# Nanobiophysical exploration of transthyretin amyloid fibrils
## Final report

**Summary**

In this work we used AFM to follow the amyloidogenetic pathway of transthyretin (TTR) by imaging the events leading to the formation of amyloid protofilaments. Single-molecule force spectroscopy (SMFS) of protofilaments was compared to naïve TTR in order to probe dynamic and structural differences. We observed that the pathway proceeds through the formation of transient amorphous aggregates, followed by the occurrence of annular oligomers (rings or doughnuts). In other types of amyloidoses similar ring structures have been implicated in cytoxicity, but their properties and involvement in the amyloid pathway are poorly understood. We show that the rings have a tendency to stack, forming tubular protofilaments. These tubular protofilaments precede the appearance of amyloid protofilaments. Their height and pitch resemble those of previous structural models for the TTR amyloid protofilament. Upon solvent exchange we also observed amyloid protofilament dissociation. The dissociation appears to proceed through an unzipping mechanism, revealing structures reminiscent of the TTR annular oligomers. SMFS of protofilaments revealed a time-dependent increase in the length of the manipulated structure, suggesting that associations between monomers stabilize with time. Force spectra of native TTR and protofilaments contained transitions spaced 4 nm apart, indicating that the component β-strands unfold sequentially. Based on our results a model of TTR protofilament assembly is proposed.

## Results and Discussion

The fibrillogenesis of transthyretin amyloid fibrils was explored in this proposal as part of a collaborative effort between the research groups of Professor Ana M. Damas of Porto (Portugal) and Miklós S.Z. Kellermayer of the University of Pécs, Hungary. Atomic force microscopic methods were utilized to gain insight into the mechanisms of TTR fibril structure and assembly. Although the effect of toxic mutations was not resolved in lieu of time, we succeeded in obtaining novel observations about the structural changes associated with the formation of TTR fibrils and protofilaments. The structural changes during the formation of fibrils was followed by examining the topographical structure as a function of incubation time. In addition, by mechanically manipulating the fibrils, the arrangement of TTR monomers within the fibril were investigated. To our knowledge, our experiments are pioneering in such nanomanipulation of TTR fibrils. In the following, the time-dependent structural changes and the results of the nanomechanical experiments are presented.

*Day 1: Native TTR*

Samples of native TTR were investigated following incubation on mica surface. The results are shown in **Figure 1**. Globular structures with an average height of 4 nm were observed. The height values correspond relatively well with the dimensions of the tetrameric native TTR units. Fibril formation was induced by lowering the pH (pH 3) and incubating the samples at 37 ˚C. At different time points aliquots from the sample were removed and analyzed by AFM.

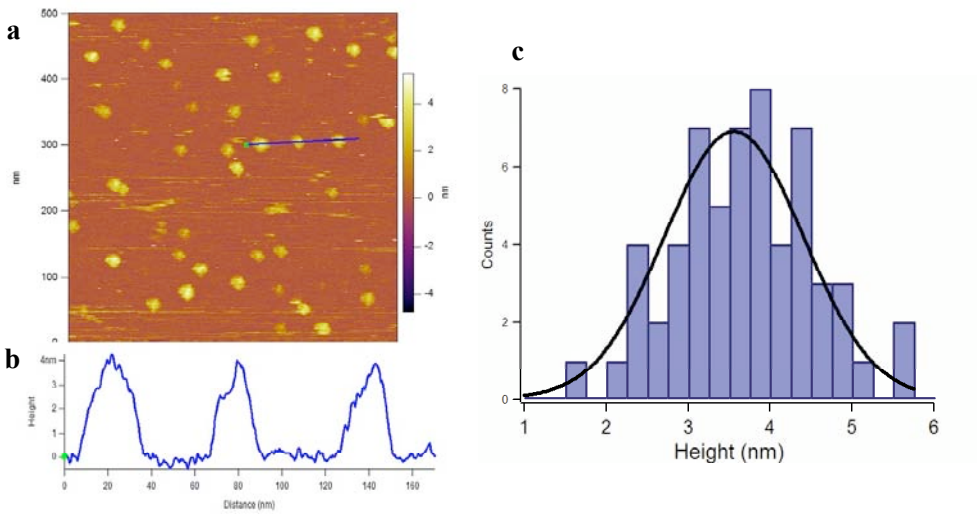


**Figure 1.** Imaging of native TTR sample with AFM. **a.** AFM image. **b.** Height profile along the line drawn in (**a**). **c.** Distribution of topographical height of native TTR.

