Biophysical Examination of Template-Controlled Amyloid Fibrillognesis

PhD Thesis

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1. INTRODUCTION

Beta-amyloids(Aß) are protein deposits in the form of filamentous aggregates implicated in Alzheimer's disease (AD). The amyloid fibrils form aggregates that are insoluble in water, the structural characterization of these fibers is limited by their insolubility and heterogeneous nature. The atomic force microscopy (AFM) is a technique to obtain images and other information at extremely high (nanometer) resolution, and therefore can successfully investigate the structure of amyloid fibrils and follow the process of fibrillogenesis.

The Aß25-35 peptide used in our experiments is thought to represent the biologically active, toxic fragment of the full-length beta1-42 peptide. This eleven amino acid long peptide forms amyloid fibrils with rapid kinetics in vitro, and the peptides neurotoxic properties correspond to that of the full-length peptide.

The amyloid fibrils show high level of structural order. The ß-strands run perpendicular to the fibre axis forming protofilaments with ß-sheet structure in which hydrogen bonding occurs along the length of the fibre. The fibril is composed of several protofilaments and can be straight or twisted.

The increasing number of diagnosed cases in neurodegenerative diseases is closely related to an ageing population. At the present time there is no treatment to cure or stop the progress of AD. Therefore the investigation of amyloid formation, structure and stability comes more and more in the foreground of scientific interest.

One of the novel therapeutic strategies for Alzheimer's disease is to block the early steps of amyloid fibril formation, The fact that several compounds may bound to the Aß polipeptide chain, preventing the peptides from aggregation, is giving hope to the patients suffering from AD. The so called beta-sheet breaker (BSB) peptides may be used as a potential therapy for AD.

But amyloids are not only the protagonists of protein folding diseases. Their self-assembly properties may provide very important tools for future nanotechnology and nanoelectronics applications.

2. OBJECTIVES

Desciphering the formation andpathomechanism of amyloid plaques may considerably contribute in understanding the molecular basics of AD and should lead to a range of therapeutic options. The Aβ25-35 peptid forms by epitaxial growth an oriented network on mica surface. In our experiments we used this ordered, easily and rapidly inducible network as an experimental model system.

Our research objectives can be summarized as follows:

I. Mapping the properties of epitaxially grown amyloid network on mica, in order to utilize it later as a model system:
    1. Morphological characterization of the network by atomic force microscopy.
    2. Nanomechanical characterization of the network by force spectroscopy.

II. Comparison of solution-grown and surface-grown amyloid fibrils. Can the epitaxially grown oriented network be considered as a good amyloid-fibril (or -plaque) model?
    1. Comparison of the morphology and nanomechanical properties of solution-grown fibrils with amyloid fibrils established on the surface.
    2. Investigation of the secondary structure of fibrils formed under different fibrillogenetic conditions by FTIR spectroscopy.

III. Does the BSB peptide „LPFFD” have generalizedbeta-sheet breaking properties? Investigation of the effect of LPFFD on the morphology and stability of the Aβ25-35 oriented network.

IV. Exploring the properties of a mutant Aβ25–35 peptide containing a Cys residue for the explicit purpose of specific chemical targeting.The Cys residue provides a specific chemical labeling of the fibrils, such a fibril network may be used in nanotechnology applications. Finding conditions in order fine-tune the properties of the network.

3. METHODS

1. THE SEQUENCE OF THE PEPTIDES USED IN OUR EXPERIMENTS

The amyloid and BSB peptides with 99% purity were produced by solid-state synthesis (SZTE-MTA Research Group on Supramolecular and Nanostructured Materials and Univerity of Szeged, Faculty of Medicine, Department of Medical Chemistry). The sequence of the wild type **Aß25-35**peptide: ^{25}GSNKGAIIGLM^{35}-amide. MW 1060,3 g/mol. The sequence of theN27C mutant Aß25-35 peptide, **Aß25-35_N27C**: ^{25}GSCKGAIIGLM^{35}-amide.MW: 1007,3 g/mol. The sequence of the BSB peptide:**LPFFD.** MW: 636 g/mol.

2. PREPARATION OF AMYLOID FIBRILS

In case of fibrils growing in solution the Aß25-35 peptides were dissolved in DMSO and further diluted Na-PBS buffer to a final concentration of 0.5 mg/ml. The Aβ25-35 fibrils were grown in solution at room temperature (24 C˚) for several days (3-14).

