

# Effect of the beta-sheet-breaker peptide LPFFD on oriented network of amyloid $\beta$ 25-35 fibrils<sup>†</sup>

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**Amyloid fibrils are self-associating filamentous structures deposited in extracellular tissue in various neurodegenerative and protein misfolding disorders. It has been shown that beta-sheet-breaker (BSB) peptides may interfere with amyloid fibril assembly. Although BSB peptides are prospective therapeutic agents in amyloidosis, there is ambiguity about the mechanisms and generality of their action. In the present work we analyzed the effect of the BSB peptide LPFFD on the growth kinetics, morphologic, and mechanical properties of amyloid  $\beta$ 25-35 ( $A\beta$ 25-35) fibrils assembled in an oriented array on mica surface.  $A\beta$ 25-35 is thought to represent the biologically active, toxic fragment of the full-length  $A\beta$  peptide. Growth kinetics and morphologic features were analyzed using *in situ* atomic force microscopy in the presence of various concentrations of LPFFD. We found that the addition of LPFFD only slightly altered the assembly kinetics of  $A\beta$ 25-35 fibrils. Already formed fibrils did not disassemble in the presence of high concentrations of LPFFD. The mechanical stability of the fibrils was explored with force spectroscopy methods. The nanomechanical behavior of  $A\beta$ 25-35 fibrils is characterized by the appearance of force staircases which correspond to the force-driven unzipping and dissociation of several protofilaments. In the presence of LPFFD single-plateau force traces dominated. The effects of LPFFD on  $A\beta$ 25-35 fibril assembly and stability suggest that inter-protofilament interactions were slightly weakened. Complete disassembly of fibrils, however, was not observed. Thus, under the conditions explored here, LPFFD may not be considered as a BSB peptide with generalized beta-sheet breaking properties. Copyright © 2011 John Wiley & Sons, Ltd.**

**Keywords:** atomic force microscopy; beta-amyloid; beta-sheet breaker peptide

## INTRODUCTION

Amyloid fibrils are filamentous aggregates which can be found in extracellular tissue-deposits in various degenerative disorders (Selkoe, 1997, 2001; Serpell *et al.*, 2000; Hardy and Selkoe, 2002). The full-length (39- to 43-residue-long) amyloid  $\beta$  ( $A\beta$ ) peptide is the major component of neuritic plaques found in Alzheimer's disease (Selkoe, 1997).  $A\beta$  forms self-associating fibrillar structures possessing predominantly cross- $\beta$  conformation (Serpell *et al.*, 2000).

The amyloid  $\beta$  25-35 peptide ( $A\beta$ 25-35) is an eleven-residue-long fragment of the full-length  $A\beta$ . It is a fibril-forming peptide that represents the biologically active, toxic form of the full-length  $A\beta$  monomer (Yankner *et al.*, 1990; Forloni *et al.*, 1993).

LPFFD (Lys-Pro-Phe-Phe-Asp) is a five-residue-long beta-sheet-breaker (BSB) peptide, also called as Soto's pentapeptide. BSB peptides are rationally designed peptides specially created to break  $\beta$ -sheets. Formation of misfolded  $A\beta$  involves the organization of several  $A\beta$  monomers in an oligomeric  $\beta$ -sheet structure stabilized by hydrogen bonds and hydrophobic interactions.  $\beta$ -sheet-breaker peptides contain a sequence similar to the region involved in protein-protein interactions (the self-recognition motif) but also include some residues unable to fit inside a  $\beta$ -sheet structure.  $\beta$ -sheet-breaker peptides may destabilize the misfolded  $A\beta$  oligomers, and consequently prevent amyloid formation (Soto *et al.*, 1998). Even though the mechanism

by which  $\beta$ -sheet-breaker peptides inhibit and reverse fibril formation has not yet been identified, two distinct molecular events are thought to be important. First, binding between  $\beta$ -sheet-breaker peptides and  $A\beta$  and second, conformational destabilization of  $\beta$ -sheet structures (Estrada and Soto, 2007).

To investigate whether the LPFFD possesses generalized beta-sheet-breaking properties, we have tested these two molecular events (assembly and disassembly) on  $A\beta$ 25-35

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peptides. Using A $\beta$ 25-35 fibrils as a model system provides several advantages. A $\beta$ 25-35 fibrils display a highly ordered trigonal arrangement on the freshly cleaved mica surface (Karsai *et al.*, 2007). The stable, ordered network of A $\beta$ 25-35 fibrils on mica is a simple, reproducible system on the nanoscale. Studying the effect of BSB peptides on this model, the changes of structural, nanomechanical properties of the A $\beta$ 25-35 fibril network can be easily monitored by atomic force microscopy (AFM).

