of fibrillar clusters implying that the hydrophobic effect plays an important role in the interfibrillar interactions.¹² This give us ground to argue that the same type of interactions are, to large extent, responsible for the observed thermal stiffening of the network. Heating up the system strengthens the hydrophobic contacts between the fibrils which ultimately results in a more densely cross-linked network. Stress-relaxation tests on an α S network at

Figure 5.9 Stress relaxation tests of a 300 μm αS network (2 mM Na^+) at different temperatures.

different temperatures support this hypothesis. Instead of speeding up the relaxation processes, heating up the sample actually slows down the relaxation dynamics (Figure 5.9) probably due to the enhanced inter-fibrillar contacts.

Temperature thus seems to enhance the formation of physical cross-links between fibrils in αS amyloid networks. In that regard, if we can translate the impact of temperature to an effective number of crosslinks present in the system, then that will enable us to incorporate this effect in the scaling relations for cross-linked semi-flexible networks and to make a direct connection between the enhanced hydrophobic interactions and the stiffening of the network. We start by introducing a two state model for the entanglements in the network $A \rightarrow B$, where A and B represent ‘free’ and “cross-linked” entanglements respectively. Assuming that hydrophobic interactions drive the cross-linking of entanglements, the equilibrium constant can be written as:^{45, 48}

$$K = K_{T_0} e^{\frac{H_0}{k_B T_0^2} (T - T_0)} \quad 5.3$$

where K_{T_0} is the equilibrium constant at a reference temperature T_0 , and $T_0 > 0$ is the endothermic binding enthalpy at temperature T_0 for two hydrophobic surfaces coming into contact. We can calculate the fraction F of entanglements that are in the cross-linked state by invoking Boltzmann statistics:

$$F = \frac{K}{1+K} = \frac{K_{T_0} e^{\frac{H_0}{k_B T_0^2}(T-T_0)}}{1 + K_{T_0} e^{\frac{H_0}{k_B T_0^2}(T-T_0)}} \quad 5.4$$

Since K_{T_0} is presumably small because at low T_0 the hydrophobic interactions are known to be weak⁴⁹, as is $|T - T_0|/T_0$ over the temperature range that we vary the temperature, we can approximate F making use of a Taylor expansion:

$$F \approx K_{T_0} e^{\frac{H_0}{k_B T_0^2}(T-T_0)} \quad 5.5$$

In a semiflexible network, contacts between filaments appear at the entanglement points. Given that i) there are no significant structural rearrangements in the network during the cross-linking process, ii) the maximum number bonds equals the number of entanglement points, we conclude that the distance between crosslinks l_c can be related to the entanglement length l_e through the fraction F :

$$l_c \sim l_e / F \quad 5.6$$

Taking into account that $\xi \sim c_p^{-2}$, where c_p represents the concentration of the filament forming protein, the entanglement length reads as:⁵⁰

$$l_e \sim l_p^{1/5} \xi^{4/5} \sim \left(\frac{\kappa}{kT} \right)^{1/5} c^{-2/5} \quad 5.7$$

Combing equations (5.6) and (5.7) with (5.2) we arrive at:

$$G'_0(T) = G'_0(T_0) e^{\frac{H_0}{k_B T_0^2}(T-T_0)} \left(\frac{T_0}{T} \right)^{2/5} \quad 5.8$$

where $G'_0(T)$ is the temperature dependent plateau modulus, which obviously has a non-trivial temperature dependence and $G'_0(T_0)$ is the plateau modulus at the reference temperature T_0 . The value of H_0 is strongly dependent on the architecture of αS fibrils. Since there is no established model for this architecture, H_0 is left as a free parameter. The

reference temperature $T_0 = 288$ K, which is the lowest temperature at which the storage modulus was measured, is used to fit equation 5.8 to the experimental data. Expression 5.8 seems to describe the experimental data very well (Figure 5.7). The fit yields $H_0 \approx 7.5 k_b T_0$. From the obtained value for H_0 (Figure 5.7) one can estimate the apparent size of the hydrophobic patches using the expression for the enthalpy at the reference state of hydrophobic contacts $H_0 \approx 2h_{h.i.}a^2$, where $h_{h.i.}$ is the energy cost per 1 nm² of exposed hydrophobic surface and a is the area.⁵¹ Taking into account that $h_{h.i.} \approx k_b T_0$ the estimate for the size of the hydrophobic patches on the fibril surface a is ≈ 0.75 nm² which is comparable to what has been found previously found in the case of viruses.^{48, 51}

5.4 Conclusion

To summarize, we have followed the temporal evolution of α S amyloid networks and showed that these network age. This aging process is characterized by slow dynamics and non-monotonic changes in the viscoelastic properties of the network. The non-monotonic nature of the mechanical changes seems to originate from the convolution of sub-processes. The depletion of residual free monomers and the formation of new fibrils is accompanied with the build-up of internal stresses. These effects enhance the elastic modulus of the network. At the same time, rearrangements in the network lead to the relaxation of accumulated stresses steering the system to its equilibrium state. The experimental results show a significant effect of temperature on the equilibration dynamics. Given enough time, and left at rest, the amyloid network eventually reaches equilibrium. However, at higher temperatures the equilibration process is significantly accelerated.

Equilibrated α S amyloid networks exhibit remarkable thermoresponsive properties. The fibrillar gel significantly stiffens at higher temperatures. Interestingly, the system completely recovers to its original state once the temperature is lowered. The thermo-stiffening of the α S network we propose is the consequence of enhanced inter-fibrillar hydrophobic contacts stimulated by the higher temperature. This is in line with previously established qualitative findings, suggesting that the hydrophobic effect plays an essential role in the interaction between α S fibrils.¹² The hydrophobic interactions between fibrils

suggest that multiple hydrophobic domains in the fibril core remain solvent exposed. At higher temperatures these hydrophobic areas become “activated”, which effectively increases the number of possible contacts points between fibrils.

An alternative explanation in which the exposure of the hydrophobic domains itself is a temperature induced phenomenon could also be considered. At higher temperature segments of the α S fibrils may unfold and reveal the hydrophobic stretches of the protein sequence to the solvent which later become anchoring points between fibrils. Such a hypothetical scenario would be consistent with previous research, suggesting that at elevated temperatures the cross-beta sheet structure of the fibrils starts to fall apart.⁵² However, the experimental findings reported here do not give any clues supporting this interpretation. Numerous unfolding events in fibrils should have become apparent in the SAXS cross-sectional Guinier analysis, as it changes the effective cross-section of the fibril. We do not observe this in our SAXS data. Moreover, compromising the structural integrity of the fibrils should also have altered the mechanical response of the network towards softening rather than towards stiffening.

Elucidating the cohesive forces holding fibrillar amyloid assemblies together is crucial for to obtain a better understanding of the pathology associated with it and the physiological role of such structures. This work provides evidence that hydrophobic interactions play essential role in the formation of α S amyloid assemblies and by that stipulates the existence of hydrophobic domains in the architecture of α S fibrils. A correlation between the exposure of hydrophobic surface in amyloid aggregates and their toxicity has been suggested by numerous studies.⁵³⁻⁵⁵ Thus the accumulation of amyloid deposits might be increasing the total hydrophobic surface present in the cell and interfere with its normal functioning.

Understanding the interfibril interactions is also imperative for the successful utilization and manipulation of this class of materials. Our data indicate that there are opportunities to harness these interactions and tune the mechanical properties of amyloid materials. Moreover, these findings indicate that it is possible to design amyloid fibrils, with engineered hydrophobic patches and synthesize materials with encoded temperature responsiveness. An important question remains however. Are these interactions generic for

amyloids or just specific for α S? Exposure of amyloid networks comprised of the disease unrelated protein β -lactoglobulin doesn't seem to provoke the same response indicating that the degree of thermo-stiffening observed for α S gels cannot be expected for all amyloid materials.⁵⁶

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References

1. Sawaya, M. R.; Sambashivan, S.; Nelson, R.; Ivanova, M. I.; Sievers, S. A.; Apostol, M. I.; Thompson, M. J.; Balbirnie, M.; Wiltzius, J. J. W.; McFarlane, H. T., *et al.*, Atomic Structures of Amyloid Cross-Beta Spines Reveal Varied Steric Zippers. *Nature* **2007**, *447*, 453-457.
 2. Sunde, M.; Serpell, L. C.; Bartlam, M.; Fraser, P. E.; Pepys, M. B.; Blake, C. C. F., Common Core Structure of Amyloid Fibrils by Synchrotron X-Ray Diffraction. *J. Mol. Biol.* **1997**, *273*, 729-739.
 3. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., Alpha-Synuclein in Lewy Bodies. *Nature* **1997**, *388*, 839-840.
 4. Barlow, D. E.; Dickinson, G. H.; Orihuela, B.; Kulp, J. L.; Rittschof, D.; Wahl, K. J., Characterization of the Adhesive Plaque of the Barnacle Balanus Amphitrite: Amyloid-Like Nanofibrils Are a Major Component. *Langmuir* **2010**, *26*, 6549-6556.
 5. Chapman, M. R.; Robinson, L. S.; Pinkner, J. S.; Roth, R.; Heuser, J.; Hammar, M.; Normark, S.; Hultgren, S. J., Role of Escherichia Coli Curli Operons in Directing Amyloid Fiber Formation. *Science* **2002**, *295*, 851-855.
 6. Maji, S. K.; Perrin, M. H.; Sawaya, M. R.; Jessberger, S.; Vadodaria, K.; Rissman, R. A.; Singru, P. S.; Nilsson, K. P. R.; Simon, R.; Schubert, D., *et al.*, Functional Amyloids as Natural Storage of Peptide Hormones in Pituitary Secretory Granules. *Science* **2009**, *325*, 328-332.
 7. Iconomidou, V. A.; Vriend, G.; Hamodrakas, S. J., Amyloids Protect the Silkworm Oocyte and Embryo. *FEBS Lett.* **2000**, *479*, 141-145.
 8. Watt, B.; van Niel, G.; Raposo, G.; Marks, M. S., Pmel: A Pigment Cell-Specific Model for Functional Amyloid Formation. *Pigm Cell Melanoma R* **2013**, *26*, 300-315.
 9. Li, J. X.; McQuade, T.; Siemer, A. B.; Napetschnig, J.; Moriwaki, K.; Hsiao, Y. S.; Damko, E.; Moquin, D.; Walz, T.; McDermott, A., *et al.*, The Rip1/Rip3 Necrosome Forms a Functional Amyloid Signaling Complex Required for Programmed Necrosis. *Cell* **2012**, *150*, 339-350.
 10. Stefani, M.; Dobson, C. M., Protein Aggregation and Aggregate Toxicity: New Insights into Protein Folding, Misfolding Diseases and Biological Evolution. *J Mol Med-Jmm* **2003**, *81*, 678-699.
 11. Krebs, M. R. H.; MacPhee, C. E.; Miller, A. F.; Dunlop, L. E.; Dobson, C. M.; Donald, A. M., The Formation of Spherulites by Amyloid Fibrils of Bovine Insulin. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14420-14424.
-

12. Semerdzhiev, S. A.; Dekker, D. R.; Subramaniam, V.; Claessens, M. M., Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. *ACS Nano* **2014**, *8*, 5543-5551.
13. Krebs, M. R. H.; Devlin, G. L.; Donald, A. M., Protein Particulates: Another Generic Form of Protein Aggregation? *Biophys. J.* **2007**, *92*, 1336-1342.
14. Corrigan, A. M.; Muller, C.; Krebs, M. R. H., The Formation of Nematic Liquid Crystal Phases by Hen Lysozyme Amyloid Fibrils. *J. Am. Chem. Soc.* **2006**, *128*, 14740-14741.
15. Gosal, W. S.; Clark, A. H.; Ross-Murphy, S. B., Fibrillar Beta-Lactoglobulin Gels: Part 1. Fibril Formation and Structure. *Biomacromolecules* **2004**, *5*, 2408-2419.
16. Manno, M.; Giacomazza, D.; Newman, J.; Martorana, V.; San Biagio, P. L., Amyloid Gels: Precocious Appearance of Elastic Properties During the Formation of an Insulin Fibrillar Network. *Langmuir* **2010**, *26*, 1424-1426.
17. Knowles, T. P. J.; Buehler, M. J., Nanomechanics of Functional and Pathological Amyloid Materials. *Nat. Nanotechnol.* **2011**, *6*, 469-479.
18. Barnes, H. A.; Hutton, J. F.; Walters, K., *An Introduction to Rheology*. Elsevier : Distributors for the U.S. and Canada, Elsevier Science Pub. Co.: Amsterdam ; New York, 1989; p ix, 199 p.
19. Shvadchak, V. V.; Claessens, M. M. A. E.; Subramaniam, V., Fibril Breaking Accelerates Alpha-Synuclein Fibrillization. *J. Phys. Chem. B* **2015**, *119*, 1912-1918.
20. Baldwin, A. J.; Knowles, T. P. J.; Tartaglia, G. G.; Fitzpatrick, A. W.; Devlin, G. L.; Shamma, S. L.; Waudby, C. A.; Mossuto, M. F.; Meehan, S.; Gras, S. L., *et al.*, Metastability of Native Proteins and the Phenomenon of Amyloid Formation. *J. Am. Chem. Soc.* **2011**, *133*, 14160-14163.
21. Sidhu, A.; Segers-Nolten, I.; Subramaniam, V., Solution Conditions Define Morphological Homogeneity of Alpha-Synuclein Fibrils. *Bba-Proteins Proteom* **2014**, *1844*, 2127-2134.
22. Sweers, K. K. M.; van der Werf, K. O.; Bennink, M. L.; Subramaniam, V., Atomic Force Microscopy under Controlled Conditions Reveals Structure of C-Terminal Region of Alpha-Synuclein in Amyloid Fibrils. *ACS Nano* **2012**, *6*, 5952-5960.
23. Sollich, P., Rheological Constitutive Equation for a Model of Soft Glassy Materials. *Phys Rev E* **1998**, *58*, 738-759.