For the study of epitaxially growing fibrils insoluble aggregates were removed by centrifugation at 250.000g and 4ºC for 2 h (Beckman Coulter Optima^{TM} MAX Ultracentrifuge). Supernatant was stored at -80C˚.

Peptide concentration was measured with the quantitative bicinchoninic acid assay(Bicinchoninic acid assay, SIGMA).

3. SUBSTRATES USED IN OUR EXPERIMENTS

We used high-grade mica sheets for imaging epitaxially grown fibrils by atomic force microscopy. In freshly cleaved mica exposed to aqueous buffer solutions K^+ions diffuse away and onto this negatively charged surface biomolecules may adhere. As a function of the K^+ concentration of the sample a dynamic equilibrium emerges between the number of free and saturated binding pockets.

4. SAMPLE PREPARATION

Prior to atomic force microscopy imaging the amyloid samples were incubated on freshly cleaved mica surface. The surface was washed to remove the unbound fibrils, we scanned the surface with AFM. In case of dried samples ultrapure (Milli-Q) water was used. The surface was dried in N_2 gas.

5. ATOMIC FORCE MICROSCOPY

5. 1. THE MFP3D ATOMIC FORCE MICROSCOPE

Non-contactmode AFM imageswereacquiredwith an Asylum Research MFP3D instrument (Santa Barbara, CA) usingsilicon-nitridecantilevers (Olympus BioLever, resonancefrequencyinbuffer ~ 9 kHz; Olympus AC160 cantilever, resonancefrequencyin air ~ 330 kHz;). 512 x 512 or 1024x1024-pixel imageswerecollectedat a typicalscanningfrequency of 0.6-1.5 Hz and with a setpoint of 0.5-0.8V.

5. 2. FORCE SPECTROSCOPY

Surface-bound fibrils were mechanically manipulated by first pressing the cantilever (Olympus BioLever, lever A) tip against the surface, then pulling the cantilever away with a constant, pre-adjusted rate. Experiments were carried out under aqueous buffer conditions (PBS buffer, pH 7.4). In situ force spectroscopy was carriedout by first scanning (under aqueous buffer conditions) the mica-boundsample surface, pressing the cantilever tip to targeted surface locationsidentified on the image, and finally rescanning the surface to test forthe effect of the mechanical perturbations.

5. 3. IMAGE ANALYSIS

AFM images (fibril height, length, angle distribution, relative fibril-covered surface area) and force spectra were analyzed using algorithms built in IgorPro v6.03 MFP3D controller software (Wavementrics, Lake Oswego, OR).

6. FTIR SPECTROSCOPY

The secondarystructure of amyloid fibrils was investigated by FTIR spectroscopy (Bruker Vertex80v FTIR spectrometer). From the sample containing solution-grown amyloid fibrils the solvent was removed by means of vacuum-centrifugation. 256 scans were collected at 2 cm^{-1} resolution. Spectral evaluation was performed by using ProteIR software.

4. RESULTS

1. CHARACTERIZATION OF THE ORIENTED AMYLOID ß25-35 MODEL-SYSTEM

The epitaxially grown Aβ25-35 network is a reproducible, stabile system with homogenous parameters. If using this network later as an experimental model system, it is essential to characterize its morphology and nanomechanical properties.

1. 1. MORPHOLOGICAL CHARACTERIZATION

We investigated by atomic force microscopy the morphology of epitaxially oriented fibrils formed from synthetic Aβ25-35 peptides.

The fibrils adsorbed onto mica surface showed a highly ordered trigonal orientation (Figure 1.). The fibril orientation angles, relative to a horizontal referenceline, revealed discrete, 60° differences between fibril orientations. The positively charged *ε*-amino groupof Lys28 anchored the peptides onto the surface of the negatively charged freshly cleaved mica.

The formation of oriented A*β*25–35 fibrils thus involvesepitaxial growth driven by mica.Depending onthe initial peptide concentration the oriented Ab25-35 network appeared withina few minutes. The height of the fibrils showed a multimodal distribution, the height of individual fibrils varied between 0,8-4 nm. The fibrils had a smooth surface, we did’nt observe topographical periodicity.