In the present work, we explored the effect of LPFFD on the growth, orientation, and force spectra of the A $\beta$ 25-35 fibrils epitaxially grown on mica. We find that LPFFD, even at high concentrations, did not significantly influence the global structural features, assembly dynamics, and stability of A $\beta$ 25-35 fibrils.

## MATERIALS AND METHODS

### Sample preparation

Lyophilized samples A $\beta$ 25-35 (<sup>25</sup>GSNKGAIIGLM<sup>35</sup>-amide) peptide produced by solid state synthesis (Zarandi *et al.*, 2007) were dissolved in DMSO (dimethyl sulfoxide) and transferred to Na-phosphate buffer ["Na-PBSA," 10 mM Na-phosphate (pH 7.4), 140 mM NaCl, 0.02% NaN<sub>3</sub>] at a final concentration of 2–2.5 mg/ml. To remove oligomeric aggregates the solution was centrifuged with 250,000g at 4°C for 2 h (Beckman Coulter Optima™ MAX Ultracentrifuge). The supernatant was diluted for further use. Peptide concentration was measured with the quantitative bicinchoninic acid assay (Smith *et al.*, 1985).

The BSB peptide LPFFD, also called as Soto's pentapeptide (Soto *et al.*, 1998), was produced by solid state synthesis. Lyophilized samples were dissolved in Na-PBSA at final concentration of 1.5–1.8 mM.

### Atomic force microscopy

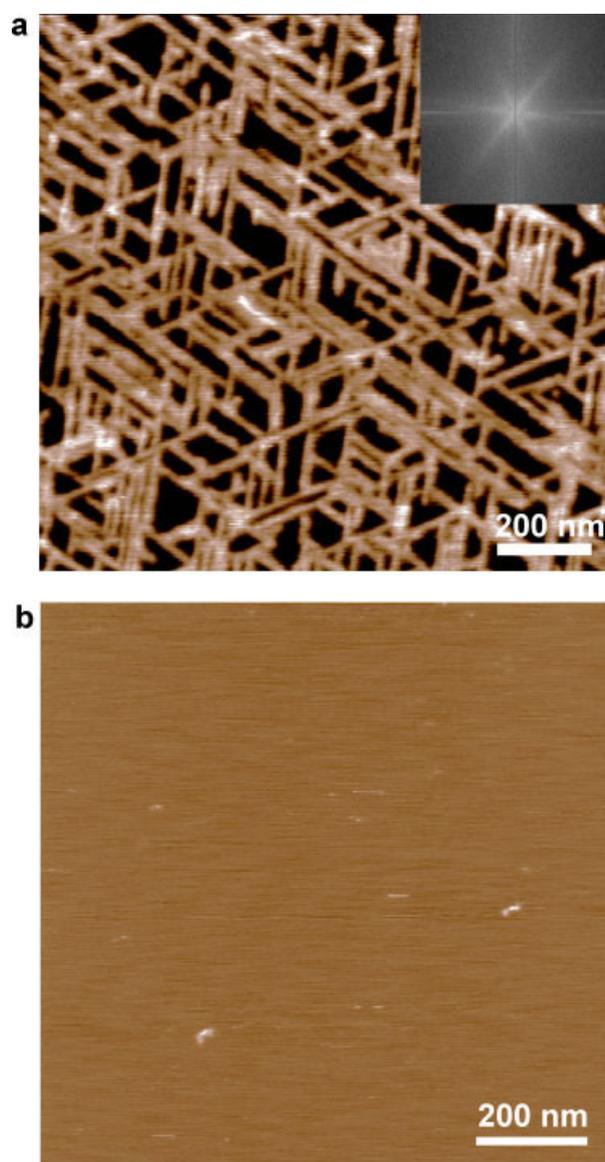
Typically 100  $\mu$ l samples were applied to a freshly cleaved mica surface. We used high-grade mica sheets (V2 grade, #52-6, Ted Pella, Inc., Redding, CA). After incubating the sample for 10 min the surface was washed gently with Na-PBSA buffer to remove unbound fibrils. The samples were imaged with AFM in buffer. Non-contact mode AFM images were acquired with an Asylum Research MFP3D instrument (Santa Barbara, CA) using silicon-nitride cantilevers (Olympus BioLever, resonance frequency in buffer  $\sim$ 9 kHz). 512  $\times$  512-pixel images were collected at a typical scanning frequency of 0.8–1.5 Hz and with a high set point (0.8–1 V).