24. Lieleg, O.; Kayser, J.; Brambilla, G.; Cipelletti, L.; Bausch, A. R., Slow Dynamics and Internal Stress Relaxation in Bundled Cytoskeletal Networks. *Nat. Mater.* **2011**, *10*, 236-242.
 25. Cipelletti, L.; Manley, S.; Ball, R. C.; Weitz, D. A., Universal Aging Features in the Restructuring of Fractal Colloidal Gels. *Phys. Rev. Lett.* **2000**, *84*, 2275-2278.
 26. Bouchaud, J. P.; Pitard, E., Anomalous Dynamical Light Scattering in Soft Glassy Gels. *European Physical Journal E* **2002**, *9*, 287-291.
 27. Cipelletti, L.; Ramos, L.; Manley, S.; Pitard, E.; Weitz, D. A.; Pashkovski, E. E.; Johansson, M., Universal Non-Diffusive Slow Dynamics in Aging Soft Matter (Vol 123, Pg 237, 2003). *Faraday Discuss.* **2003**, *123*, 423-423.
 28. Broedersz, C. P.; MacKintosh, F. C., Modeling Semiflexible Polymer Networks. *Rev Mod Phys* **2014**, *86*, 995-1036.
 29. Gardel, M. L.; Shin, J. H.; MacKintosh, F. C.; Mahadevan, L.; Matsudaira, P.; Weitz, D. A., Elastic Behavior of Cross-Linked and Bundled Actin Networks. *Science* **2004**, *304*, 1301-1305.
 30. Lin, Y. C.; Broedersz, C. P.; Rowat, A. C.; Wedig, T.; Herrmann, H.; MacKintosh, F. C.; Weitz, D. A., Divalent Cations Crosslink Vimentin Intermediate Filament Tail Domains to Regulate Network Mechanics. *J. Mol. Biol.* **2010**, *399*, 637-644.
 31. Yao, N. Y.; Broedersz, C. P.; Lin, Y. C.; Kasza, K. E.; MacKintosh, F. C.; Weitz, D. A., Elasticity in Ionically Cross-Linked Neurofilament Networks. *Biophys. J.* **2010**, *98*, 2147-2153.
 32. Lin, Y. C.; Koenderink, G. H.; MacKintosh, F. C.; Weitz, D. A., Viscoelastic Properties of Microtubule Networks. *Macromolecules* **2007**, *40*, 7714-7720.
 33. Huisman, E. M.; Wen, Q.; Wang, Y. H.; Cruz, K.; Kitenbergs, G.; Erglis, K.; Zeltins, A.; Cebers, A.; Janmey, P. A., Gelation of Semiflexible Polyelectrolytes by Multivalent Counterions. *Soft Matter* **2011**, *7*, 7257-7261.
 34. Kouwer, P. H. J.; Koepf, M.; Le Sage, V. A. A.; Jaspers, M.; van Buul, A. M.; Eksteen-Akeroyd, Z. H.; Woltinge, T.; Schwartz, E.; Kitto, H. J.; Hoogenboom, R., *et al.*, Responsive Biomimetic Networks from Polyisocyanopeptide Hydrogels. *Nature* **2013**, *493*, 651-655.
 35. Hough, L. A.; Islam, M. F.; Janmey, P. A.; Yodh, A. G., Viscoelasticity of Single Wall Carbon Nanotube Suspensions. *Phys. Rev. Lett.* **2004**, *93*.
 36. Heussinger, C.; Frey, E., Floppy Modes and Nonaffine Deformations in Random Fiber Networks. *Phys. Rev. Lett.* **2006**, *97*.
-

37. Uversky, V. N.; Li, J.; Fink, A. L., Evidence for a Partially Folded Intermediate in Alpha-Synuclein Fibril Formation. *J. Biol. Chem.* **2001**, *276*, 10737-10744.
38. Ahmad, B.; Chen, Y. J.; Lapidus, L. J., Aggregation of Alpha-Synuclein Is Kinetically Controlled by Intramolecular Diffusion. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 2336-2341.
39. Vilar, M.; Chou, H. T.; Luhrs, T.; Maji, S. K.; Riek-Loher, D.; Verel, R.; Manning, G.; Stahlberg, H.; Riek, R., The Fold of Alpha-Synuclein Fibrils. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 8637-8642.
40. Fitzpatrick, A. W. P.; Vanacore, G. M.; Zewail, A. H., Nanomechanics and Intermolecular Forces of Amyloid Revealed by Four-Dimensional Electron Microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 3380-3385.
41. Chen, M.; Margittai, M.; Chen, J.; Langen, R., Investigation of Alpha-Synuclein Fibril Structure by Site-Directed Spin Labeling. *J. Biol. Chem.* **2007**, *282*, 24970-24979.
42. Niggemann, G.; Kummrow, M.; Helfrich, W., The Bending Rigidity of Phosphatidylcholine Bilayers - Dependences on Experimental-Method, Sample Cell Sealing and Temperature. *Journal De Physique II* **1995**, *5*, 413-425.
43. Pan, J.; Tristram-Nagle, S.; Kucerka, N.; Nagle, J. F., Temperature Dependence of Structure, Bending Rigidity, and Bilayer Interactions of Dioleoylphosphatidylcholine Bilayers. *Biophys. J.* **2008**, *94*, 117-124.
44. Tang, J. X.; Fraden, S., Nonmonotonic Temperature Dependence of the Flexibility of Bacteriophage Fd. *Biopolymers* **1996**, *39*, 13-22.
45. van der Schoot, P., Theory of Supramolecular Polymerization. In *Supramolecular Polymers*, 2nd ed.; Ciferri, A., Ed. Taylor & Francis: Boca Raton, 2005; pp xiii, 761 p.
46. Lieleg, O.; Schmoller, K. M.; Claessens, M. M. A. E.; Bausch, A. R., Cytoskeletal Polymer Networks: Viscoelastic Properties Are Determined by the Microscopic Interaction Potential of Cross-Links. *Biophys. J.* **2009**, *96*, 4725-4732.
47. Chandler, D., Interfaces and the Driving Force of Hydrophobic Assembly. *Nature* **2005**, *437*, 640-647.
48. Kegel, W. K.; van der Schoot, P., Competing Hydrophobic and Screened-Coulomb Interactions in Hepatitis B Virus Capsid Assembly. *Biophys. J.* **2004**, *86*, 3905-3913.
49. Dapoian, A. T.; Oliveira, A. C.; Silva, J. L., Cold Denaturation of an Icosahedral Virus - the Role of Entropy in Virus Assembly. *Biochemistry* **1995**, *34*, 2672-2677.
50. Degennes, P. G.; Pincus, P.; Velasco, R. M.; Brochard, F., Remarks on Polyelectrolyte Conformation. *J Phys-Paris* **1976**, *37*, 1461-1473.

51. Phillips, R.; Kondev, J.; Theriot, J., *Physical Biology of the Cell*. Garland Science: New York, 2009; p xxiv, 807 p.
52. Ikenoue, T.; Lee, Y. H.; Kardos, J.; Saiki, M.; Yagi, H.; Kawata, Y.; Goto, Y., Cold Denaturation of Alpha-Synuclein Amyloid Fibrils. *Angew Chem Int Edit* **2014**, *53*, 7799-7804.
53. Bemporad, F.; Chiti, F., Protein Misfolded Oligomers: Experimental Approaches, Mechanism of Formation, and Structure-Toxicity Relationships. *Chem. Biol.* **2012**, *19*, 315-327.
54. Olzscha, H.; Schermann, S. M.; Woerner, A. C.; Pinkert, S.; Hecht, M. H.; Tartaglia, G. G.; Vendruscolo, M.; Hayer-Hartl, M.; Hartl, F. U.; Vabulas, R. M., Amyloid-Like Aggregates Sequester Numerous Metastable Proteins with Essential Cellular Functions. *Cell* **2011**, *144*, 67-78.
55. Mannini, B.; Mulvihill, E.; Sgromo, C.; Cascella, R.; Khodarahmi, R.; Ramazzotti, M.; Dobson, C. M.; Cecchi, C.; Chiti, F., Toxicity of Protein Oligomers Is Rationalized by a Function Combining Size and Surface Hydrophobicity. *ACS Chem. Biol.* **2014**, *9*, 2309-2317.
56. Gosal, W. S.; Clark, A. H.; Ross-Murphy, S. B., Fibrillar Beta-Lactoglobulin Gels: Part 2. Dynamic Mechanical Characterization of Heat-Set Systems. *Biomacromolecules* **2004**, *5*, 2420-2429.
57. Rooijen, B. D. v. Structural and Functional Insights into Interactions of Oligomeric Alpha-Synuclein with Lipid Membranes. PhD Thesis, University of Twente, Enschede, 2009.
58. Petoukhov, M. V.; Franke, D.; Shkumatov, A. V.; Tria, G.; Kikhney, A. G.; Gajda, M.; Gorba, C.; Mertens, H. D. T.; Konarev, P. V.; Svergun, D. I., New Developments in the Atsas Program Package for Small-Angle Scattering Data Analysis. *J. Appl. Crystallogr.* **2012**, *45*, 342-350.
59. Lamour, G.; Kirkegaard, J. B.; Li, H.; Knowles, T. P.; Gsponer, J., Easyworm: An Open-Source Software Tool to Determine the Mechanical Properties of Worm-Like Chains. *Source Code for Biology and Medicine* **2014**, *9*, 1-6.

CHAPTER 6

IONICALLY CROSSLINKED α -SYNUCLEIN AMYLOID NETWORKS

Abstract

Electrostatic interactions play an essential role in the phase behavior of alpha-synuclein (α S). In the presence of mono- and divalent ions, α S assembles into higher order fibrillar structures. While it is clear that monovalent counterions just suppress the inter-fibril repulsive electrostatic interactions, experimental evidence cast doubts if ions of higher valence solely carry this function. In this study, we further investigate the effect of multivalent ions on the inter-fibril interactions of α S amyloids. We do that by monitoring the mechanical response of α S fibril networks in the presence of ions with different valence and concentration. The additional attraction between fibrils in the presence of multivalent counterions manifests itself as a drastic increase of the storage modulus of the α S amyloid network as function of ion valence at constant ionic strength. Multivalent counterions seem to bridge adjacent fibrils and act as an effective cross-linkers. Scaling concepts for cross-linked semiflexible networks accurately describe the mechanical response of the ionically cross-linked α S amyloid networks. The stronger Coulombic interactions between the negatively charged groups in the presence of trivalent ions compared to ions of lower valence causes them to form the most effective cross-links. Additionally, the release of a larger amount of fibril condensed monovalent ions with higher counterion valence most probably contributes to the cross-link strength.