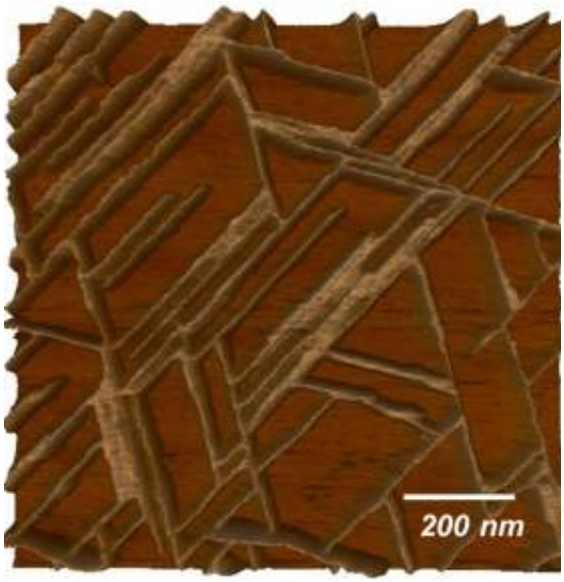

Figure 1. *Mica-adsorbedAβ25-35 fibrilsshowingspecific trigonal arrangement*

1. 2. FORCE SPECTROSCOPY

By using force spectroscopy methods we are able to explore the mechanics and structural dynamics of amyloid fibrils. Similar to the nanomechanical response of other Aβ fibrils, epitaxially grown, oriented Aβ25-35fibrils also showed characteristic force plateaus (Figure 2). The force plateau isinterpreted to arise from the unzipping of a β-sheet from thefibril.

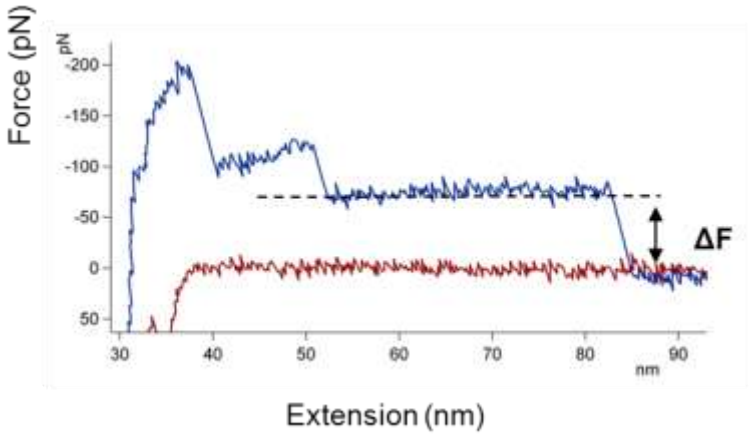

Figure 2. *Characteristicforce-lengthcurve*

2. COMPARISION OF SOLUTION-GROWN AND EPITAXIALLY GROWN AMYLOID FIBRILS

2. 1. MORPHOLOGY

Although both fibrils have the same chemical composition; they have been investigated using the same mica surface, their surface topography differed significantly. In contrast to the ordered trigonal arrangement of epitaxially grown fibrils, the fibrils assembled in solution showed a random orientation on mica (Figure 3) and they displayed a topographical periodicity. Epitaxially grown fibrils were homogeneous. AFM images of fibrils assembled in solution indicated a high degree of polymorphism: some of them displayed a presumably helical structure, but we also observed striated ribbons.

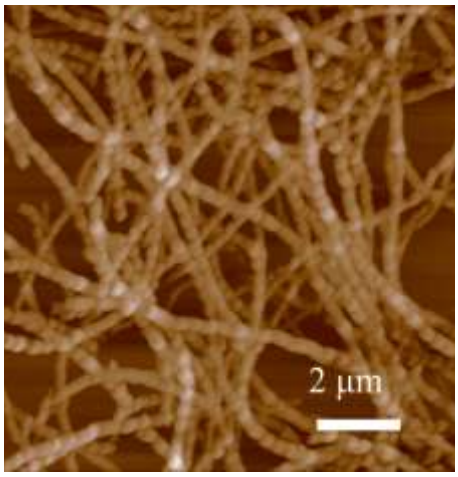

Figure3. *Morphology of solution-grownAß25-35fibrils*

In AFM images we observed fibrils of different heights. The average fibril height was 0.8 - 3 nm for the epitaxially-grown fibrils, whereas fibrils grown in solution were 7- 40 nm thick. Typically not more than five β- sheets build up one epitaxially-grown oriented fibril, whereas solution-grown fibrils may contain then times or even more β- sheet units.