### In situ AFM

Time-lapse AFM images of A $\beta$ 25-35 fibril growth on mica were recorded by repetitively scanning 2  $\mu$ m wide areas following the addition of the peptides. 512  $\times$  512 pixel images were typically scanned at a rate of 2 Hz. Usually at least ten images were collected with no interleave pause. In control experiments a 100- $\mu$ l A $\beta$ 25-35 solution (8  $\mu$ M) was pipetted onto a freshly cleaved mica surface, then the recording of time-lapse AFM images was immediately started. For studying the effect of the LPFFD peptide on A $\beta$ 25-35 fibril assembly kinetics, a 100- $\mu$ l sample of A $\beta$ 25-35/LPFFD mixture (8  $\mu$ M A $\beta$ 25-35, 300  $\mu$ M LPFFD) was applied to the freshly cleaved mica surface, and time-lapse AFM images were immediately recorded.

### Force spectroscopy

Force spectroscopy measurements were carried out according to procedures published before (Kellermayer *et al.*, 2005; Karsai *et al.*, 2006b). Briefly, 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. Typical stretch rate was 500 nm/s. Experiments were carried out under aqueous buffer conditions (Na-PBS buffer, pH 7.4). Stiffness was determined for each cantilever by using the thermal method (Hutter and Bechhoefer, 1993). To explore the effect of LPFFD on the nanomechanical behavior of A $\beta$ 25-35, the fibrils were manipulated in the presence of the BSB peptide. A 100- $\mu$ l sample of A $\beta$ 25-35/LPFFD mixture (8  $\mu$ M A $\beta$ 25-35 and 196  $\mu$ M LPFFD) was pipetted on freshly cleaved mica, then allowed to incubate for



**Figure 1.** AFM images of control specimens. (a) AFM image of oriented A $\beta$ 25-35 fibril network on mica surface. Inset: 2D-FFT of AFM image. (b) AFM image of mica surface following the incubation of a 1 mM solution of LPFFD for 45 min.

90 min. Unbound fibrils were removed by washing gently with Na-PBSA buffer. A $\beta$ 25-35 fibrils without LPFFD served as control.

### Image processing and data analysis

AFM images and force spectra were analyzed using built-in algorithms of the IgorPro v6.03 MFP3D controller software (Wavemetrics, Lake Oswego, OR). Orientation angles of fibrils were measured manually between a line fitted to the fibril axis and a horizontal reference line. 2D FFT was carried out with ImageJ software (public domain). For data analysis, we used IgorPro v6.0 and KaleidaGraph 4.0 software packages.