6.1 Introduction

In aqueous environments electrostatic interactions are fundamental to various phenomena observed in the fields of chemistry, soft condensed matter and biology. The most intuitive approach is to consider electrostatic interactions in the spirit of Columbic behavior, which instructs that objects with like-charges should repel and those with opposite charges should attract. The introduction of a simple electrolyte in an aqueous solution of charged objects (colloids or polymers) modulates these interactions. This phenomenon is successfully described by mean field theories such as the Poisson-Boltzmann and adaptations of it. The power of the Poisson-Boltzmann formalism is best demonstrated for solutions with moderately charged objects and monovalent salts. The descriptive power of this theory however, breaks down once highly charged objects are immersed in an environment populated with multivalent (counter)ions. Contrary to the established convention, like-charged objects might start to attract. With his seminal work, Oosawa pointed out that at certain conditions, correlated movements of condensed multivalent counter ions could generate attraction between adjacent like-charged rods.¹ This unusual behavior has received significant attention and extensive theoretical treatment.²⁻⁵ Numerous experimental demonstrations of like-charge attraction between biopolyelectrolytes followed. Charged biopolymers such as DNA, F-actin, microtubules and filamentous viruses exhibit this anomalous behavior in the presence of multivalent ions.⁶⁻⁷ This anomalous interaction also results in a complex phase behavior of these biopolymers. The structures that are found *in vitro* range from bundles to rafts and toroids.⁸⁻¹⁰

Besides the well-known cytoskeletal proteins and DNA, fibrils of alpha-synuclein (α S), exhibit a rich phase behavior in the presence of salt. The neuronal protein α S self-assembles, in the presence of ions, into charged amyloid fibrils which in turn - depending on the ionic strength – form soft networks/suspensions or higher order structures with different morphologies.¹¹ This *in vitro* self-assembly phenomenon is very interesting because it can provide a better understanding of the pathological formation and the role of *in vivo* amyloid assemblies such as Lewy bodies.¹² Additionally, a better understanding of α S phase behavior can generate design principles for novel amyloid based materials with tunable properties which enjoy an increasing attention.¹³⁻¹⁷ To that end, a more detailed

understanding of the interactions responsible for this self-assembly at multiple length scales is essential. We have shown that the phase behavior of α S fibrils (morphology wise) is very similar in the presence of mono- and divalent ions and that it is dictated by the interplay between short range attraction and long range repulsion provided by hydrophobic and electrostatic interactions respectively. The role of monovalent ions in this interaction scheme is to simply screen the electrostatics, which falls completely into the picture that is drawn in the classical Poisson-Boltzmann framework. However, in our previous work an extra contribution of multivalent ions to the attractive potential, which is not captured by the classical formalism, could not be excluded.¹¹ A difference in α S aggregation kinetics in the presence of ions with different valence but identical ionic strength indicated that valence might have a role beyond simple screening (Chapter 2). This is further supported by the mapped phase behavior of α S. Compared to monovalent ions, the first signs of fibril cluster formation appear at somewhat lower ionic strength in the presence of divalent ions.

Here we use α S amyloid networks to study the effect of ion valence on the inter-fibril interactions. We choose this system for two reasons: i) besides from disease perspective, amyloid networks are interesting in the context of materials since they gel at low concentration and possibly form stimuli-responsive networks; ii) the effect of ion valence can be easily identified in these networks by monitoring its mechanical response in the presence of different ions. We do that by means of oscillatory rheology. In addition, we use small angle x-ray scattering (SAXS) to monitor structural changes in the network induced by the changing experimental conditions. We successfully deploy an approach for α S amyloid gel preparation that seems to keep the network geometry fixed (or restrain the changes bellow the detection limit) after the increment of the ionic strength or the administration of ions with different valence. This significantly simplifies the interpretation of the experimental observations. Finally, we deploy scaling relations for crosslinked semi-flexible networks to model the mechanical response of α S amyloid gels and to interpret the changes in their properties induced by the presence of multivalent ions.

6.2 Materials and methods

6.2.1 Preparation of α S soft networks, seeds and suspension samples.

α S soft networks. The preparation of the recombinant α S is described elsewhere (see materials and methods, Chapter 2). Soft α S networks were prepared by incubating 5 mol% (of the total α S concentration) α S seeds with α S monomers in 10 mM Tris (Sigma), 2 mM NaCl (Sigma), pH=7.4 and incubated for 7 days at 37 °C in quiescent conditions. After 7 days, the samples were stored for at least 40 days at room temperature. The final ion concentration (for SAXS measurements see Table 6-1) of the sample was adjusted just prior the measurements (rheology) or the insertion in glass capillaries (Hilgenberg GmbH, L=80, OD=1.5, Wall=0.01 mm).

Table 6-1

	1	2	3
C_{NaCl} [mM]	60	120	600
C_{CaCl_2} [mM]	20	40	-
C_{GdCl_3} [mM]	5	10	50

α S suspensions. Solution conditions for the preparation of the suspensions were identical as the one used for the α S network but with shaking at 900 RPM (Heidolph Int., Titramax 100). The aggregation was seeded with 5% seeds and total α S concentration was kept 100 μ M. Once the aggregation was completed (within 10 days) the final salt concentration (Table 6-2) was adjusted at the targeted level. CsCl, BaCl₂ and GdCl₃ salts were used to adjust the final ion concentrations.

Table 6-2

	1	2	3
C_{NaCl} [mM]	1	20	200
C_{CaCl_2} [mM]	0.1	5	50
C_{GdCl_3} [mM]	0.05	2.5	25

α S seeds. 100 μ M of α S was incubated in 10 mM Tris (Sigma), 2 mM NaCl (Sigma), pH=7.4, 37 °C and shaking at 900 RPM. Once the aggregation was completed, the fibril solution was sonicated (Branson, Sonifier 250) on ice. The solution was sonicated at the lowest power for 5 seconds and left at rest for another 55 second. The cycle was repeated

five times. Subsequently, the sonicated samples were tested for seeding efficiency by incubation with fresh α S monomers (data not shown).

6.2.2 SAXS measurements

Experiments were performed at the BM26 DUBBLE (Dutch-Belgian Beamline, ESRF, and Grenoble, France). Two dimensional images were collected using Pilatus 1M photon counting detector. The sample to detector distance was 6.6 m. The wavelength for the incident X-ray was 0.1 nm and beam cross-section with dimensions 2.5 mm $\times$ 4.5 mm. The energy of the x-rays was 12 eV. The attained q range was 0.03–1.5 nm⁻¹. The ATSAS 2.6.0 software package was used for post-acquisition processing and analysis of the SAXS data.¹⁸

6.2.3 Rheology

A small volume of the α S soft networks samples were slowly pulled out from the storage tubes using a truncated pipette tip to minimize shearing and network disruption. The sample was then carefully deposited on the rheometer's stage. The salt concentration was adjusted to the targeted final level by carefully administering a small volume of a concentrated stock salt (NaCl, CaCl₂ or GdCl₃) solution with a gel loading tip and the measurements were commenced. The rheology measurements were performed on an Anton Paar MCR 301 rheometer using a flat-plate geometry $d = 25$ mm. The gap size was kept at 0.16 mm and the sample was covered with mineral oil to avoid evaporation during the measurements. First the storage modulus was measured at constant strain and frequency of 0.5% and 0.5 Hz respectively, until it attained a constant value. Once the network equilibrated, the frequency sweeps were initiated.

6.2.4 Co²⁺ binding

3 mM of α S were incubated with seeds at different concentrations of CoCl₂ (Sigma) for 3 weeks at 37 °C. After that, the aggregated samples were spun down for an hour at 20 000 G. No measurable quantities of monomeric α S were detected in the supernatant. Aliquot of the supernatant was pipetted in a quartz cuvette and the absorbance at 510 nm (Shimadzu UV-2400PC) was measured. The concentration of the residual (unbound) Co²⁺

was determined in each sample from a calibration line. The latter was constructed using 15 samples with known Co^{2+} concentrations.

6.3 Results and discussion

6.3.1 Counterion induced stiffening of αS amyloid networks

To obtain networks of net negatively charged alpha-synuclein (αS) fibrils, protein seeds (short fragments of αS fibrils) were incubated in quiescent conditions in the presence of counterion species with different valence. At low salt concentrations, the samples evolved into transparent self-supporting networks for all types of salts used (Figure 6.1). At higher ionic strength I however, the samples failed to form macroscopically homogenous gels. Incubation with high concentrations of NaCl resulted in a two phase gel with one of

Figure 6.1 αS amyloid gels formed in different conditions. Gels formed in the presence of monovalent counterions (panel A). In 1 mM NaCl solution, αS monomers ($C_{\alpha\text{S}} = 200$ mM) form soft self-supporting amyloid gels (1) whereas at 60 mM NaCl (2) a two phase gel is formed with one of the phases appearing significantly denser (see arrows). Similarly, with divalent counterions (panel B), αS monomers form a homogeneous amyloid gel (3) in a 0.6 mM CaCl_2 solution. In contrast, at 8 mM CaCl_2 viscous suspensions of macroscopic aggregates are formed (4). First forming soft gels in the presence of low amounts of monovalent counterions (panel C, 2 mM NaCl, 100 mM αS) and subsequently adding bivalent (5, 6 mM CaCl_2 in total) or trivalent (6, 3 mM GdCl_3 in total) ions yields stiff homogenous gels.

the phases appearing denser than the other (Figure 6.1, panel a, 2). With CaCl_2 , the final state of the sample diverged even more from a homogenous gel. The protein aggregation resulted in a viscous and turbid suspension of macroscopic protein aggregates (Figure 6.1 panel B, 2).¹¹ The significant difference in the phase behavior of the samples induced by the different types of salts implies sensitivity of the system towards the valence of the counter ions used. This sensitivity to the counter ion valence also manifests itself in a difference between the ionic strengths at which the final state of the system starts to deviate from a homogenous network. These findings are in line with previous studies investigating the phase behavior of αS as function of ionic strength and salt type (Chapter 2).¹¹ The inability of the system to form homogenous gels at elevated ionic strengths significantly shortens the range of ionic strengths at which the effect of counterion valence on αS amyloid networks can be studied. To overcome this obstacle a different strategy was deployed. First, soft gels were formed by incubating αS seeds and monomers at a very low concentration of monovalent salt (NaCl). Once an equilibrated self-supporting network had formed, the ionic strength of the sample was adjusted by the addition of extra salt and left to equilibrate. This approach yielded stiff transparent gels without any visual signs of phase separation (Figure 6.1 panel C) irrespective of the salt concentration or species used. A visual inspection in combination with mechanical agitation of the equilibrated samples indicated increasing stiffness of the gels with the valence of the counterions at identical ionic strength. To quantify this observation, the changes in viscoelastic properties of the soft αS networks were monitored, immediately after the addition of extra salt, using oscillatory rheology. These measurements confirm a significant change in the viscoelastic properties of the αS networks. Introducing additional Na^+ counterions in the system induced an instant jump in the storage modulus $G'(\omega)$. After this initial jump, G' continued to grow and after an equilibration period – varying in length for the different experimental conditions – it reached a stationary value. The values of the equilibrium G' as a function of the Na^+ concentration covers a range spanning one order of magnitude. The significant change in the absolute value of G' is accompanied by a noticeable drop in the damping factor (G''/G' where G'' is the loss modulus). This indicates that the viscoelastic response of the network is increasingly dominated by the elastic portion (Figure 6.2). Interestingly, all the

forementioned changes in the viscoelastic properties of the network are significantly amplified when counterions with higher valence are introduced in the system. In the presence of Ca^{2+} the range of equilibrium G'' values covers two orders of magnitude and

Figure 6.2 Temporal evolution of equilibrated gels incubated with ions of different valence. The protein concentration used for the whole set of ionic strength conditions was 300 mM. Columns 1, 2 and 3 correspond to networks treated with mono-, di- and trivalent counterions respectively. Rows a, b and c show the storage moduli, the damping factors ($\tan \delta$) and the instantaneous moduli (G'_{Ins}) respectively for networks incubated with ions of different valence and ionic strengths. Row C also shows the colour coding legend for the ionic strength levels used in the different measurements.

almost three orders of magnitude changes in G' are observed in the presence of Gd^{3+} . This sensitivity of the system to the ionic strength underlines the major role that electrostatics play in the inter-fibril interactions. Screening the long range electrostatic repulsion with monovalent salts allows for the formation of short ranged hydrophobic contacts between fibrils (Chapters 2 and 5) and facilitates the agglomeration of αS fibrils into giant clusters. However, this rationale is not enough to explain the much stronger effect that Ca^{2+} and Gd^{3+} have on the fibrillar networks at identical ionic strengths. The sensitivity of the system to the valence of the counterions indicates that the observed electrostatic effects go beyond charge screening and additional mechanism(s) need to be invoked to explain the experimental observations with multivalent ions.