Another important difference was the growth kinetics of fibrils: while fibril assembly in solution occurred on a time scale of hours to days, on mica surface fibrils appeared within a few minutes. The mica surface might act as a catalyzer accelerating the process of fibrillogenesis.

2. 2. FORCE SPECTROSCOPY

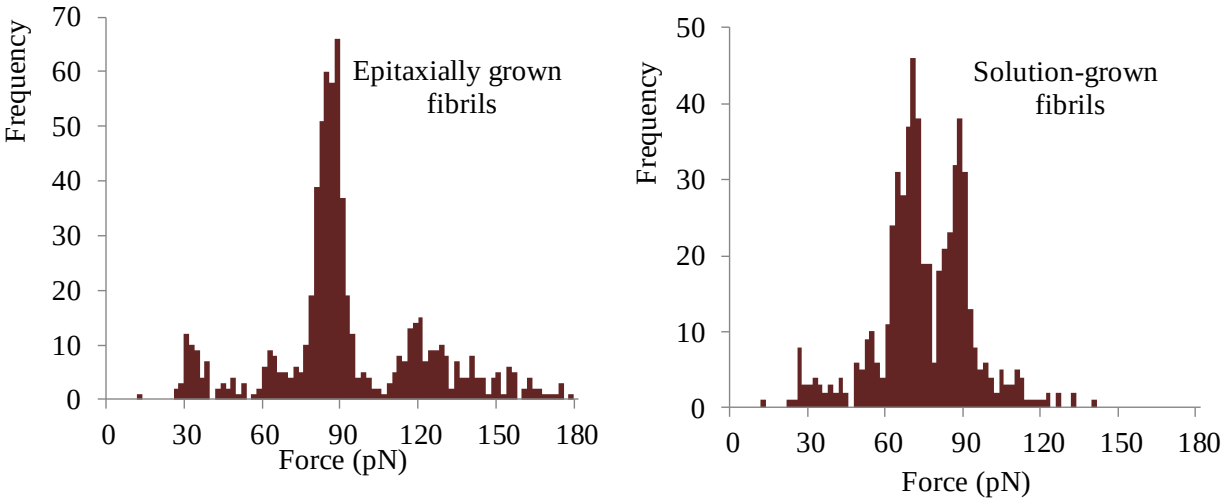

Figure 4.*Plateauheightdistribution*

The nanomechanical investigations of both types of fibrils showed similar plateau-like nanomechanical responses. The nanomechanical behavior of Aß25-35 fibrils is characterized by the appearance of force plateaus which correspond to the force-driven unzipping of protofilaments. Therefore, plateau forces are related to the mechanical stability of the fibril. The histograms obtained from the collected force data showed similar multimodal distribution (Figure 4.), the discrete maximum-values on the force distribution histograms corresponded to the force necessary to unzip 1-2-3-…protofilaments.The elementary plateau force was 30 pN.

2. 3. INVESTIGATING THE SECONDARY STRUCTURE OF SOLUTION-GROWN AND EPITAXIALLY GROWN AMYLOID FIBRILS

Fourier transform infrared (FTIR) spectroscopy was used to detect the presence of β-sheet secondary structure in fibrils.Bands between 1600 and 1700 cm^{-1} can be assigned to amide I modes (essentially C=O stretching vibrations of the amide group) and are sensitive to the secondary structure of proteins.