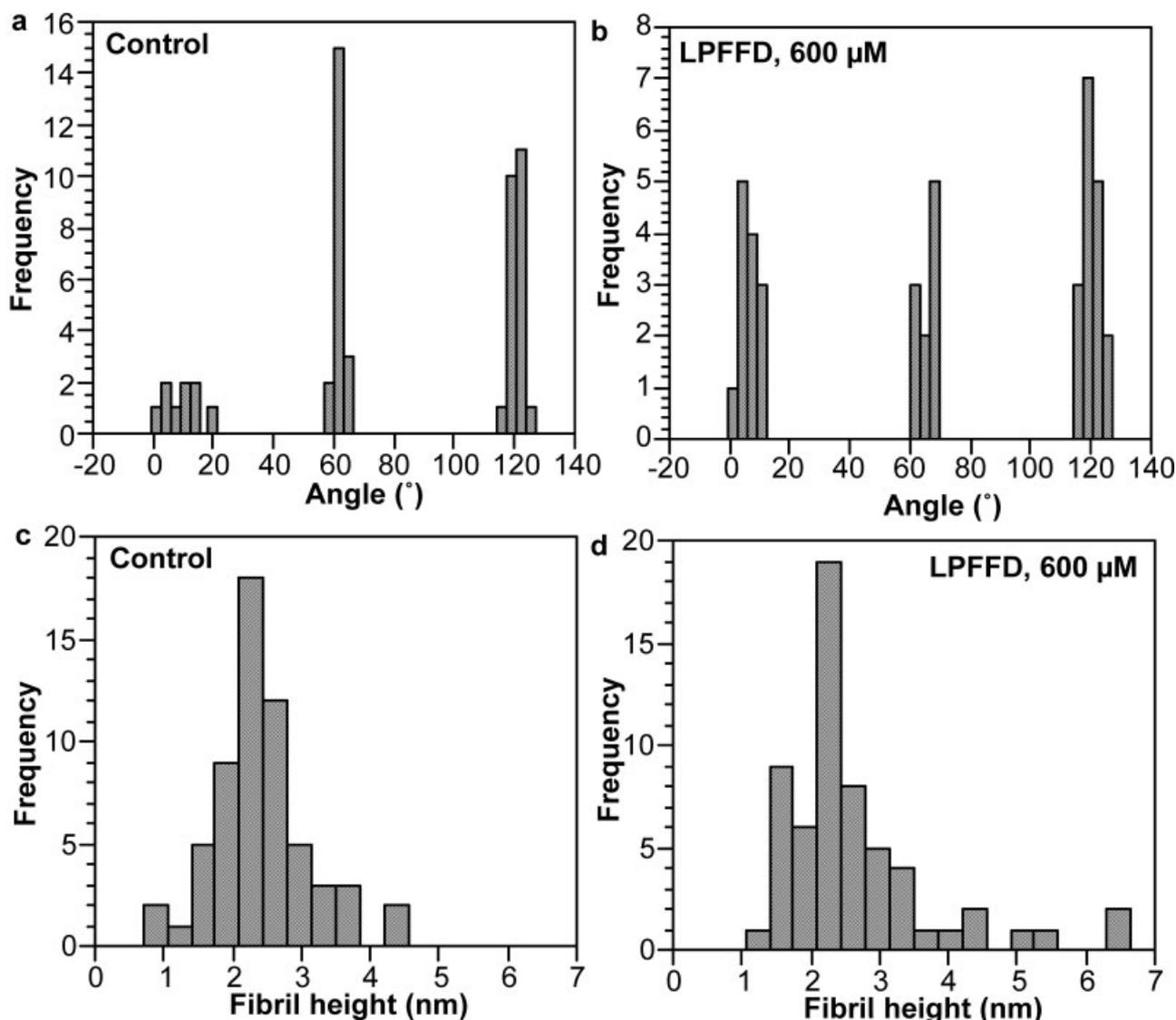
## RESULTS

### Control experiments

The oriented network of A $\beta$ 25-35 fibrils grown epitaxially on mica was used in the present work as a model system for rapid amyloid

fibril assembly. Figure 1a shows this oriented network model used as control. The fibrils are highly oriented with an angle of 60° between individual fibrils, and 120° between each of the three main orientation directions. These three directions are represented in a regular hexagonal pattern on the 2D FFT image (Figure 1a inset). The fibrils have reached lengths so that they touch each other; therefore, very few free fibril ends are observed. Depending on the initial peptide concentration the oriented A $\beta$ 25-35 network forms with different rates on the time scale of minutes to hours. Because of the accelerated fibrillogenesis, the effect of physical and chemical parameters on amyloid fibrillogenesis can be easily explored. The effect of the LPFFD peptide was investigated on various parameters of the structure and formation of the oriented A $\beta$ 25-35 network.

To test whether the LPFFD peptide itself forms fibrils on the mica surface, a 1 mM LPFFD solution was incubated on freshly cleaved mica for 45 min at room temperature. After washing the surface with buffer to remove the excess LPFFD solution, we scanned the surface with AFM (Figure 1b). The AFM image shows a mica



**Figure 2.** Angle-distribution of oriented A $\beta$ 25-35 fibrils (8  $\mu$ M) on mica in the absence (a) and presence (b) of LPFFD peptides (600  $\mu$ M). Topographical height-distribution of oriented A $\beta$ 25-35 fibrils (8  $\mu$ M) on mica in the absence (c) and presence (d) of LPFFD peptides (600  $\mu$ M).