The enhancement of the storage modulus is not continuous throughout the whole concentration range of multivalent counterions. This non-monotonic behavior is marked by a downturn in the trend of the equilibrium modulus (G'_{eq}) at the highest ionic strengths. This is observed for the 300 μM αS networks incubated with $GdCl_3$ and $CaCl_2$ (a similar effect is observed for the 600 μM but it not shown) but not for the networks with $NaCl$ (Figure 6.2 and 6.3). The fact that a non-monotonic behavior of G'_{eq} with ionic strength is only observed in presence of multivalent cations implies that overcharging might be the mechanism responsible for this observation.¹⁹ Overcharging or charge inversion is a process that can take place in a solution once highly charged colloids or polyelectrolytes are exposed to sufficiently high concentration of multivalent (counter) ions.²⁰⁻²¹ In such an environment, the surface charge neutralizing counterions might be confined in excessive quantities in the ionic atmosphere of the charged species. This can induce a change in sign of the overall charge and renders the inter-particle or inter-chain potential again more repulsive (or less attractive). Increased inter-fibrillar repulsion could indeed explain the inflection point observed in the $G'_{eq} = f(I)$ curve. If a decrease in the effective attraction between the fibrils is indeed responsible for the observed inflection of $G'_{eq} = f(I)$, the instantaneous modulus (G'_{Ins}) should behave identically. However, $G'_{Ins}(I)$ does not show a peak value (Figure 6.3) but continuously increases for the whole range of ionic strengths. Therefore the reversal of the $G'_{Ins}(I)$ requires an alternative explanation. Aggregating systems and

networks of semi-flexible filaments are known to explore complex energy landscapes during their formation and by doing so such networks are susceptible to kinetic arrests (Chapter 5).²²⁻²⁴ It has previously been shown that fibrillar α S networks can also get kinetically trapped (Chapter 5). Kinetic trapping of the network provides a consistent explanation for the mismatch between the behavior of G'_{Ins} and G'_{eq} . By varying the concentration of

Figure 6.3 Instantaneous and equilibrium storage moduli as function of ionic strength. Both G' and $G'(f)$ are normalized to their maximum values. (Left) In the presence of NaCl both moduli continuously increase for the whole set of experimental conditions while with CaCl₂ and GdCl₃ (middle and right) the equilibrium moduli show a downturn at higher ionic strength.

ions or their valence, the interfibrillar attraction is significantly affected as is apparent from the continuous growth of G'_{Ins} . The G'_{eq} however, fails to follow the lead as the enhanced attraction at the highest ionic strengths (Gd³⁺ and Ca²⁺) ‘freezes’ the system before it can attain its minimum energy state. Significant retardation in the equilibration kinetics can be experimentally observed at higher (di- and trivalent) salt concentrations (Figure 6.2). In such conditions, the network needs much more time to attain its equilibrium modulus which is in line with the notion of a system that is heading towards a kinetic arrest. It is worth noting that the observations in Figure 6.1 also support the idea that the networks are kinetically trapped. At high ionic strengths, the system phase separates if the aggregation is started from the monomeric state of the protein. However, no signs of phase separation are

observed if the ionic strength is increased when the network of fibrillar α S is already formed.

6.3.2 Time – crosslinker concentration superposition

Despite the large variations in magnitude, the elastic response (G'_{eq}) of all α S networks remains nearly frequency independent over the whole range of experimental conditions (Figure 6.4), indicating that the system remains in the rubbery plateau characterized by the plateau modulus $G_0 = G'_{eq}(f) = const.$ In contrast, the G''_{eq} is not frequency invariant (Figure 6.4). Additionally, $G''_{eq}(f)$ seem to depend on the ionic strength and type of salt used (Figure 6.4). In the presence of NaCl, the shape of $G''_{eq}(f)$ is insensitive to the changing ionic strength. However, this doesn't hold for the networks that have been incubated with multivalent salts. At low $GdCl_3$ concentrations the shape of G''_{eq} resembles that of α S networks in NaCl. At 10.4 mM however, a minimum appears around 0.05 Hz which shifts towards higher frequencies with increasing I (Figure 6.4). This feature in the dissipative part of the viscoelastic response has also been observed for other semi-flexible networks. Physically crosslinked actin networks exhibit a well-defined minimum in the loss modulus followed by a maximum at lower frequency. More interestingly, this minimum shifts to higher frequencies as the cross-linking protein concentration is increased.^{19, 25-26} It has been argued and experimentally supported that this minimum is the result of the competition between two dissipative mechanisms each with its own characteristic timescale, namely: i) viscous drag that filaments experience while performing thermally excited undulations; ii) stress release by the unbinding of the cross-links, a consequence of the finite life-time of the bond between f-actin and the cross-linking protein. The position of the minimum is dictated by the timescales of both process and has been shown to be also sensitive to the degree of cross-linking.²⁶ The similar trend in the viscoelastic behavior of f-actin and α S networks in the presence of cross-linking proteins and multivalent ions respectively, suggests there is an analogy between the roles of cross-linking proteins and multivalent ions in the two systems. Ions of higher valence seem to effectively cross-link the α S fibrils. It is therefore instructive to check if there is an

Figure 6.4 Time – crosslinker concentration superposition. Frequency sweeps and master curves for Na^+ (a, b) and Gd^{3+} (c, d) incubated fibrillar networks. $C_{\alpha S} = 300 \mu\text{M}$. Red triangles (c) designate the position of the dissipation minimum. (Inset, d) Scaling between the frequency and elastic modulus shift factors.

equivalence between ion concentration and time for the mechanical response of αS networks. Such superposition of variables, proves to be a ubiquitous phenomenon for polymer networks. Principles such as time - temperature, time - cure, strain-rate – frequency and salt – time superposition have been previously established and demonstrated in different types of networks.²⁷⁻³¹ Despite the combinations of variables being different, their conjugation ultimately permits the same thing, namely the exploration of frequency regimes which otherwise remain experimentally inaccessible. With the appropriate rescaling, it is possible to construct a master curve for the viscoelastic response of the system over a much larger span of frequencies. To construct a master curve, we use the frequency sweeps for the two most contrasting cases in terms of viscoelastic response: the networks incubated with mono- and trivalent counterions. In both cases the minimum

in $G''_{eq}(f)$ for each ionic strength was used as an “anchor” to superimpose the frequency spectra. The shift factors a , b and c were subsequently used to rescale the frequency f , G'_{eq} and G''_{eq} respectively. In the absence of a minimum in $G''_{eq}(f)$, frequency spectra are shifted until a best overlay is obtained. For the networks cross-linked with Na^+ , the minimum in $G''_{eq}(f)$ is lacking at low I and at higher I it exhibits a minor shift which extends the frequency window only marginally (Figure 6.4). In contrast, the distinct minimum in the $G''_{eq}(f)$ for the Gd^{3+} cross-linked networks and its frequency dependence permits the reconstruction of a master curve extending the frequency window with three orders of magnitude (Figure 6.4). The scaling parameters follow a power law $b \sim a^x$, $c \sim a^y$ where $x \approx 1$ and $y \approx 0.9$. Similar scaling exponents have been observed for other soft matter systems.^{25, 32} Despite the extended range of accessible frequencies, the system remains in the rubbery plateau in terms of its viscoelastic response. A crossover at which the viscous dissipation becomes dominant over the elastic component is anticipated once the mechanical response of the system is probed at sufficiently high frequencies. The dynamics at these time scales are governed by the bending fluctuation modes of polymer segments locked between two entanglement points separated by a distance l_e .³³ The relaxation processes mediated by these entangled strands are associated with a characteristic time scale τ_e known as the entanglement time. This time scale in turn sets the crossover frequency $f_c \sim \tau_e^{-1}$.³⁴ Additionally, two point microrheology and bulk rheology experiments with entangled and crosslinked actin networks respectively have also identified a frequency regime $f_c < \tau_e^{-1}$ preceding the crossover. In this frequency domain, a nearly asymptotic rise of both moduli is observed as $G'(f) \sim G''(f) \sim f^{1/2}$.^{19, 35} No signs of a crossover or a preceding rise of both moduli are observed in the high frequency region of the reconstructed master curve for Gd^{3+} incubated αS networks (Figure 6.4). The successful application of the cross-linker concentration – time superposition (CCTS) does not suffice to access short enough time scales where the frequency regimes mentioned above become evident. This is in line with bulk rheology measurements for f-actin/fascin networks in which the cross-over between the rescaled $G'(f)$ and $G''(f)$ appears at much

higher rescaled frequencies ($f_c = 10^5$).¹⁹ The onset of the $G'(f) \sim G''(f) \sim f^{1/2}$ scaling is also observed at frequencies $f_c = 10^3$ which remain out of reach in the current experimental setup. In addition, the higher stiffness of α S fibrils as compared to actin filaments ($l_p^{\alpha S} = 85 \mu\text{M}$ vs $l_p^{act} = 17 \mu\text{M}$) also slightly shifts the higher frequency regimes since the entanglement time is coupled to l_p by the expression $\tau_e \approx \xi l_e^4 (kT l_p)^{-1}$.³³ Nevertheless, the successful deployment of the CCTS for Gd^{3+} cross-linked α S networks and the failure to do so for the Na^+ cross-linked networks underlines the different effect that multivalent counterions have on the fibrillar networks compared to monovalent counterions.

6.3.3 Scaling relation for ionically cross-linked α S amyloid networks

The equivalence between cross-linked semi-flexible networks (CSNs) and fibrillar α S/salt gels has thus been implied by our findings. An adequate description of the viscoelastic behavior of α S cross-linked (α SCNs) by established scaling concepts would further justify the adoption of the CSNs model. Before attempting a more quantitative treatment of the α SCNs mechanical response, we need a better understanding of the effect that counterions have on the higher order organization of α S fibrils. Ions and cross-linking proteins can induce self-organization of biopolymers such as microtubules and actin into semi-crystalline bundled structures.^{9, 36} It is imperative to know if counterions can induce similar behavior in α S fibrils suspended in solution and more importantly in already formed networks. First we monitor possible structural reorganizations in suspensions of α S fibrils with different types of counterions. The suspensions are formed by incubating seeds of α S fibrils with monomers (total $100 \mu\text{M}$) at low concentration of monovalent salt. The aggregating solution was mechanically agitated to ensure fibril breakage which facilitates the formation of viscous suspension of short fragmented fibrils rather than a percolating network. After adjusting the ionic strength by introducing the counterions of interest, the samples are examined by small angle x-ray scattering (SAXS). F-actin and microtubule bundles generate sharp peaks in 1d SAXS curves at different q values corresponding to length scales associated with the high degree of ordering inside these bundle structures.^{9, 37} No such peaks were detected in any of the $I(q)$ curves despite the wide range of

experimental conditions that α S suspensions were subjected to (Figure 6.5). α S fibrils don't seem to organize into bundles, at least not in the way that f-actin and microtubules do. The SAXS curves corresponding to the higher ionic strength suspensions however seem to differ from their low ionic strength counterparts (Figure 6.5). At high ionic strength a somewhat steeper descent in intensity is observed for the whole q range. We further quantify fibril organization by means of a cross-sectional Guinier analysis. This approach allows the estimation of the cross-section size of long anisotropic particles. The cross-sectional radius of gyration (R_g) of the fibrils in low ionic strength suspensions (all salt types) revolves around ~ 6 nm which agrees with single α S fibril heights obtained with AFM.³⁸ At elevated salt levels,

Figure 6.5 SAXS measurements for α S fibrillar suspensions and networks. 1D scattering curves for 100 μ M suspension (a) and 300 mM network (b) of α S fibrils. (Insets) Cross-sectional Guinier fits from which the R_g 's for different experimental conditions have been extracted and summarized in c and d. Blue, red and black symbols correspond to suspensions and networks incubated with mono-, di- and trivalent counterions respectively (see materials and methods). Curves are vertically shifted for a more clear presentation.

a significant increase in R_g is observed implying a certain degree of lateral fibril association. The onset of R_g increases in the order $[\text{NaCl}] > [\text{CaCl}_2] > [\text{GdCl}_3]$ which is not surprising given the experimentally established effect that counterion valence has on these type of networks

(Figure 6.5). Interestingly, an increase in the cross-section dimensions of the fibrils is not observed once they are incorporated in a network. Elevating the salt concentration or adding different salt type to an equilibrated gel doesn't induce changes in R_g (except for 600 mM NaCl) (Figure 6.5). This observation is in agreement with the previous experimental findings and provides an additional support for the idea that the network is kinetically trapped. In a suspension, fibrils are able to aggregate and this effectively causes R_g to increase to an extent that can be detected with a cross-sectional Guinier analysis. In an entangled/weakly cross-linked network, their mobility is significantly hindered. The fibril mobility is even more severely impaired if a cross-linker is introduced in the system. Even though counter ions enhance interfibrillar attraction, this only results in a stronger bonding at the interfibrillar contact points rather for a dramatic reorganization in the network. This finding enables us to treat the counterion-induced stiffening of the network as an effect caused by isotropic cross-linking at a fixed network geometry.

