Amyloids: from molecular structure to mechanical properties

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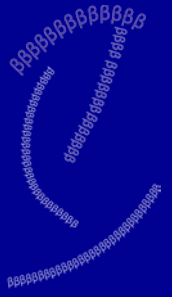
Appendix



Summary

Approximately 15 to 20% of the human body consists of proteins and peptides, and a similar percentage is observed for bacteria. Proteins serve a wide range of functions in nature, for instance in transport, storage, movement, support and stability, or as antibodies, enzymes or hormones. Proteins are composed of strings of amino acids and can fold into complex three-dimensional structures. This molecular (or secondary) structure is essential for the execution of their function. A special case of a three-dimensional structure of proteins are amyloids. Amyloids are aggregates which are formed upon self-assembly of peptides and proteins of diverse sequence, native structure and function. It is thought that the amyloid state is an intrinsic characteristic of polypeptide molecules under denaturing conditions. Amyloid fibrils are generally several nanometers in diameter and up to micrometers in length. They are remarkably rigid and strong, properties which are thought to have their origin in the hydrogen-bonded β-sheet core. In nature, amyloids are wide spread, in organism ranging from bacteria and fungi to invertebrates and man. In many cases they serve a useful purpose, for example by providing protection or stability, in cell adhesion or aggregation. However, amyloids are also related to a broad range of human diseases, including Alzheimer's and Parkinson's disease and type II diabetes mellitus, in which oligomers and/or fibrils accumulate in tissue and cause cell death. From an engineering perspective, the excellent mechanical properties and the tunable properties of amyloids make that (non-toxic) amyloids are potential candidates for applications in foods, tissue engineering and materials science.

The central goal in this thesis is to investigate the biophysical properties of amyloid fibrils over a range of length scales: from molecular structure to single fibril rigidity and network mechanics. Insights into the relation between the molecular structure and the mechanical properties are essential to understand and control the assembly process of amyloid fibrils. This is important both for rational therapeutic treatment of amyloid-related diseases and for potential applications of amyloids. In this thesis, I described the study of the molecular structure of model amyloid fibrils using various vibrational spectroscopy techniques. The morphology and rigidity of amyloid fibrils were determined using atomic force microscopy, fluorescence microscopy and scanning transmission electron microscopy. Network mechanics were measured by macroscopic rheometry. I investigated on all these length scales distinct types of amyloid fibrils, with the aim to get a better understanding of the origin of structural polymorphism of amyloid fibrils. Furthermore, control of amyloid formation and disaggregation by the polyphenol epi-gallocatechin-3-gallate (EGCG), a natural compound which potentially can be used for treatment of amyloid-related diseases, was studied.

In the first part of this thesis I focused on the molecular structure of amyloid fibrils and their relation to the structural polymorphism observed for amyloids. In Chapter 2, the relation between the molecular structure, morphology and rigidity of amyloid fibrils was investigated. The model protein β-lactoglobulin (β-lg), a milk protein, forms distinct types of amyloid fibrils dependent on the protein concentration during incubation at acidic pH and high temperature. At low β-lg concentration, the fibrils that are formed are long and straight, as observed with atomic force microscopy (AFM). The persistence length, a measure for rigidity, was several micrometers, in the order of the contour length. At high β-lg concentrations, short, worm-like fibrils are formed with a 40 times lower persistence length. Using vibrational sum-frequency generation spectroscopy (VSFG), we showed that the β-sheet content of the straight fibrils was higher than that of the worm-like fibrils. In Chapter 3 I investigated the differences in molecular structure and composition of the two fibril types in more detail. Mass spectrometry experiments show that the composition of the two fibril types is similar. The molecular structure of the fibrils was investigated with various bulk spectroscopy techniques (circular dichroism, Fourier transform infrared and Raman spectroscopy). All three techniques showed a large contribution of the β-sheet core of the amyloid fibrils. Surprisingly, no differences in molecular structure were observed for the two distinct fibril types, in contrast to the VSFG measurements. A drawback of the bulk spectroscopic techniques is that it is not possible to distinguish between different polymorphic forms of amyloid fibrils, or between their core and the surface. Therefore the molecular structure of the surface of the fibrils was probed using tip-enhanced Raman spectroscopy (TERS). In TERS, AFM and Raman spectroscopy are combined, which provides the possibility to probe the molecular structure of the fibrils with nanometer resolution. I showed that the surface of both types of amyloids formed from β-lg mainly contains unordered or α-helical structures, in contrast to their β-sheet core. Although no differences between distinct fibril types could be observed using the bulk-spectroscopic techniques, TERS experiments showed that the β-sheet content on the surface of the long, straight fibrils was higher than that on the worm-like fibrils. To elucidate the origin of the difference in morphology and molecular structure, the kinetics of fibril formation were followed using mass spectrometry and AFM. With mass spectrometry the hydrolysis of β-lg monomers into peptide fragments was investigated. The ratio between intact monomers and peptide fragments decreased faster for samples at high than at low β-lg concentration. Probably this difference is caused by the incorporation of intact monomers into worm-like fibrils formed at high β-lg concentration, while straight fibrils are composed of peptides. Using AFM, fibrils were observed after shorter incubation times for samples incubating at high β-lg concentration than for samples at low

concentration. In summary, the origin of the difference in morphology and molecular structure of amyloid fibrils is likely related to their formation kinetics.