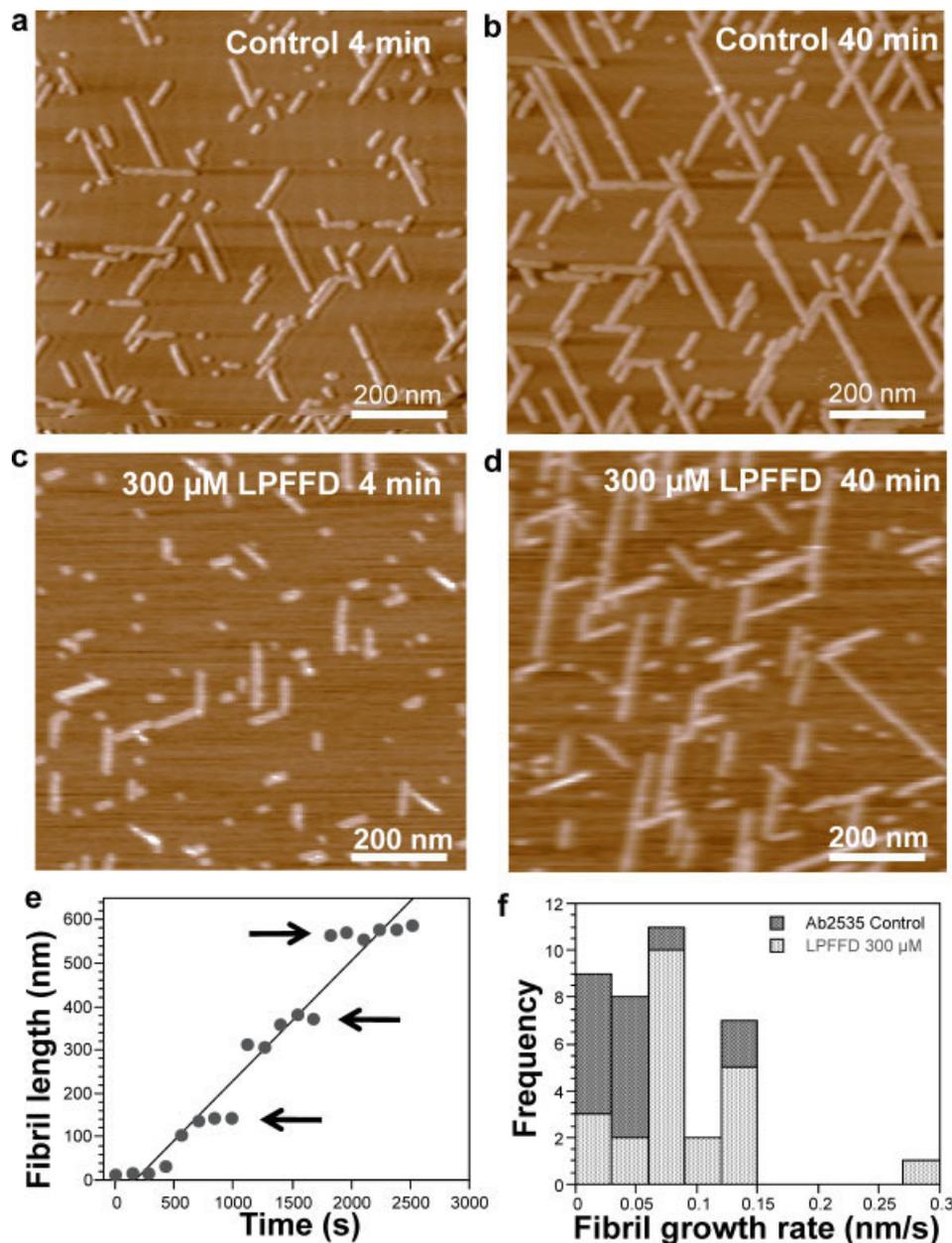
surface devoid of filamentous structures. Thus, the LPFFD peptide by itself did not form aggregates or fibrils on the mica surface.

### Effect of LPFFD on the orientation, structure, and assembly dynamics of A $\beta$ 25-35 fibrils on mica

To investigate the effect of LPFFD on the global structure of the A $\beta$ 25-35 network, we first measured the fibril orientation angles in LPFFD-treated samples. Figure 2 shows the results. The orientation angles in the control sample (Figure 2a) were

narrowly distributed around 0, 60, and 120°, thus revealing discrete, 60° differences between fibril orientations. The orientation angles of fibrils formed in the presence of 600  $\mu$ M LPFFD display a similar distribution (Figure 2b).

The overall structural features of A $\beta$ 25-35 fibrils were assessed by measuring their topographical height in the absence and presence of LPFFD. The height distributions of control and LPFFD-treated fibrils are shown in Figure 2c and d, respectively. The mean fibril height was 2.41 nm ( $\pm 0.09$  nm SEM,  $n = 60$ ) and 2.64 nm ( $\pm 0.14$  nm SEM,  $n = 60$ ) for the control and



**Figure 3.** Effect of LPFFD on the growth rate of A $\beta$ 25-35 fibrils. (a) and (b) are the first and last images, respectively, of the control sample during an *in situ* AFM experiment. The oriented network of A $\beta$ 25-35 was formed at a peptide concentration of 8  $\mu$ M. (c) and (d) are the first and last images, respectively, of the LPFFD-treated sample during an *in situ* AFM experiment. The fibril network, formed at an A $\beta$ 25-35 peptide concentration of 8  $\mu$ M, was allowed to grow in the presence of 300  $\mu$ M LPFFD. (e) Increase of A $\beta$ 25-35 fibril length as a function of time. Arrows point at periods of pauses during fibril growth. The fibril length versus time data were fitted with linear function so as to obtain average fibril growth rate from the slope. (f) Distribution of fibril growth rate for control (dark gray) and LPFFD-treated (light gray) samples.

LPFFD-treated samples, respectively. There was no significant difference between the mean fibril heights ( $t$  probability 0.2).