With this clarification in mind we can now relate interfibrillar cross-linking to the network stiffening in a more quantitative manner. The mechanical properties of an α S semi-flexible network can be interpreted in the light of the non-affine floppy mode model.³⁹ This framework was successfully deployed to describe the elasticity of bundled actin networks and the mechanical properties of simulated composite networks.⁴⁰⁻⁴¹ In contrast to affine approaches the network deformation follows the macroscopic strain in non-affine manner in this model.⁴² The transition between these two regimes of deformation is sensitive the ratio l_p / l_c , where l_p and l_c are the persistence length and the distance between neighboring cross-link points respectively. In a system where $l_p / l_c \ll 1$ the elasticity of the network derives from entropic stretching of the excess length stored in the segments between cross-link points with an effective spring constant.⁴³⁻⁴⁴ In the other limit where $l_p / l_c \gg 1$, the segments appear stiff on the length scale of the cross-link distance. For these stiff filaments entropic stretching cannot be the dominant deformation mode, instead bending deformations with wavelengths comparable to the length l_c of the segments interlocked between cross-link points will dominate.^{39, 42} The spring constant for this transverse type of nonaffine deformations reads as $k_b \sim l_c$. Comparing the two spring constants and given that

$l_p / l_c \gg 1$ for the α S networks, it is evident that the bending deformations are energetically less expensive than the entropic stretching. We estimate that, even at the lowest protein concentration used, the deformations in α S fibrillar networks fall into the range of the floppy mode regime. The mesh size for a 100 μ M fibrillar network was estimated by $\xi^2 = 3(c_n l)^{-1}$ where c_n and l are the protein monomer number concentration and length per monomer in a fibril respectively. Assuming a four filament fibril with inter-monomer spacing of ≈ 0.45 nm we arrive at a mesh size of $\xi^2 \approx 0.6$ μ m. With the persistence length of α S fibrils $l_p \approx 85$ μ m (Chapter 5) this tips the balance in favor of the non-affine deformation mode. Raising the cross-linker and protein concentrations, which we do experimentally, pushes the system even deeper into the realm of the non-affine deformations. In the floppy mode model, the plateau modulus for a α S fibril network scales as:

$$G'_{eq} \sim \frac{k_b \delta_{na}^2}{\xi^2 l_c} \sim \frac{\kappa \delta_{na}^2}{\xi^2 l_c^4} \quad 6.1$$

where κ is the bending modulus of α S fibrils and δ_{na} is the non-affine strain. Since the strain is kept constant throughout all of the experimental conditions we can omit it along with the other constants which simplifies the scaling to $G'_{eq} \sim \xi^{-2} l_c^{-4}$. Using this expression the effect of ion crosslinking can be tested by relating ξ and l_c to the protein concentration $c_{\alpha S}$ and ionic strength I respectively. Subsequently we can check if those relations hold experimentally. If we consider the counterions as effective cross-linkers then the cross-link distance l_c should be dependent on the ionic strength and the entanglement length l_e which sets the minimal possible distance between cross-link points. We can connect the latter quantities through the scaling:

$$l_c \sim I^{-y} l_e \sim I^{-y} \left(\frac{\kappa}{kT} \right)^{1/5} c_{\alpha S}^{-2/5} \quad 6.2$$

Plugging this into eq.6.1 and omitting the constants yields:

$$G'_{eq} \sim I^x c_{\alpha S}^{13/5} \quad 6.3$$

This expression predicts a power law dependence between G'_{eq} and ionic strength. The value of the scaling exponent $x = 4y$ depends on the interaction strength at the interfibrillar contact points, i.e. the efficiency of the crosslinker. The stronger the latter is, the higher value the exponent should attain. Indeed, the experimentally obtained storage modulus scales with ionic strength as a power law for all protein concentrations (Figure 6.6, a-d). The order of the scaling exponents for the different counterions increases as $x^{Na^+} < x^{Ca^{2+}} < x^{Gd^{3+}}$. The effect that monovalent ions have on the G'_{eq} is modest compared to the effect of polyvalent counterions. For the Ca^{2+} and Gd^{3+} crosslinked networks the scaling for

Figure 6.6 Viscoelastic response of α S fibrillar networks in the presence of different counterions.

$r_{\alpha S/i} = C_{\alpha S}/C_i$ designates the ratio between protein and counterion concentration where $i = Na^+$, Ca^{2+} or Gd^{3+} .

both salts changes in non-systematic way for the different α S concentrations (Figure 6, a-d). The cause of this non-monotonic can be recognized as a convolution of two effects. As it is obvious from scaling relation 6.2, G'_{eq} also has a power law dependence on the protein concentration, which hasn't been taken into account. Simply rescaling G'_{eq} with $C_{\alpha S}^{13/5}$ however, is not sufficient to correct for the non-monotonic change in the scaling. In the search for rationale explaining this phenomenon we may have to take into account the

polyelectrolyte nature of α S fibrils. As negatively charged semi-flexible polymers with supposedly high negative charge density, α S fibrils most probably confine significant amount of counterions in the ionic atmosphere surrounding them. To be able to measure the resulting changes in counterion concentration in the bulk solution we have induced gel formation by incubating α S seeds and monomers with CoCl_2 . The concentration of Co^{2+} can be determined spectroscopically which allows for the quantification of the amount of bound counter ions. We have previously shown that the phase behavior of α S is sensitive to the valence of the counterions but not to the chemical species. That allows us to translate the findings we obtain with Co^{2+} binding to other divalent species like Ca^{2+} . At sufficiently high Co^{2+} concentration the aggregating α S solution evolves into a two phase gel (Figure 6.7) similarly to what was observed earlier with NaCl (Figure 6.1, 1a). The intensity of magenta coloring seems to correlate with the protein density in the gel which implies significant “binding” of Co^{2+} to α S fibrils. This is confirmed by the spectroscopic determination of bound Co^{2+} . At saturation, the number of bound counterions amounts to $\approx 4 \text{ Co}^{2+}$ per α S.

Figure 6.7 Co^{2+} binding by α S. (Left) A two phase gel formed at 3 mM α S concentration and 11 mM CoCl_2 . The fibril rich region shows magenta coloring indicative for the higher local density of Co^{2+} . (Right) Spectroscopically determined bound Co^{2+} as a function of the total Co^{2+} concentration.

taking into account the fibril architecture, we estimate the ion to fibril length ratio, which adds up to ≈ 32 counterions per nm of fibril length. The significant accumulation of

counterions near the fibril suggests that the ionic strength alone is not an appropriate quantity to establish a scaling relation between the storage modulus and salt concentration. The amount of confined counter ions does not depend solely on the total ion concentration but rather on the ratio $r_{\alpha S/i} = c_{\alpha S/i} / c_i$ where $i = \text{Na}^+$, Ca^{2+} or Gd^{3+} . Since, the interfibrillar interactions at the cross-link points are probably mediated through these confined ions, their strength is expected to scale with the amount of confined counterions (especially at low salt concentrations) and thus with $r_{\alpha S/i}$ since the ions will be equally distributed around all available fibrils. Rearranging Eq. 6.3 to scale out the protein concentration contribution and incorporating $r_{\alpha S/i}$ yields:

$$\frac{G'_{eq}}{c_{\alpha S}^{13/5}} \sim r_{\alpha S/i}^x \quad 6.4$$

This relation collapses the data on a single line for di- and trivalent counterions (Figure 6.6, e, f) and by that validates the considerations above. Re-examining the scaling coefficients confirms that trivalent ions are the most potent cross-linkers.

Despite the successful identification and description of salt treated αS fibrillar gels as cross-linked semi-flexible networks, it is not yet clear through which physico-chemical mechanisms multivalent ions mediate the inter-fibril attraction. Monovalent ions seem to induce cross-linking through screening the electrostatic repulsion and by that making the hydrophobic interactions more prominent at fibril intersection points (Chapters 2 and 5).¹¹ Thus, when ions are referred to as cross-linkers the clarification that monovalent salts do not participate directly in the cross-linking should be made. The picture with multivalent ions is distinctively different. Charge screening cannot be held responsible for the stiffening of the network as the scaling of G'_{eq} with I has shown. Ion coordination is an unlikely mechanism since a linear polycation such as spermine induces stiffening (data not shown) similar to Ca^{2+} and Gd^{3+} . The experimental clues for the mechanisms by which multivalent salts crosslink αS networks point towards an electrostatic mechanism. That limits the choice to two possible mechanisms: i) counter ions bridge the negatively charged carboxylic groups in the c-termini of intersecting fibrils similarly to what has been observed in ion crosslinked polysaccharide gels;⁴⁵⁻⁴⁷ ii) attraction of the charged like rods is generated by the correlated

movement of condensed counterions. The attractive forces generated by the latter mechanism would be rather weak given the random orientation of interacting fibrils in the network. The parallel alignment of the charged α S fibrils would have facilitated the correlations between a larger number of condensed counterions resulting in a stronger attraction between the fibrils. However, such geometrical reorganization of fibrils is not observed in the α SCNs. There is no evidence for bundling in the ionically crosslinked α S networks. Since it is unlikely that the weak interactions resulting from the counterion correlations at the entanglement points of α SCNs can generate the observed substantial increase in the storage modulus, counter-ion bridging is the most likely source responsible for the stiffening of the network at elevated concentration of multivalent cations.

6.4 Conclusion

We have demonstrated a substantial stiffening of α S amyloid networks as a response to different concentrations and species of salt. The effect is mild in the presence of monovalent counterions but significant in the presence of di- and trivalent ions. Even though in general this effect seems to be electrostatic in nature, there is a marked difference between the mechanisms that generate the network stiffening in the presence of mono- versus multivalent counterions. Monovalent ions screen the repulsion between the negatively charged α S fibrils which facilitates the hydrophobic interactions at the entanglement points and effectively increases the degree of cross-linking in the network. In contrast, the multivalent ions themselves seem to act as cross-linkers and mediate the attractive interaction at the inter-fibrillar junctions. In search for the mechanism responsible for the stiffening of α S amyloid networks in the presence of multivalent counterions we have dismissed counter-ion correlations as a possible cause for the network stiffening. In the absence of parallel alignment of the interacting fibrils, the net attraction at the entanglement points generated by counterion correlations would not be sufficient to produce the observed strong enhancement of the storage modulus. Counterion bridging is therefore the most likely mechanism causing the enormous stiffening of the network. The floppy mode model based scaling relation for cross-linked semi-flexible networks adequately captures the mechanical response of the multivalent cross-linked α S amyloid

networks. It also outlined the trivalent counter-ions as the most potent cross-linkers. We propose a simple interpretation of this observation in the light of the adopted ion bridging mechanism. Since the magnitude of Coulombic interactions is strongly dependent on the size of the electrical charge, counter ions with higher valence most likely generate the strongest attraction to the negative charges of α S fibrils and by that, they cross-link entangled fibrils most efficiently. Additionally, counter ions with higher valence might trigger the release of a larger amount of condensed monovalent ions which probably also contributes to the magnitude of the attractive inter-fibrillar interaction.