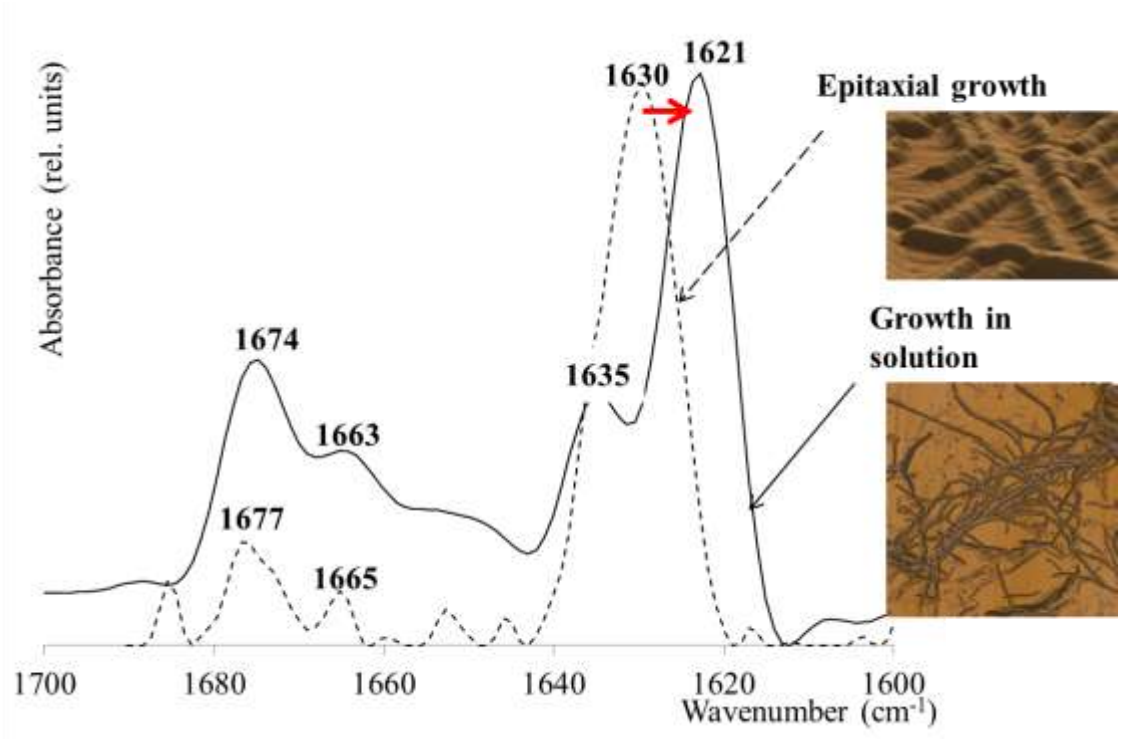

Figure5. *Comparative FTIR spectrum of solutiongrown and epitaxially grown amyloid fibrils.*

Comparing the IR spectra of epitaxially-grown and mature (14 days old) fibrils, we have found in both cases, that the IR spectra contained an intense peak indicative of beta-sheet structure: 1630 cm^{-1} and 1623 cm^{-1} for epitaxially grown fibrils and for fibrils assembled in solution respectively. The shift in the amide I band from 1630 cm^{-1} to 1623 cm^{-1}(Figure 5, red arrow) indicates a more compact structure for solution-grown amyloid fibrils.

3. EFFECT OF THE LPFFD BETA-SHEET BREAKER PEPTIDE ON ORIENTED Aß25-35 NETWORK

In the present work, we explored with AFM the effect of LPFFD on thegrowth, orientation, and force spectra of the Aß25-35 fibrilsepitaxially grown on mica. The stability of fibrils was determined by force spectroscopy.

3. 1. EFFECT OF LPFFD PEPTIDE ORIENTATION, STRUCTURE, AND ASSEMBLYDYNAMICS OF Aß25-35 ON MICA

To investigate the effect of LPFFD on the global structure of theAß25-35 network, we first measured the fibril orientation anglesin LPFFD-treated samples.We first measured the fibril orientation anglesin LPFFD-treated samples and then compared it with the orientation angles obtained for the control sample. Theorientation angles in the control sample revealed discrete, 60° differences between fibril orientations. Theorientation angles of fibrils formed in the presence of LPFFD display a similar distribution. The height distributions of control and LPFFD-treated fibrils were similar: the mean fibril height was 2,41 nm (±0,09 nm SEM, n=513) and 2.64 nm (±0,14 nm SEM, n = 60) for the control and LPFFD-treated samples, respectively. There was no significant difference between the mean fibril heights.

The effect of LPFFD on the kinetics of Aß25-35 fibril growth was investigated by monitoring the length of individual fibrils as a function of time in the presence of LPFFD during time-lapse in situ AFM experiments. The growth of individual fibrils in function of time could be captured in both cases. The average growth was fitted with a linear function, the mean fibril growth rates were calculated: 0,1 nm/s and 0,08 nm/s for control and LPFFD-treated samples respectively. The LPFFD didn’t exert any significant effect on the growth of individual fibrils.