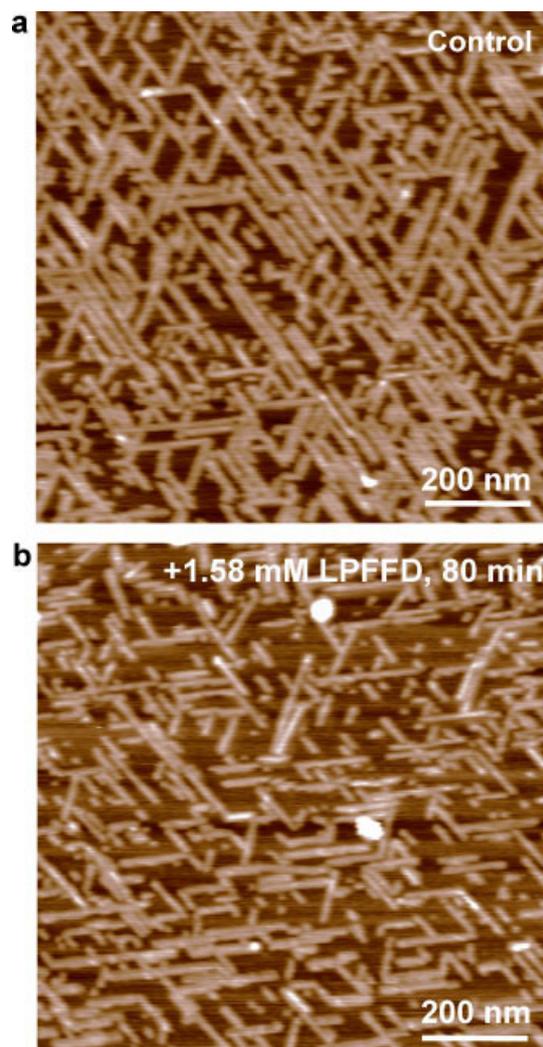
The effect of LPFFD on the kinetics of A $\beta$ 25-35 fibril growth was investigated by monitoring the length of individual fibrils as a function of time in the presence of LPFFD during time-lapse *in situ* AFM experiments (Figure 3). A 100  $\mu$ l sample from a mixture of 300  $\mu$ M LPFFD and 8  $\mu$ M A $\beta$ 25-35 was applied to the freshly cleaved mica surface, then AFM images were continuously recorded of the same sample surface. The acquisition of an image took 100 s. The evolution of fibrillar network during the examined time period was similar in the case of the control (Figure 3a and b) and LPFFD-treated (Figure 3c and d) samples. The growth of individual fibrils could be captured in both cases. Fibrils grew until they reached another fibril lying across their growth path. The instantaneous length of a single fibril as a function of time is shown in Figure 3e. Although steps and pauses can be discerned in the trace (arrows) the average growth can be fitted with a linear function. Figure 3f shows the distribution of fibril velocities for control and LPFFD-treated samples across the range of 0.3 nm/s. The mean fibril growth rates were 0.16 nm/s ( $\pm 0.08$  nm/s SEM,  $n = 17$ ) and 0.08 nm/s ( $\pm 0.01$  nm/s SEM,  $n = 23$ ) for the control and LPFFD-treated samples, respectively. Given the deviation from normal distribution we compared the fibril growth rates with the non-parametric Mann-Whitney test, which showed that there are no significant differences between them.

#### Effect of LPFFD on the disassembly of A $\beta$ 25-35 fibrils *in vitro*

The fibril dismantling effect (Sigurdsson *et al.*, 2000) of the LPFFD peptide was investigated by treating a mature A $\beta$ 25-35 fibril network with large concentrations of LPFFD (Figure 4). First a dense, oriented amyloid network was generated (Figure 4a), then the surface was washed gently with PBSA buffer to remove the unbound fibrils, and finally the network was incubated in the presence of 1.58 mM LPFFD solution at room temperature for 80 min (Figure 4b). The relative surface coverage was calculated. Although a 10% decrease in surface coverage is evoked by the addition of LPFFD, similar effects are achieved by washing the surface with buffer solution.