*3 to 5-day-old TTR*

In samples 3 to 5 days old, curvilinear, fibril-like aggregates were observed (**Figure 2**). The fibrils were 50-100 nm long and 3 nm in height. Quite interestingly, the fibrillar structures appeared to be constructed of annular oligomers. Such annular intermediates have been shown to appear in the case of other types of amyloid fibrils. Most likely, protofibrillar aggregates result from oligomer lateral association.

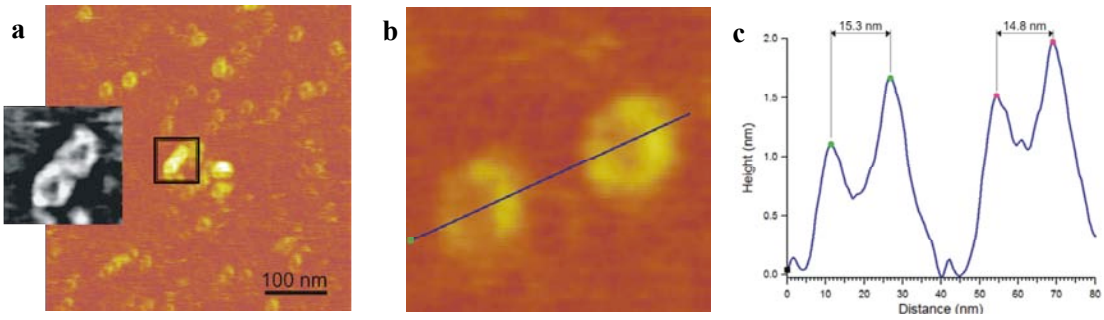


**Figure 2.** Images of annular oligomeric structures. **a.** AFM image of fibrillar structures. **b.** Enlarged view of two doughnut-shaped oligomers. (**c**) Height profile along the line drawn in (b).

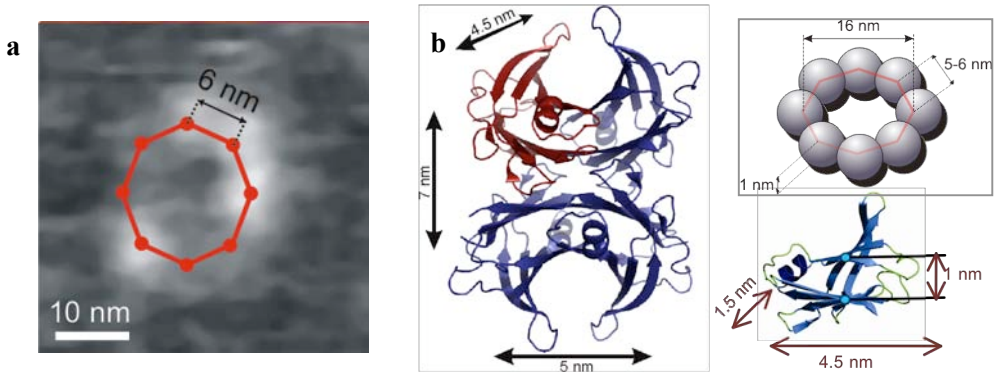


**Figure 3.** Annular TTR oligomer structure and its schematic interpretation. **a.** High-magnification AFM image. **b.** Structural model of native TTR. **Inset,** schematics of annulus formation.

Average dimensions of the annular oligomeric structures were (**Figure 3**): diameter: 15.8 ± 2.3 nm, height: 0.97± 0.04 nm. Each unit of the TTR annular oligomers could then be composed of a monomer or an organized cluster of monomer molecules.

*5 to 7-day-old-samples*

Annular structures were also observed in older samples as well. Apparently, the rings can bind to each other so as to form stacks (**Figure 4**). The stacking of TTR annular structures may be an important step in the process of TTR fibril formation.

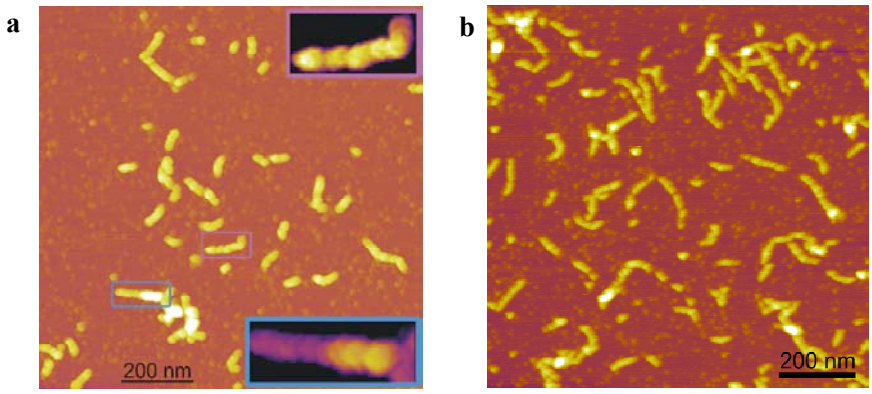


**Figure 4.** AFM images of 5-7 day old TTR samples. The fibrils aparently grow by the stacking of annuilar structures (**a**), which merge to form continually growing fibrils (**b**).