The polyvalent interactions that α S fibrils can realize provides a source of inspiration for the design of novel multi-responsive materials. It has been already shown that the mechanical response of α S networks can be varied by an order of magnitude as a function of temperature (Chapter 5). Here we show that additionally counter ions can be used to tune – in a similar way that nature does with cross-linking proteins - the mechanical response of α S networks within a remarkable ‘dynamic range’ spanning almost 3 orders of magnitude.

The polyvalent/multimodal nature of the interfibrillar interactions may also have implications for amyloid formation and accumulation in protein aggregation diseases. Additionally, it may have implications for how amyloid deposits interfere with the normal functioning of a cell. The different ways through which α S fibrils can interact perhaps also significantly extends the pool of possible interaction partners (other proteins) in the cytosol and by that enhancing the unwanted sequestering abilities of amyloid deposits. That might explain the growing number of biomolecules that are identified in the composition of Lewy bodies.⁴⁸⁻⁴⁹ Furthermore, the ability of α S to ‘abduct’ multivalent counterions could interfere with the ion balance once the accumulation of α S in amyloid deposits becomes significant. The physiological balance of trace elements (multivalent) may be most vulnerable in this respect.

References

1. Oosawa, F., Interaction between Parallel Rodlike Macroions. *Biopolymers* **1968**, *6*, 1633-1647.
2. Ha, B. Y.; Liu, A. J., Counterion-Mediated Attraction between Two Like-Charged Rods. *Phys. Rev. Lett.* **1997**, *79*, 1289-1292.
3. Podgornik, R.; Parsegian, V. A., Charge-Fluctuation Forces between Rodlike Polyelectrolytes: Pairwise Summability Reexamined. *Phys. Rev. Lett.* **1998**, *80*, 1560-1563.
4. Gronbech-Jensen, N.; Mashl, R. J.; Bruinsma, R. F.; Gelbart, W. M., Counterion-Induced Attraction between Rigid Polyelectrolytes. *Phys. Rev. Lett.* **1997**, *78*, 2477-2480.
5. Shklovskii, B. I., Wigner Crystal Model of Counterion Induced Bundle Formation of Rodlike Polyelectrolytes. *Phys. Rev. Lett.* **1999**, *82*, 3268-3271.
6. Tang, J. X.; Wong, S.; Tran, P. T.; Janmey, P. A., Counterion Induced Bundle Formation of Rodlike Polyelectrolytes. *Berichte der Bunsengesellschaft für physikalische Chemie* **1996**, *100*, 796-806.
7. Gosule, L. C.; Schellman, J. A., Compact Form of DNA Induced by Spermidine. *Nature* **1976**, *259*, 333-335.
8. Wong, G. C. L.; Lin, A.; Tang, J. X.; Li, Y.; Janmey, P. A.; Safinya, C. R., Lamellar Phase of Stacked Two-Dimensional Rafts of Actin Filaments. *Phys. Rev. Lett.* **2003**, *91*.
9. Needleman, D. J.; Ojeda-Lopez, M. A.; Raviv, U.; Miller, H. P.; Wilson, L.; Safinya, C. R., Higher-Order Assembly of Microtubules by Counterions: From Hexagonal Bundles to Living Necklaces. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 16099-16103.
10. Hud, N. V.; Downing, K. H.; Balhorn, R., A Constant Radius of Curvature Model for the Organization of DNA in Toroidal Condensates. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 3581-3585.
11. Semerdzhiev, S. A.; Dekker, D. R.; Subramaniam, V.; Claessens, M. M., Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. *ACS Nano* **2014**, *8*, 5543-5551.
12. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., Alpha-Synuclein in Lewy Bodies. *Nature* **1997**, *388*, 839-840.
13. Bolisetty, S.; Mezzenga, R., Amyloid-Carbon Hybrid Membranes for Universal Water Purification. *Nat. Nanotechnol.* **2016**.
14. Shimanovich, U.; Efimov, I.; Mason, T. O.; Flagmeier, P.; Buell, A. K.; Gedanken, A.; Linse, S.; Akerfeldt, K. S.; Dobson, C. M.; Weitz, D. A., *et al.*, Protein Microgels from Amyloid Fibril Networks. *ACS Nano* **2015**, *9*, 43-51.

15. Yolamanova, M.; Meier, C.; Shaytan, A. K.; Vas, V.; Bertoncini, C. W.; Arnold, F.; Zirafi, O.; Usmani, S. M.; Muller, J. A.; Sauter, D., *et al.*, Peptide Nanofibrils Boost Retroviral Gene Transfer and Provide a Rapid Means for Concentrating Viruses. *Nat. Nanotechnol.* **2013**, *8*, 130-136.
 16. Knowles, T. P. J.; Oppenheim, T. W.; Buell, A. K.; Chirgadze, D. Y.; Welland, M. E., Nanostructured Films from Hierarchical Self-Assembly of Amyloidogenic Proteins. *Nat. Nanotechnol.* **2010**, *5*, 204-207.
 17. Reynolds, N. P.; Charnley, M.; Mezzenga, R.; Hartley, P. G., Engineered Lysozyme Amyloid Fibril Networks Support Cellular Growth and Spreading. *Biomacromolecules* **2014**, *15*, 599-608.
 18. Petoukhov, M. V.; Franke, D.; Shkumatov, A. V.; Tria, G.; Kikhney, A. G.; Gajda, M.; Gorba, C.; Mertens, H. D. T.; Konarev, P. V.; Svergun, D. I., New Developments in the Atsas Program Package for Small-Angle Scattering Data Analysis. *J. Appl. Crystallogr.* **2012**, *45*, 342-350.
 19. Lieleg, O.; Bausch, A. R., Cross-Linker Unbinding and Self-Similarity in Bundled Cytoskeletal Networks. *Phys. Rev. Lett.* **2007**, *99*.
 20. Grosberg, A. Y.; Nguyen, T. T.; Shklovskii, B. I., Colloquium: The Physics of Charge Inversion in Chemical and Biological Systems. *Rev Mod Phys* **2002**, *74*, 329-345.
 21. Bungenberg de Jong, M. G., Colloid Science, 2. Reversible Systems, Kruyt, G.R. In New York: Elsevier: 1949; pp 333-432.
 22. Cipelletti, L.; Ramos, L.; Manley, S.; Pitard, E.; Weitz, D. A.; Pashkovski, E. E.; Johansson, M., Universal Non-Diffusive Slow Dynamics in Aging Soft Matter. *Faraday Discuss.* **2003**, *123*, 237-251.
 23. Cipelletti, L.; Manley, S.; Ball, R. C.; Weitz, D. A., Universal Aging Features in the Restructuring of Fractal Colloidal Gels. *Phys. Rev. Lett.* **2000**, *84*, 2275-2278.
 24. Lieleg, O.; Kayser, J.; Brambilla, G.; Cipelletti, L.; Bausch, A. R., Slow Dynamics and Internal Stress Relaxation in Bundled Cytoskeletal Networks. *Nat. Mater.* **2011**, *10*, 236-242.
 25. Lieleg, O. In Vitro Model Systems of the Actin Cortex. PhD Thesis, Technische Universität München, München, 2008.
 26. Lieleg, O.; Claessens, M. M. A. E.; Luan, Y.; Bausch, A. R., Transient Binding and Dissipation in Cross-Linked Actin Networks. *Phys. Rev. Lett.* **2008**, *101*.
 27. Tobolsky, A. V., Stress Relaxation Studies of the Viscoelastic Properties of Polymers. *J. Appl. Phys.* **1956**, *27*, 673-685.
-

28. Spruijt, E.; Sprakel, J.; Lemmers, M.; Stuart, M. A. C.; van der Gucht, J., Relaxation Dynamics at Different Time Scales in Electrostatic Complexes: Time-Salt Superposition. *Phys. Rev. Lett.* **2010**, *105*.
29. Wyss, H. M.; Miyazaki, K.; Mattsson, J.; Hu, Z. B.; Reichman, D. R.; Weitz, D. A., Strain-Rate Frequency Superposition: A Rheological Probe of Structural Relaxation in Soft Materials. *Phys. Rev. Lett.* **2007**, *98*.
30. Adolf, D.; Martin, J. E., Time Cure Superposition During Cross-Linking. *Macromolecules* **1990**, *23*, 3700-3704.
31. Ferry, J. D., *Viscoelastic Properties of Polymer*. Wiley: New York, 1980.
32. Trappe, V.; Weitz, D. A., Scaling of the Viscoelasticity of Weakly Attractive Particles. *Phys. Rev. Lett.* **2000**, *85*, 449-452.
33. Isambert, H.; Maggs, A. C., Dynamics and Rheology of Actin Solutions. *Macromolecules* **1996**, *29*, 1036-1040.
34. Rubinstein, M.; Colby, R. H., *Polymer Physics*. Oxford University Press: Oxford, 2003; p xi, 440 p.
35. Liu, J.; Gardel, M. L.; Kroy, K.; Frey, E.; Hoffman, B. D.; Crocker, J. C.; Bausch, A. R.; Weitz, D. A., Microrheology Probes Length Scale Dependent Rheology. *Phys. Rev. Lett.* **2006**, *96*.
36. Derosier, D. J.; Tilney, L. G., How Actin-Filaments Pack into Bundles. *Cold Spring Harbor Symp. Quant. Biol.* **1981**, *46*, 525-540.
37. Claessens, M. M. A. E.; Semmrich, C.; Ramos, L.; Bausch, A. R., Helical Twist Controls the Thickness of F-Actin Bundles. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 8819-8822.
38. Sweers, K. K. M.; Segers-Nolten, I. M. J.; Bennink, M. L.; Subramaniam, V., Structural Model for Alpha-Synuclein Fibrils Derived from High Resolution Imaging and Nanomechanical Studies Using Atomic Force Microscopy. *Soft Matter* **2012**, *8*, 7215-7222.
39. Heussinger, C.; Frey, E., Floppy Modes and Nonaffine Deformations in Random Fiber Networks. *Phys. Rev. Lett.* **2006**, *97*.
40. Lieleg, O.; Claessens, M. M. A. E.; Heussinger, C.; Frey, E.; Bausch, A. R., Mechanics of Bundled Semiflexible Polymer Networks. *Biophys. J.* **2007**, 139a-139a.
41. Huisman, E. M.; Heussinger, C.; Storm, C.; Barkema, G. T., Semiflexible Filamentous Composites. *Phys. Rev. Lett.* **2010**, *105*.
42. Wilhelm, J.; Frey, E., Elasticity of Stiff Polymer Networks. *Phys. Rev. Lett.* **2003**, *91*, 108103.

43. Mackintosh, F. C.; Kas, J.; Janmey, P. A., Elasticity of Semiflexible Biopolymer Networks. *Phys. Rev. Lett.* **1995**, 75, 4425-4428.
44. Heussinger, C.; Schaefer, B.; Frey, E., Nonaffine Rubber Elasticity for Stiff Polymer Networks. *Phys Rev E* **2007**, 76.
45. Rees, D. A., Structure, Conformation, and Mechanism in the Formation of Polysaccharide Gels and Networks. In *Adv. Carbohydr. Chem. Biochem.*, Melville L. Wolfrom, R. S. T.; Derek, H., Eds. Academic Press: 1969; Vol. Volume 24, pp 267-332.
46. Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. J. C.; Thom, D., Biological Interactions between Polysaccharides and Divalent Cations - Egg-Box Model. *FEBS Lett.* **1973**, 32, 195-198.
47. Ravanat, G.; Rinaudo, M., Investigation on Oligogalacturonic and Polygalacturonic Acids by Potentiometry and Circular-Dichroism. *Biopolymers* **1980**, 19, 2209-2222.
48. Beyer, K.; Ariza, A., Protein Aggregation Mechanisms in Synucleinopathies: Commonalities and Differences. *Journal Of Neuropathology and Experimental Neurology* **2007**, 66, 965-974.
49. Wakabayashi, K.; Tanji, K.; Mori, F.; Takahashi, H., The Lewy Body in Parkinson's Disease: Molecules Implicated in the Formation and Degradation of Alpha-Synuclein Aggregates. *Neuropathology* **2007**, 27, 494-506.