#### Effect of LPFFD on the nanomechanics of A $\beta$ 25-35 fibrils

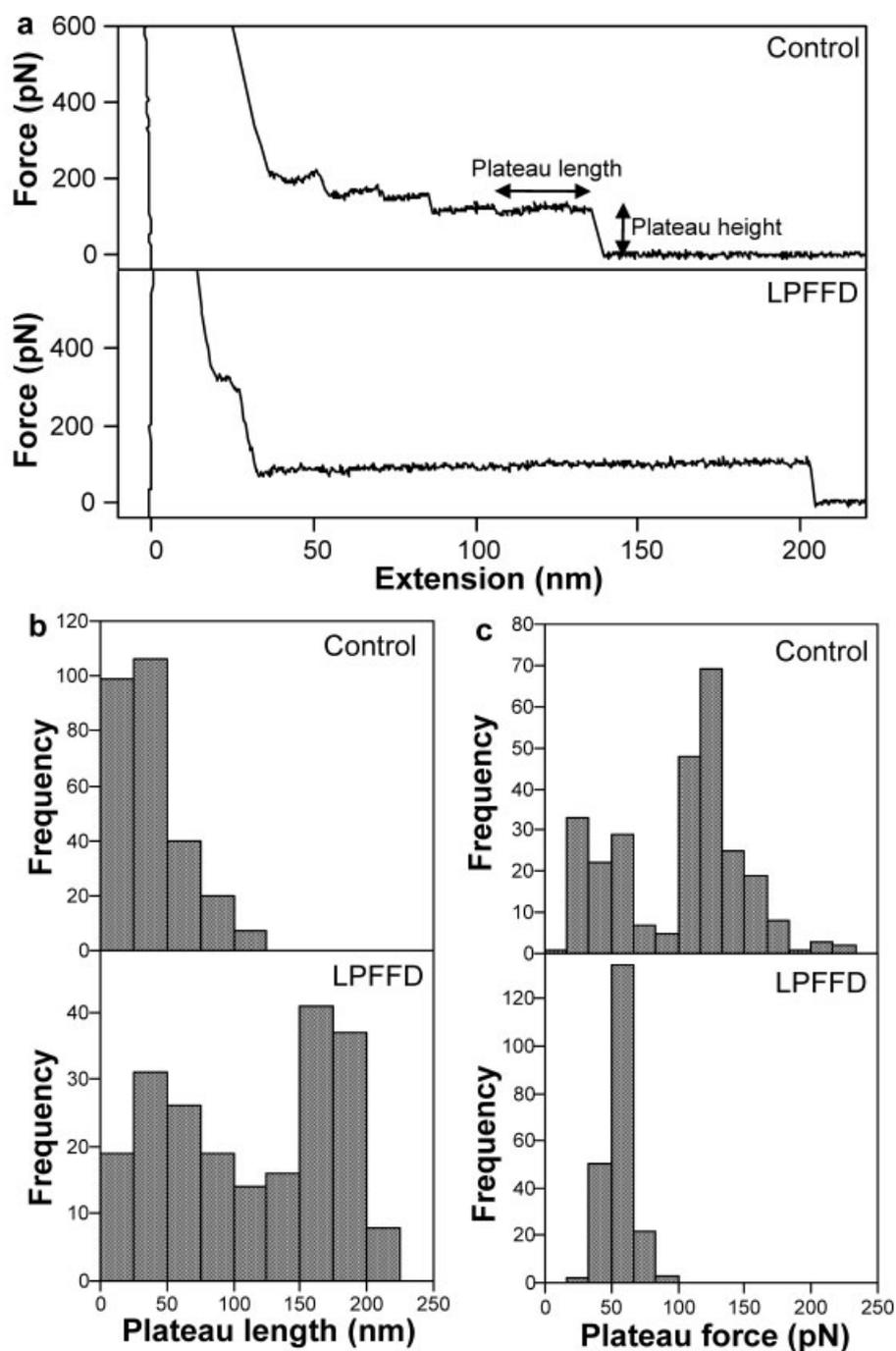
The effect of LPFFD on the mechanical stability of oriented A $\beta$ 25-35 fibrils was explored by manipulating the fibrils in the presence of the peptide. The nanomechanical behavior of A $\beta$ 25-35 fibrils is characterized by the appearance of force plateaus which correspond to the force-driven unzipping of protofilaments (Figure 5a top). In the presence of 196  $\mu$ M 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 (Figure 5a bottom). The distribution of plateau lengths for the control and LPFFD-treated samples is shown in Figure 5b. In the presence of LPFFD a wider distribution of plateau lengths is observed, with lengths up to 220 nm. Figure 5c shows the distribution of plateau forces for the control and LPFFD-treated samples. Whereas a multimodal distribution is clearly seen in the control, the plateau forces in the LPFFD-treated sample are distributed relatively narrowly around a single maximum at  $\sim 60$  pN.



**Figure 4.** Effect of LPFFD on the stability of already existing A $\beta$ 25-35 network. (a) Control sample. (b) A $\beta$ 25-35 network treated with 1.58 mM LPFFD for 80 min.

## DISCUSSION

We have recently shown that the A $\beta$ 25-35 peptide, which is thought to represent the toxic fragment of the full-length amyloid beta-peptide (Yankner *et al.*, 1990; Forloni *et al.*, 1993), and its mutant form (A $\beta$ 25-35\_N27C) forms a highly oriented network on the surface of mica (Karsai *et al.*, 2007, 2008; Kellermayer *et al.*, 2008). The growth of the fibrils is epitaxially driven and occurs on a time scale significantly shorter than in bulk solution (Kellermayer *et al.*, 2008). The rapid formation of fibrils, and the possibilities of parameterizing global and local fibril structure as well as following the kinetics of individual fibril growth make the oriented A $\beta$ 25-35 fibril network an appealing experimental model system. In the present work we investigated the effect of the LPFFD beta-sheet-breaking (BSB) peptide (Soto *et al.*, 1998) on the structure, dynamics, and nanomechanics of the oriented A $\beta$ 25-35 fibril network. Although BSB peptides are designed according to targeted amyloid peptide sequences (Tjernberg *et al.*, 1996, 1999; Soto *et al.*, 1998; Estrada and Soto, 2007), they are expected to possess generalized beta-sheet disrupting properties, particularly because amyloid fibrils are held together primarily by peptide backbone interactions



**Figure 5.** Effect of LPFFD on the force spectra of epitaxially grown A $\beta$ 25-35 fibrils. (a) Examples of force curves control (top) and LPFFD-treated (196  $\mu$ M, bottom) samples. Only the cantilever retraction data are shown. (b) Distribution of plateau length for control (top) and LPFFD-treated (bottom) samples. (c) Distribution of plateau force for control (top) and LPFFD-treated (bottom) samples.