3. 2. EFFECT OF LPFFD ON THE DISASSEMBLY OF Aß25-35 FIBRILS

The fibril dismantling effect of the LPFFDpeptide was investigated by treating a mature Ab25-35 fibrilnetwork with large concentrations of LPFFD. First adense, oriented amyloid network was generated, thenthe surface was washed gently with PBSA buffer to remove theunbound fibrils, and finally the network was incubated in the presence of LPFFD solution. The relative surface coverage was calculated.Although a 10% decrease in surface coverage is evoked by theaddition of LPFFD, similar effects are achieved by washing thesurface with buffer solution.

3. 3. EFFECT OF LPFFD ON THE NANOMECHANICS OF Aß25-35 FIBRILS

The effect of LPFFD on the mechanical stability of oriented Aß25-35 fibrils was explored by manipulating the fibrils in the presence of the peptide. In the presence of LPFFD the global appearance of the force curves was similar to that of the control, but the number of force steps was reduced, and longer force plateaus could be observed. The observation that the average plateau forces decreased in the presence of LPFFD suggest that the peptide may weaken the interaction between protofilaments.

4. CHARACTERIZATION OF THE ORIENTED NETWORK FORMED BY N27C MUTANT PEPTIDE

Amyloid fibrils may be used in nanotechnology applications, because of their self-assembly properties and stability, if their growth and orientation can be controlled. Therefore, the Aß25-35 network formed on mica itself could be used for nanobiotechnological applications.

We have developed an epitaxially oriented network from mutant Aß25-35 peptides for the explicit purpose of specific chemical targeting.The sulfhydryl group of the Cys^{27} residue of the mutant Aß25-35_N27C peptide isoriented toward the solution and is chemically reactivereactive, thus paving the way toward sophisticated nanotechnological utilization of amyloid fibrils. The fine-tuning of the oriented network formed by mutant Aß25-35 peptide is shown in the following experiments.

4. 1. ORIENTATION OF Aß25-35_N27C FIBRILS ON MICA

Aß25–35_N27C fibrils displayed a highly ordered trigonal arrangement on freshly cleaved mica surface. The fibril orientation angles, relative to a horizontal reference line, were essentially identical to those obtained with the wild-type Aß25–35 peptide. Thus, the exchange of Gln27 to Cys did not interfere with the orientation of fibrils on mica surface.

The average height of the fibrils was 0.8 - 1 nm. This value most likely corresponds to the thickness of individual ß-sheets formed of Aß25-35_N27C peptides.

4. 2. EFFECT OF CATIONS ON FIBRIL BINDING

The binding of Aß25–35_N27C to mica was sensitive to the presence of cations. We tested the effect of NaCl and KCl on fibril binding.

Increasing NaCl concentration above 140 mM resulted in reduced binding of fibrils to the mica surface. In the presence of 200 mM NaCl, only a few short fibrils and oligomers were observed. In contrast, wild-type peptides were able to grow even in the presence of 640 mM NaCl.

KCl-dependent binding experiments also revealed a weakened binding of Aß25–35_N27C to mica. Reduced binding of Aß25–35_N27C fibrils was observed at KCl concentrations as low as 3 mM, concentrations of ~20 mM KClcompletely inhibited fibril growth (Figure 6.).

Thus, the N27C mutation, although did not affect fibril orientation, weakened the interaction with the mica surface. Our results also indicate that fibril binding to mica is more sensitive to K^+ than to Na^+.