*Nanomanipulation of TTR fibrils*

In order to gain further insight into the structural arrangement within the TTR fibrils, we carried out nanomechanical manipulations. The AFM cantilever was lowered onto specific points along fibrillar structures; subsequently, the cantilever was pulled away at preadjusted, constant velocity. The forces during the procedures were measured by using cantilevers calibrated for stiffness. Examples of force curves are shown in **Figure 5**. In the force curves, transitions are observed in teh form of sawtooth-like peaks. Such force transitions correspond to structural transitions within the fibril evoked by the imposed mechanical forces.

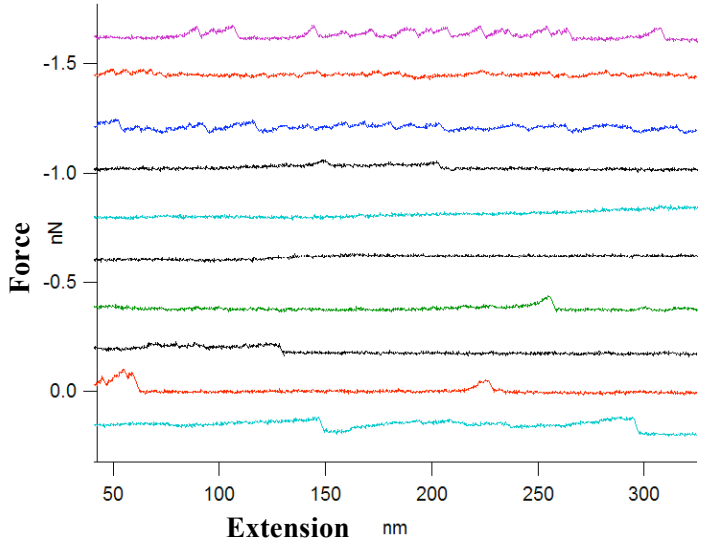


**Figure 5.** Collection of force versus extension curves obtained during nanomanipulation experiments of TTR fibrils.

From the force curves analyses were made according to the maximum stretch (total extension) and the separation between the consecutive force peaks (**Figure 6**).

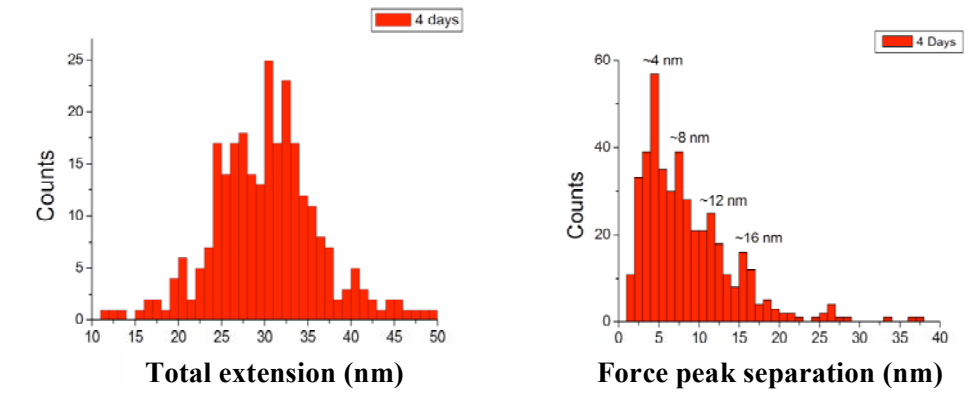


**Figure 6.** Histograms of total extension and force peak separation obtained from force versus extension curves of TTR fibrils.

Native TTR and early aggregates (4 days) were stretched up the the unfolded contour length of the TTR monomer. In aggregates of 14 days, 19% of records show pullings above the TTR monomer unfolded contour length. Possibly, monomer-monomer interface stabilization within the fibrils resulted in the longer stretches. In the force curves a 4 nm peak separation dominated. The 4 nm corresponds well to the length of a single ß-strand. Thus, the nanomechanical perturbations likely caused a sequential unfolding of the ß-strands that build up the TTR structure. The presence of force patterns with an underlying 4 nm repeat observed in more mature fibrillar aggregates indicate a fairly regular structure of these TTR aggregates.