CHAPTER 7

DONE AND TO DO

The self-assembly of a protein into amyloid fibrils and the formation of inclusion bodies is a common denominator of many diseases. The formation of alpha-synuclein (α S) amyloid fibrils and their subsequent accumulation is the pathological signature of Parkinson's disease. Details on how exactly these fibrillar deposits assemble *in vivo* remain obscure and so does their pathological role. Besides their role in diseases amyloids have also a less sinister side. They are very interesting from a material perspective. Protein fibrils are mechanically and chemically very stable and many organisms seem to have exploited these beneficial properties to design functional amyloid materials. Amyloids have therefore been enjoying increasing interest from many different fields of material research. For example, fibrils have been utilized as templates for metallic nanowires with potential application in electronics, as vehicles in the field of drug delivery and as scaffolds for tissue engineering.

In order to explore further the properties of amyloid fibrils for the design of novel biomaterials and to understand the pathological appearance of inclusion bodies we need to understand the self-assembly process responsible for their formation and the interactions driving it. In this thesis we systemically study the self-assembly of α S into fibrils, higher order structures and macroscopic materials. We continuously interpret our findings in the context of both: disease conditions or material science.

Mapping the interactions: Formation of suprafibrillar aggregates (SFAs). In Chapter 2 we show that by introducing the appropriate physico-chemical conditions, α S undergoes a multiscale self-assembly process to form higher order fibrillar structures in a cell free environment. The presence of counterions in a sufficiently high concentration triggers the formation of SFAs, which underlines the importance of electrostatic interactions in this self-assembly phenomenon. Both, monovalent and divalent counterions successfully induce the assembly of SFAs. The formation of SFAs in the presence of monovalent ions imply a source of inter-fibril attraction different from the one that is responsible for higher order-

organization of other biopolymers such as DNA, microtubules and actin, i.e. attraction due to ion correlations or bridging. Temperature induced clustering of α S fibrils suggests a hydrophobic origin of the attractive interactions. In contrast to monovalent ions, with divalent ions SFAs form at lower ionic strength. This observation suggests that the role of multivalent ions in the α S interfibril (and inter-monomer) interactions might be diverging from a simple charge screening. Thus, an attraction generated from counterion correlations or bridging cannot be ruled out when multivalent ions are present in the system. In addition, we have shown that electrostatic interactions could play a regulatory role for the size of the aggregates. All these findings are interesting in the context of materials and disease. α S could act as an inspiration for the design of proteins/peptides that inherit its ability to self-assemble across length scales under the influence of simple physico-chemical triggers such as counter ions. Moreover, controlling the interactions driving the self-assembly phenomenon might allow us to tune the micro- and perhaps the macroscopic properties of the resulting amyloid based materials. The ability of α S to form SFAs under a wide range of conditions may also be linked to the appearance of Lewy bodies and neurites in Parkinson's disease. The conditions (ionic strength and presence of multivalent ions) in the cytosol are most likely in the range where the fibrils have an enhanced propensity to aggregate. Thus, once formed *in vivo* the ultimate fate of fibrils might be to cluster and form inclusion bodies.

Despite the gained insights on the *in vitro* hierarchical self-assembly of α S, there is not much of the mechanistic details on how these SFAs evolve in time, especially in the early stages of formation process, are known. Understanding the first steps of the assembly process could bring us closer to a more complete understanding of this complex phenomenon. Moreover, analyzing snapshots from the different stages of the aggregation process could provide us with clues why the SFAs end up in different and often anisotropic morphologies. A composite approach deploying the combination of microfluidics, fluorescence microscopy and atomic force microscopy (AFM) might prove sufficient to monitor the whole self-assembly process. Placing an aggregating sample (with ThT) in a microfluidic chamber accompanied with a micro-magnetic stirring device and subsequently acquiring a real-time video of the aggregation process might provide details on how the

aggregates develop in time. Because of the limited resolution of conventional fluorescence microscopy, AFM or super resolution microscopy techniques can be used to study the aggregates in their most early stage of development.

A zoo of morphologies. In **Chapter 3** we use an extended set of experimental conditions to underline the sensitivity of SFAs formation to electrostatic interactions. The electrostatic interactions were fine-tuned using a combination of approaches, namely: i) inducing SFAs formation in an extended range of salt concentrations; ii) formation of SFAs in the presence α S WT and 1-108 truncated version missing the majority of the negative charges at the C-terminal part; iii) variation of the pH of the solution. These approaches generated SFAs with a many different morphologies. The markedly different aggregation kinetics in the presence of Ca^{2+} (compared to Na^+) further support the findings (initially conceived in **Chapter 2**) that multivalent ions might play a role in the attractive interactions on inter-fibrillar and possibly on inter-monomer level. The formation of SFAs greatly influences the aggregation kinetics. That is well illustrated by the progressive suppression of the seeding efficiency of pre-formed fibrils with increasing ionic strength. The tight packing of fibrils inside the aggregates probably suppresses processes such as secondary nucleation, fibril fragmentation and monomer addition. The tighter packing has a pronounced effect on the SFAs morphology as well. With increasing salt concentration the overall size of the SFAs becomes smaller. The findings in this chapter raise an interesting point for discussion regarding the potential *in vivo* role of Lewy bodies. If the fibrils incorporated in the aggregates are unable to recruit monomers fast enough, then it is disputable if the inclusion bodies would adopt a protective form; they cannot prevent the formation of primary nuclei and potentially toxic species of aggregates. It is perhaps safe to say however, that it is unlikely that the inclusion bodies facilitate proliferation of fibrils (oligomers) since they obviously slow down the aggregation kinetics.

A tighter packing of fibrils inside the aggregates would explain some of the main findings in this chapter, so an experimental validation of this hypothesis would be of use. Sedimentation velocity analysis of the aggregates could provide clues for differences in density among the various types of SFAs. AFM image height analysis of dried SFAs could also

prove useful in estimating the average density of the SFAs in a similar fashion to what was done in **Chapter 4**.

Structure. In **Chapter 4 I** focused on the architecture of the cylindrical SFAs. More than 90% of the SFAs volume could be assigned to water making them a true hydrogel particles. We have exploited the optical properties of the fibrils to analyze the architecture of these type of aggregates. Polarized light microscopy (PLM) images suggest a different architecture of the SFAs at the exterior and peripheral parts. The fibrils located in the internal parts of the aggregates have a random distribution of orientations while the fibrils at the periphery seem to have a preference for high crossing angles. We hypothesize that this is due to the difference in the ionic environments at which the different parts of the aggregates form. Since αS seem to 'bind' multivalent counterions, the availability of 'free' counterions changes with the progress of the aggregation process. The SFAs need to reset the way they grow in order to comply with the ionically poorer environment. A higher crossing angle between fibrils is established which minimizes the electrostatic repulsion and allows for the aggregates to grow further. We have also investigated the structure of the SFAs using an alternative approach deploying αS monomers coupled with a solvatochromic dye. In line with the PLM findings, this approach shows that the interior and periphery of the SFAs are indeed different.

Hydrophobic interactions: a closer look. In **chapter 5** we quantify the inter-fibrillar hydrophobic interactions by studying αS amyloid network with oscillatory rheology. First we study the aging process of αS amyloid gels. The networks show unusual non-monotonic changes in the mechanical response with time. Slow dynamics mark the aging of the network. These dynamics seem to be driven by two overlapping processes: i) monomer depletion; ii) buildup of stress by the formation of pre-strained fibrils and a subsequent relaxation. These dynamics could be significantly sped up by heating the system up and driving it faster towards its equilibrium state. This approach conveniently shortens the time scale of this type of experiments since it could take over a month for a network to equilibrate at room temperature. As anticipated the equilibrated fibrillar networks exhibit a spectacular temperature response. In contrast to most biopolymer (semi-flexible) networks, αS network stiffen significantly upon the administration of higher temperatures.

Even more remarkably, the stiffening seems to be completely reversible (least the time scales of the experiments). This finding agrees very well with the temperature induced clustering of fibrils observed in **Chapter 2**. Temperature stimulates the inter-fibril hydrophobic interactions and by that effectively cross-links the network. To quantitatively describe this behavior of α S networks, we applied a novel expression that is based on an established scaling relation (floppy mode model) and the Boltzmann statistics of hydrophobic ‘bonds’. The expression describes the experimental data very well which gives us extra confidence that the physico-chemical description of the interfibril interactions that we have hypothesized is plausible. The model allows us also to estimate the apparent size of the hydrophobic patches that mediate the inter-fibrillar interactions. The size of the patches is in good agreement with what has been estimated for capsid proteins of viruses.

The work in this chapter illustrates how we can ‘tame’ hydrophobic interactions and tune the mechanical properties of a cross-linker free amyloid network. Our results show that the storage modulus of the network can be increased with almost an order of magnitude by simply elevating the temperature. The findings from this chapter could also serve as source of inspiration for new design principles of thermo-responsive materials. We expect that the thermo-stiffening effect can be further tuned by properly engineering the size (and perhaps density) of the patches at the surface of protein fibrils.

The existence of hydrophobic patches in the α S fibril architecture may also link to a possible toxic effect of amyloid fibrils. A connection between the exposure of hydrophobic surface in amyloids and their toxicity has been proposed by numerous investigations. Thus the formation and accumulation of amyloid deposits might increase the total hydrophobic surface present in the cell and by that establishing toxicity.

The applicability of the approach used in this chapter to study inter-fibril hydrophobic interactions could be extended to many other amyloidogenic systems. Beta-lactoglobulin (β L) amyloid gels for example, do not show thermo-stiffening. Interestingly, β L fibrils do not induce toxic effects either. Thus, it would be interesting to check if there is a correlation between fibril networks of different amyloidogenic proteins that exhibit (or don’t) thermo-stiffening and the toxicity of their individual fibrils. If there is a clear correlation, i.e. the fibrils of most of the proteins whose networks exhibit thermo-stiffening

are toxic (or the reverse), than the connection between hydrophobic surface exposure and toxicity could be further verified.

Multivalent ions: more than just charge screening. In the final experimental **Chapter 6** we again use oscillatory rheology and α S amyloid networks but to study the interfibril interactions in the presence of counterions with different valence. Indications that multivalent ions may mediate attractive interfibril interactions were already provided by the work done in **Chapters 2 and 3**. Here we show in detail the different effect that mono- and multivalent ions have on the interactions between α S fibrils. The α S amyloid network significantly stiffens upon the administration of different concentrations and species of salt. The stiffening effect however, is considerably stronger in the presence of multivalent ions. While monovalent ions simply screen the electrostatic interactions and facilitated the hydrophobic contacts at the junction points of the fibril network, multivalent counterions seem to act as cross linkers themselves. Counterions bridging fibrils at the junction points is the most likely mechanism causing the enormous stiffening of the network. We quantitatively link the stiffening with the concentration of multivalent counterions by using a scaling relation for cross-linked semi-flexible networks (floppy mode model). The modified scaling seems to adequately capture the mechanical response of the α S amyloid networks cross-linked with multivalent ions. Trivalent ions stand out as the most potent cross-linkers. Since the strength of Coulombic interactions depends on the size of the electrical charge, counter ions with higher valence most likely provide stronger attraction between the fibrils.

The findings in chapter 5 and 6 clearly outline α S amyloid networks as multistimuli responsive materials. Compared to temperature, ions can modulate the viscoelastic response of α S network in an even larger dynamic range spanning almost 3 orders of magnitude. The ability of α S fibrils to 'bind' multivalent counter may be related to how amyloid deposits interfere with the normal functioning of a cell. The progress of amyloid deposit accumulation could seriously disturb the ion balance within the cell especially that of trace elements which are present in very low concentrations. The ability of α S fibrils to bind ions might be seen as a beneficial property for material applications. It has been demonstrated that β L based materials can be used as heavy metal binding membranes in which every β L molecule is able to sequester one heavy metal ion. Since α S fibrils bind

multivalent ions in a better stoichiometry (approximately 3.5 ions per α S and given that β L is a bigger protein) it could turn to be a much more efficient material for such type of applications.