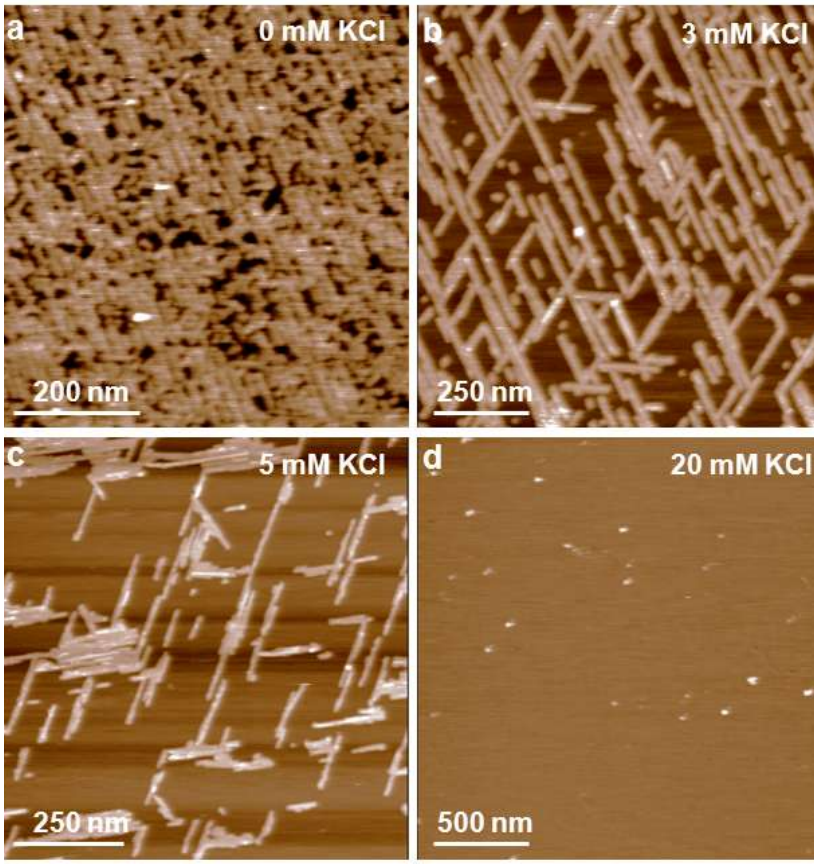

Figure6.*AFM images of Aß25–35N27C fibrils bound to mica in presenceof increasing concentrations of added KCl.*

4. 3. NANOMECHANICAL PROBING OF Aß25-35_N27C FIBRILS

We probed the surface of the fibrils with a gold-coated AFM cantilever tip with in situ nanomechanical manipulation. The cantilever tip was pressed against a fibril at predetermined locations, and then pulled away with a 500 nm/s velocity. Subsequently, the surface was scanned to investigate the effect of the mechanical perturbation. The results are shown in Figure 7.Figure7.a .shows the AFM image of fibrils prior to nanomechanical manipulation. Black arrowheads point at the target points of manipulation. On Figure 7.b. the AFM image was obtained following nanomechanical manipulation. Gray arrowheads point at the manipulated sites, where fibril discontinuity can be discerned.

The local nanomechanical manipulation of the Aß25–35_N27C fibrils resulted in the removal of small fibril fragments.

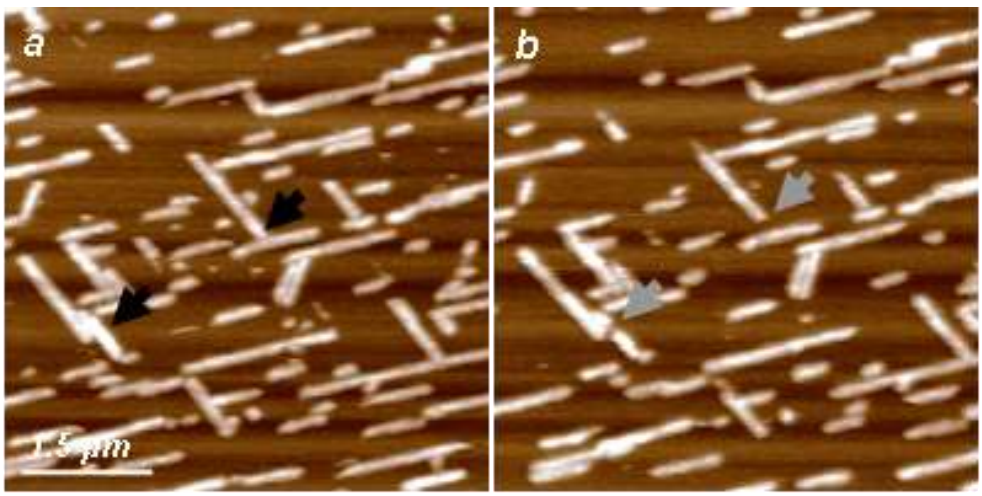

Figure 7.*In situ nanomechanical manipulation of mica-associated Aß25–35_N27C fibrils*

The most plausible explanation for the removal of fibrils is that a covalent bond has formed between Au atoms on the cantilever tip and the S atom of Cys27. Our observations suggest that the sulfhydryl group of Cys27 is indeed in an exposed position within the oriented fibril network and is chemically accessible and reactive.

5. CONCLUSIONS AND THESES

The Aß peptides form self-assembled fibrillar structures in Alzheimer's disease.The Aß25-35 peptide used in our experiments is the biologically active, toxic fragment of the full-length Aß peptide.