In Chapter 4 TERS was used to investigate amyloid fibrils formed from the diabetes-related peptide hIAPP in detail. To understand amyloid toxicity, it is important to know the surface structure of amyloids. The molecular structure and amino acid composition of the surface of the fibrils was probed with nanometer resolution. Similar to β-lg amyloids, the surface of the hIAPP fibrils is mainly composed of unordered or α-helical structures, in contrast to the core, which is composed of β-sheets. Dependent on the pH during fibril formation, fibrils with a different surface structure and amino acid residue composition are formed. Furthermore, the molecular structure of hIAPP fibrils formed at a lipid interface was probed, because it is thought that hIAPP forms amyloids at the plasma membrane *in vivo*.

In the second part of my thesis I focused on the effect of the green tea polyphenol EGCG on amyloid formation, morphology and rigidity. EGCG is one of the most promising therapeutic agents for treatment of amyloid-related diseases. In Chapter 5 the effect of EGCG on the formation of hIAPP amyloid fibrils at a lipid interface was studied. Although EGCG is able to inhibit amyloid formation efficiently in bulk solution, at lipid interfaces the inhibition is less efficient. The formation of amyloids was followed by probing the molecular structure of hIAPP using VSFG and by imaging the amyloids using AFM. EGCG is not able to disaggregate mature fibrils at a phospholipid interface, in contrast to fibrils in bulk solution, which were disaggregated upon incubation with EGCG. In Chapter 6 the effect of EGCG at neutral and acid pH on amyloid fibrils was studied. As a model protein, hen egg white lysozyme (HEWL) was used. I showed that at acidic pH EGCG does not affect the morphology or rigidity of the amyloid fibrils. However, at neutral pH a subpopulation of thick fibrils was formed upon incubation with EGCG. AFM and electron microscopy imaging revealed that the thick fibrils formed large fibril aggregates. In the second part of the thesis I showed that EGCG is able to disaggregate and bundle amyloid fibrils, but that the efficacy is highly dependent on the conditions, like solution pH and the presence of lipid interfaces. It is therefore important to consider the treatment conditions during the design and testing of amyloid inhibitors.

The third part of my thesis focused on the mechanics of amyloid fibrils and networks thereof. In Chapter 7 the rigidity of HEWL amyloid fibrils was investigated using different approaches. The first approach was the determination of the persistence length based on fibril conformations of a large ensemble of amyloid fibrils imaged using AFM. An average persistence length between 2.5 and 4.4 μm was measured, depending on fibril hydration and the use of glass or mica as imaging substrate. The disadvantage of this

approach is that the fibrils are immobilized and dried on a surface, which potentially can affect their conformation and structure. Therefore I also used a second approach: fibrils were labeled with a fluorescent dye and the thermal bending undulations of freely fluctuating fibrils suspended in water were imaged using fluorescence microscopy. The range of persistence lengths that was observed for 8 fibrils was remarkably large: between 0.7 and 6.7 μm. For 2 other fibrils, the persistence length was even too large to measure by video microscopy. In summary, the two different techniques give similar average values of the persistence length in the range of several micrometers, but time-lapse imaging reveals a large variability for fibrils within the same sample. This variability is a signature of the structural polymorphism of amyloid fibrils.

In the last chapter of this thesis, Chapter 8, I investigated suspensions of amyloid fibrils on the largest length scale. The rheological behavior of amyloid fibril suspension is particular relevant for their applications, for instance in materials science and food texturing. I compared the viscoelastic properties of suspensions of the two distinct types of β-lg amyloid fibrils described in Chapters 2 and 3: long, rigid versus short, worm-like fibrils. Both fibril types behave in suspension as soft solids with an apparent yield stress. Strong shear-thinning behavior over a large range of shear rates was observed. By using a combination of rheology and small-angle neutron scattering (rheo-SANS) it was shown that long, rigid fibrils align under shear, while for short, worm-like fibrils no change in alignment was observed, even at high shear rates. This suggest that the strong shear-thinning behavior of short, worm-like fibrils is caused by weak attractive forces between the fibrils. In conclusion, I showed that suspensions of β-lg amyloid fibrils behave rheologically as suspensions of weakly attractive, semi-flexible rods.

The research in this thesis is a tiny step forward towards a better understanding of the structure and mechanics of amyloid fibrils. However, many open questions remain yet unresolved. In all research on amyloid fibrils, their polymorphic nature should be kept in mind, because this can highly affect bulk measurements both of molecular structure and mechanical properties. To avoid this problem, techniques which can be used to study single amyloid fibrils are highly promising to resolve the properties of amyloid fibrils. For instance, to get a better understanding of the molecular structure of amyloid fibrils, it would be interesting to study amyloid fibrils formed from short peptides with known sequence, for example tri-peptides, with high spatial resolution using TERS. Probably this can lead to the development of a structural model of amyloid fibrils, which can be compared to models resolved from nuclear magnetic resonance (NMR) measurements. In this thesis I showed the proof of principle of measurements of the rigidity of single amyloid fibrils by analysis of their thermal undulations. This could be used to study distinct types of amyloids in more detail, to compare fibrils formed from

different peptides or proteins or to test the effect of molecules that interact with amyloids, for instance polyphenols. By combining fluorescence microscopy with AFM imaging, for example using microfluidic devices, it would be possible to relate high resolution morphologic information to fibril mechanics.

For the therapeutic treatment of amyloid-related diseases there is still a long way to go. Although recent results obtained with natural compounds such as polyphenols look promising, understanding of the interactions of EGCG on amyloid oligomers, fibrils and the effect on formation kinetics is still limited. Furthermore, as long as the origin and mechanism of amyloid-related diseases is not yet elucidated, a rational design of drugs to avoid or stop amyloid toxicity remains challenging.