Since all the rheology measurements were performed in the regime of linear viscoelastic response, it would be interesting to perform experiments in the non-linear regime and test if the network stiffens at higher deformations similarly to many other biopolymers such as actin, fibrin, collagen and others. It would be also be interesting to investigate if such response can be tuned by applying the same physico-chemical stimuli that were used in the studies carried out for this thesis.

SAMENVATTING

De zelf-assemblage van een eiwit in een amyloïde fibril is bekend bij veel ziektes. Talloze ziektebeelden gaan gepaard met de aggregatie van een bepaald eiwit in amyloïde fibrillen en de ophoping hiervan in weefsel in de vorm van inclusion bodies. In het geval van de ziekte van Parkinson is alpha-synucleïne (α S) het eiwit dat de amyloïde fibrillen vormt. De amyloïde afzettingen die gevormd worden door deze fibrillen worden ook wel Lewy bodies genoemd en ze worden gezien als een typisch kenmerk voor de ziekte van Parkinson. De pathologische betekenis van deze super fibrillaire structuren en het mechanisme waardoor ze gevormd worden is nog steeds onbekend.

Amyloïde vorming en organisatie hiervan op een hoger niveau is niet noodzakelijk gerelateerd aan een ziektebeeld. De natuur heeft de voordelen van de eigenschappen van amyloïden gebruikt om functionele materialen te maken. Bijvoorbeeld, amyloïde netwerken worden gebruikt als opslagsysteem voor hormonen in klieren en andere organen en als kleefmiddel bij zeepokken of E. Coli om hun kolonies op verschillende oppervlaktes af te zetten. Sommige vissen- en insectensoorten gebruiken de chemische en mechanische stabiliteit van amyloïde fibrillen – vergelijkbaar met die van zijde en staal – door ze in te zetten als een versterkend frame om hun eieren te beschermen tegen schade. Deze en vele andere voorbeelden classificeren amyloïden als een aantrekkelijke kandidaat voor het ontwerpen van nieuwe biologisch geïnspireerde materialen.

Om de eigenschappen te ontdekken van amyloïde fibrillen voor het ontwerp van nieuwe biomaterialen of om de pathologische vorming van inclusion bodies te doorgronden moeten we het proces van zelf assemblage achter hun vorming begrijpen en de interacties die dit reguleren. In dit proefschrift bestuderen we systematisch de formatie van α S in fibrillen in hogere order structuren. Hierna bespreken we onze bevindingen in de context van ziekte condities en materiaalkunde.

In **hoofdstuk 1** geven we een algemene α S introductie, over zijn voorlopige in vivo functies en zijn rol in ziekte pathologie. In dit gedeelte wordt een groot gedeelte van de relevante wetenschappelijke literatuur besproken.

In **hoofdstuk 2** laten we zien dat α S hiërarchisch zelf assembleert in supra fibrillaire aggregaten (SFAs) van een beperkte grootte die de kloof overbruggen tussen nano- en microschaal. Het zelf-assemblage proces is een consequentie van de wisselwerking tussen de langeafstand afstotende en de korte afstand aantrekkende interacties. Het afstotende deel van de interacties stamt van de hoge negatieve netto lading van de α S fibrillen. De aantrekkende interacties lijken een hydrofobe oorsprong te hebben. Bovendien, de lading op de fibrillen heeft een regulerende rol in het groeiproces van de aggregaten. De in vitro gegenereerde supra fibrillaire aggregaten lijken te ontstaan in fysiologisch relevante eiwit concentraties en ion sterktes. De totale ion sterkte in het cytosol is hoogstwaarschijnlijk in het gebied waar de fibrillen een verhoogde neiging tot zelf aggregatie hebben. Dus, zodra fibrillen zijn gevormd in vivo zijn ze voorbestemd om te klusteren en supra fibrillaire structuren te vormen zoals Lewy bodies en neurieten.

In **hoofdstuk 3** demonstreren we dat α S aggregatie en SFAs vorming zeer gevoelig zijn voor elektrostatistische interacties. Elektrostatistische interacties vormen SFAs in een overvloed van verschillende morfologie. Deze morfologie hangt af van de lading van het eiwit en de zout condities. In de aanwezigheid van multivalente tegenionen, is lading screening alleen niet voldoende om de experimentele observaties te beschrijven, die impliceren dat bijkomende elektrostatistische interactie mechanismes een rol spelen. De formatie van SFAs beïnvloedt ook de aggregatie kinetiek. De formatie van SFAs veroorzaakt significante vertraging in de aggregatie kinetiek. De verschijning van SFAs verandert de aggregatie dynamiek aanzienlijk, doordat in zulke hogere orde structuren snelheidsbepalende processen zoals secundaire nucleatie, fibril fragmentatie en het toevoegen van monomeren aan actieve fibrillen, waarschijnlijk worden onderdrukt. Er moet dus goed opgepast worden als het gaat om de interpretatie van kinetische data die verkregen zijn onder de condities waarbij SFAs zijn gevormd. Deze bevinding maakt ook ruimte voor enkele interessante overwegingen in een fysiologisch kader. Als amyloïde fibrillen er niet in slagen om snel genoeg α S monomeren te binden dan kunnen ze niet voorkomen dat primaire nucleatie en dus de formatie van potentieel toxische oligomeren en fibrillen gevormd worden in oplossing. Als fibrillen in SFAs de monomeren niet efficiënt

opnemen, hoe beschermend kan dan de formatie van LB en andere amyloïde afzettingen zijn in eiwit aggregatie ziektes?

In **hoofdstuk 4** bestuderen we de structuur van de cilindrische SFAs. De SFAs bestaan voor meer dan 90% uit water, waardoor ze geclassificeerd kunnen worden als hydrogel deeltjes. We hebben ook laten zien dat α S fibrillen, net als fibrillen van andere amyloïde-achtige eiwitten, birefringent zijn. We hebben deze optische eigenschap gebruikt om de architectuur van de SFAs te onderzoeken en hebben de modulaire opbouw, ontstaan door de verschillend georganiseerde fibrillen in het binnenste en de buitenkant van de SFAs, zichtbaar gemaakt. De fibrillen gelokaliseerd in het binnenste van gedeelte van de aggregaten hebben een willekeurige oriëntatie distributie terwijl de fibrillen aan de buitenkant een voorkeur hebben voor kruising onder een grote hoek.

In **hoofdstuk 5** kwantificeren we de interfibrillaire hydrofobe interacties door α S amyloïde netwerken te bestuderen met behulp van oscillerende reologie. α S amyloïde netwerken in evenwicht vertonen opmerkelijke thermoresponsieve eigenschappen. De α S fibrillaire gel verstijft aanzienlijke bij hogere temperaturen. Interessant is dat het systeem compleet terug gaat naar de oorspronkelijke staat zodra de temperatuur is gezakt. De thermische verstijving van het α S netwerk is een consequentie van de verhoogde interfibrillaire contacten gestimuleerd door de hogere temperatuur. Dit is in overeenstemming met de voorheen vastgestelde kwalitatieve bevindingen, suggererend dat de hydrofobe effecten een belangrijke rol spelen in de interactie tussen α S fibrillen. De hydrofobe interactie tussen fibrillen suggereert dat meerdere hydrofobe domeinen in het binnenste van de fibril niet blootgesteld worden aan het oplosmiddel. Bij hogere temperaturen worden deze hydrofobe gebieden geactiveerd, waardoor het aantal mogelijke contactpunten wordt verhoogd tussen de fibrillen. Uit onze gegevens blijkt dat er mogelijkheden zijn om deze interacties af te schermen en de mechanische eigenschappen van de amyloïde materialen te beïnvloeden. Een correlatie tussen de blootstelling van hydrofobe oppervlaktes in amyloïde aggregaten en hun toxiciteit is gesuggereerd in talloze studies. De opeenhoping van amyloïde afzettingen zou het totale aantal hydrofobe oppervlaktes in de cel toe laten nemen en kunnen interfereren met de normale werking.

Het bestaan van hydrofobe domeinen in de architectuur van α S fibrillen zou ook gevolgen kunnen hebben in de context van de ziekte condities.

In **hoofdstuk 6** laten we op een meer kwantitatieve manier de verschillende rol die mono- en divalente ionen hebben op de interfibrillaire interacties zien door α S netwerken te bestuderen oscillerende reologie. α S amyloïde netwerken verstijven aanzienlijk als reactie op verschillende concentraties en soorten zout. Het effect is klein in de aanwezigheid van monovalente tegenionen, maar significant in de aanwezigheid van di- en trivalente ionen. Monovalente ionen verlagen de elektrostatische afstoting tussen de negatief geladen α S fibrillen, waardoor hydrofobe interacties kunnen plaatsvinden. In tegenstelling, de multivalente ionen lijken zich als cross-linkers te gedragen en stimuleren de interactie tussen fibrillen op de kruispunten. Overbrugging door tegenionen is het meest voordehand liggende mechanisme dat verstijving van het netwerk veroorzaakt. De bevindingen in dit hoofdstuk laten zien dat tegenionen gebruikt kunnen worden om de mechanische respons van α S netwerken af te stemmen, binnen een opmerkelijk dynamisch bereik van bijna 3 ordes van grootte. De natuur doet dit op eenzelfde wijze met behulp van cross-linkers. In fysiologische context, het vermogen van α S om multivalente tegenionen te gebruiken kan de ionen balans verstoren, wanneer de ophoping van α S in amyloïde afzettingen significant wordt. Het fysiologische evenwicht van sporenelementen (tegenionen) kan in dit opzicht het meest kwetsbaar zijn.

Tot slot, **hoofdstuk 7** laat een overzicht zien van het werk dat is gedaan voor dit proefschrift en de hoogtepunten van de bevindingen komende hieruit.

LIST OF PUBLICATIONS:

Semerdzhev, S.A.; Roelof, D.R.; Subramaniam,V.; Claessens, M.M.A.E.; *Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. ACS Nano*, 2014, 8(6), PP 5543-5551
DOI: 10.1021/nn406309c

Stefanovic, A. N. D.; Lindhoud, S.; Semerdzhev, S.A; Claessens, M.M.A.E.; Subramaniam,V.; *Oligomers of Parkinson's Disease-Related α -Synuclein Mutants Have Similar Structures but Distinctive Membrane Permeabilization Properties. ACS Biochemsitry*, 2015, 54 (20), pp 3142-3150
DOI: 10.1021/bi501369k

Publications in preparation/ to be submitted:

Semerdzhev, S.A; Lindhoud, S.; Stefanovic; A. N. D.; Subramaniam,V.; van der Scoot, P.; Claessens, M.M.A.E.. *Aging and thermal stiffening of alpha-synuclein amyloid networks.* (to be submitted)

Semerdzhev, S.A.; Lindhoud, S.; Subramaniam,V.; Claessens, M.M.A.E. *Ionicly cross-linked amyloid networks.* (in preparation)

Semerdzhev, S.A.; Subramaniam,V.; Claessens, M.M.A.E. *Electrostatic interactions control apparent alpha-synuclein aggregation kinetics and the morphology of alpha-synuclein supra-fibrillar assemblies.* (in preparation)

Raiss, C.C.; Semerdzhev, S. A.; Grabmayr, H.; Konings, I.B.M; Bausch, A.R.; Subramaniam, V.; Claessens, M. M. A. E. *Alpha-Synuclein is an F-actin binding protein associated with filopodial and cortical actin filaments.* (in preparation)

Shvadchak, V.; Semerdzhev, S.; Chaudhary, H.; Claessens, M. M. A. E. *Role of alpha-synuclein N-terminus in fibril formation.* (in preparation)

Thangaraj, S.K.; Semerdzhev, S.A.; Dijk, L.; Claessens, M.M.A.E.; *Selective binding and sequestration of cytoskeleton proteins by α -synuclein amyloid assemblies* (in preparation)

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