(Tycko, 2004). Thus, the investigation of the effect of such peptides in our experimental model system is well warranted. LPFFD (Lys-Pro-Phe-Phe-Asp) is a five-residue long BSB peptide designed towards the hydrophobic core region (residues 17-21) of the full-length A $\beta$ 1-40 peptide (Soto *et al.*, 1998). Although the targeted sequence is not present in A $\beta$ 25-35, based on the generalized BSB effect LPFFD may in principle influence the global appearance and assembly/disassembly dynamics of A $\beta$ 25-35 fibrils.

We find in numerous experiments that the global structural features, assembly-dynamics, and stability of the A $\beta$ 25-35 network are not influenced significantly even in the presence of high concentrations of the LPFFD peptide. The orientation direction of A $\beta$ 25-35 fibrils was not affected by LPFFD (Figures 2a and b), indicating that the interaction between A $\beta$ 25-35 peptides and the mica surface, which are the primary determinants of the oriented arrangement, are not influenced by the presence of the LPFFD peptide. The global structure of the fibrils, assessed

by the mean fibril height, was also unaffected by the LPFFD treatment (Figure 2c and d). The assembly of A $\beta$ 25-35 fibrils on mica proceeded with similar kinetics regardless of whether the LPFFD peptide was present or not (Figure 3). The observation suggests that LPFFD peptide does not significantly interfere with the binding of A $\beta$ 25-35 peptides along the axis of the fibril. As a result, high concentrations of the LPFFD peptide did not have significant effect on the global structure of an already formed, mature network of A $\beta$ 25-35 fibrils (Figure 4). The only finding that suggests a modulatory effect of LPFFD on A $\beta$ 25-35 properties was seen in nanomechanical experiments (Figure 5). In the presence of LPFFD longer force plateaus were observed with fewer force steps. Force plateaus in nanomechanical traces of amyloid fibrils are attributed to the unzipping of protofilaments from the fibril (Karsai *et al.*, 2005, 2006a; Kellermayer *et al.*, 2005). The height of the plateaus is related to the force necessary to unzip the protofilaments. Therefore, plateau forces are related to the mechanical stability of the fibril. The observation that the average plateau forces decreased in the presence of LPFFD suggest that the peptide may weaken the interaction between protofilaments. The length of the force plateau corresponds to the distance between consecutive protofilament rupture events. The longer the plateau, the longer it takes for the protofilament to rupture, along its length or at its attachment points, during mechanical unzipping. We can only speculate about the reasons of increased plateau length observed in the presence of LPFFD. Conceivably, the decrease in inter-protofilament interaction strength was coupled with an increase in the axial stability of the protofilament. However, more detailed experimentation will be necessary to unravel the exact mechanisms behind the finding.

Our results indicate that the LPFFD peptide does not have a significant beta-sheet-breaking effect in the amyloid assembly system utilized in the present experiments. The lack of a marked inhibitory effect of the LPFFD peptide on the structure and

dynamics of the oriented network of A $\beta$ 25-35 fibrils can be attributed most likely to the sequence mismatch between the LPFFD and A $\beta$ 25-35 peptides. Although the LPFFD peptide may have a generalized beta-sheet-breaking property, it is likely that the strong interactions between the mica surface and the A $\beta$ 25-35 peptide outcompeted any minor effects. The oriented A $\beta$ 25-35 fibril network nevertheless continues to serve as a model system for more specific physical and chemical factors that may interfere with amyloid fibrillogenesis. The multiple parameters extracted in our experiments using scanning force microscopy underline the utility of this experimental model system.

## CONCLUSIONS

The effect of the BSB peptide LPFFD was investigated in an amyloid model system of oriented A $\beta$ 25-35 fibrils on mica. The addition of LPFFD did not significantly alter the orientation and assembly kinetics of A $\beta$ 25-35 fibrils. Already formed fibrils did not disassemble in the presence of high concentrations of LPFFD. The mechanical stability of the fibrils was only slightly decreased, suggesting that LPFFD may moderately weaken inter-protofilament interactions. Complete disassembly of fibrils, however, was not observed. Thus, under the conditions explored here, LPFFD may not be considered as a BSB peptide with generalized beta-sheet breaking properties.

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