I. We constructed a highly ordered, stable network from Aß25-35 peptides showing trigonal orientationon mica.The same peptide can assemble itself into amyloid fibrils in solution as well. We mapped the morphological and nanomechanical features of the oriented network used later as an experimental model system.

II. We were the first to compare the properties of epitaxially-grown and solution-grown amyloid fibrils.The morphology, nanomechanics and secondary structure of fibrils were investigated by means of atomic force microscopy, force spectroscopy and FTIR spectroscopy.

1. Both types of fibrils displayed an underlying fibrillar morphology. Nevertheless, in contrast to the homogeneity structure of epitaxially-grown fibrils, solution-grown fibrils showed higher structural hierarchy, polymorphism and topographical periodicity. Epitaxially grown fibrils formed linear, sheet-like structures.
2. The fibrils differed in their growth kinetics: while fibril assembly in solution occurred on a time scale of hours to days, on mica surface fibrils appeared within a few minutes. The mica surface might act as a catalyzer accelerating the process of fibrillogenesis. Because of the accelerated fibrillogenesis, the effect of physical and chemical parameters on amyloid fibrillogenesis can be easily explored.
3. Both types of fibrils displayed identical nanomechanical responses in form of force plateaus. The elementary force plateau was ~30 pN.
4. Both types of fibrils displayed an underlying beta-sheet structure. The FTIR spectra also showed that fibrils assembled in solution have a more compact structure.
5. We demonstrated that theepitaxially grown oriented network be considered as a good amyloid-fibril model.

III. The stable, ordered network of Aß25-35 fibrils on mica is a simple, reproducible system on the nanoscale. Studying on this model the effect of different factors interfering with fibrillogenesis, the changes of structural, nanomechanical properties of the Ab25-35 fibril network can be easily monitored by atomic force microscopy.The effect of the BSB peptide LPFFD was investigated in an amyloid model system of oriented Aß25-35 fibrils on mica.

1. From force spectroscopy measurements we concluded that the LPFFD peptide may weaken the interaction between protofilaments.
2. Our results indicate that the LPFFD peptide does not have a generalized beta-sheet-breaking effect in the amyloid assembly system utilized in the present experiment.

IV. By using mutant Aß25–35 peptides (Aß25–35_N27C) containing amino acid residues with specific chemical reactivity the labeling of the oriented network with various biomolecules or conducting metals can be accomplished. For future nanotechnological, nanoelectrical applications the fine-tuning of the network's properties (e.g., average fibril length, number of branching points) was needed.

1. We succeded in fine-tuning the properties of the Aß25–35_N27C network by varying the cation- and peptide-concentration.
2. We demonstrated, that the sulfhydryl group of Cys27 within the oriented fibril network is indeed exposed to the solution and is chemically accessible and reactive.
3. We therefore demonstrated, that the properties of the mutant Aß25-35 network may provide important tools for future nanotechnological applications.

6. LIST OF PUBLICATIONS

Publications related to the present doctoral thesis:

1. **Ünige Murvai**, KatalinSoós, BotondPenke and Miklós S. Z. Kellermayer.Effect of the beta-sheet-breaker peptide LPFFD on oriented network of amyloid ß 25-35 fibrils, J. Mol. Recognit. 2011. 24 (3): p. 453-60.
2. Árpád Karsai, **Ünige Murvai**, KatalinSoós, BotondPenke and Miklós S.Z. Kellermayer.Oriented epitaxial growth of amyloid fibrils of the N27C mutant ß25-35 peptide.European Biophys.J., 2008. 37 (7): p. 1133-7.
3. Árpád Karsai, László Grama, **Ünige Murvai**, KatalinSoós, BotondPenke and Miklós S Z Kellermayer: Potassium-dependent oriented growth of amyloid ß25–35 fibrils on mica, Nanotechnology. 2007. 18 345102

Other publications

Éva M. Szego, Attila Csorba, TamásJanáky, Katalin A. Kékesi, István M. Ábrahám, Gábor M. Mórotz, BotondPenke, Miklós Palkovits, **Ünige Murvai**, Miklós S.Z. Kellermayer, JózsefKardos, Gábor D. Juhász. Effects of Estrogen on Beta-Amyloid-Induced Cholinergic Cell Death in the Nucleus BasalisMagnocellularis.Neuroendocrinology. 2011. 93 (2): p. 90-105.