## Model of TTR amyloid assembly

Based on our findins a model of TTR assembly may be proposed (**Figure 7**). In this model native TTR assembles, via partially unfolded intermediates, into amorphous aggregates, followed by the formation of soluble oligomeric species. Soluble oligomers then assemble into annular structures which form the basis of subsequent assembly steps. The annular structures coalesce by stacking into protofibrillar assemblies. Conceivably, structural rearrangements at this stage permit the formation of helical protofilaments. Understanding the topological and energetic constraints of such a structural rearrangement await further experimentation.

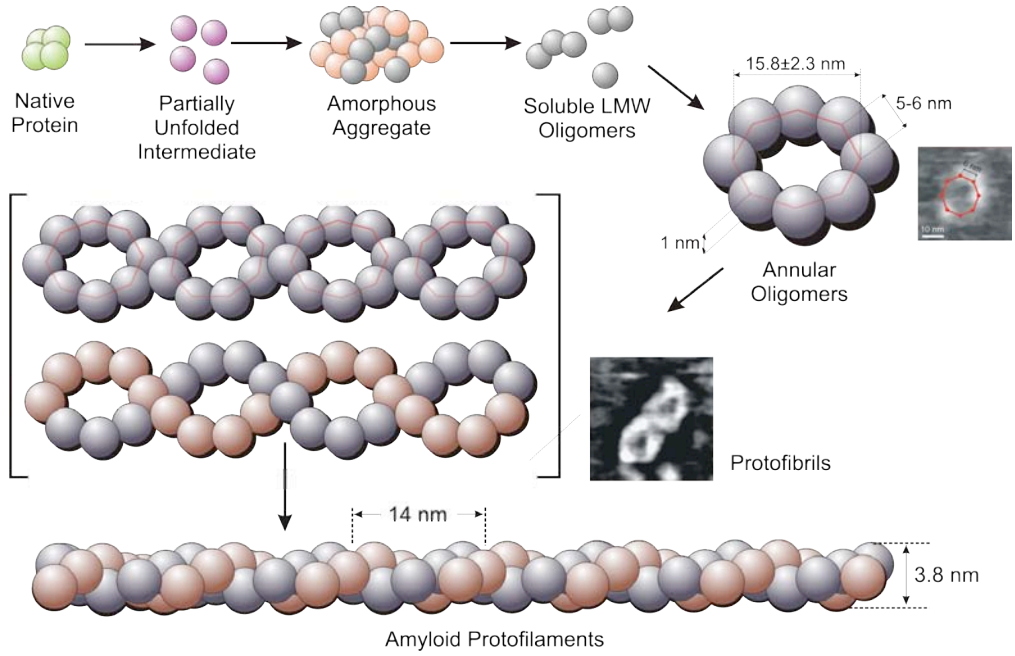


**Figure 7.** Schematics of the structural model of transthyretin amyloid fibril assembly.

## Publications

1. Pires RH, Karsai A, Saraiva MJ, Kellermayer MSZ, Damas (2008) *Understanding the Transthyretin Amyloidogenesis Pathway*. Revista Portuguesa de Farmácia LII(3): 36.
2. Pires RH, Karsai A, Saraiva MJ, Kellermayer, MSZ and Damas AM. "Understanding the Transthyretin Amyloidogenesis Pathway." I National Meeting on Medicinal Chemistry, 13-15th Nov. 2008, Porto - PORTUGAL
3. Pires RH, Karsai A, Saraiva MJ, Damas AM and Kellermayer, MSZ. "Structure Of Transthyretin Amyloid Fibrils Explored With AFM Imaging and Nanomanipulation." XVI National Congress of Biochemistry , 22-26th October 2008, Azores - PORTUGAL
4. Pires RH, Karsai A, Saraiva MJ, Kellermayer, MSZ and Damas AM. "Transthyretin Amyloidogenesis Pathway: insights from AFM and force spectroscopy". EURAMY Meeting, 17-18th Oct. 2008. Póvoa do Varzim - PORTUGAL
5. Pires RH, Karsai A, Saraiva MJ, Damas AM and Kellermayer, MSZ. "Assembly and Structure of Transthyretin Amyloid Protofilaments revealed by AFM Imaging and Single Molecule Force Spectroscopy". EBSA Congress 6-9 July, 2009, Genova, Italy.