Nanoscale

Cite this: *Nanoscale*, 2011, **3**, 3049

www.rsc.org/nanoscale

COMMUNICATION

Assembly of glucagon (proto)fibrils by longitudinal addition of oligomers†

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Received 29th March 2011, Accepted 13th May 2011
DOI: 10.1039/c1nr10332f

The process of glucagon peptide aggregation was studied with high resolution atomic force microscopy (AFM). The statistical analysis of *ex situ* AFM images in combination with *in situ* AFM observation suggests that it is more likely that (proto)fibrils are formed *via* direct longitudinal growth of oligomers, instead of the lateral association of two or more filaments.

Self-assembly of amyloid nanofibrils has attracted tremendous attention due to their close association with a variety of neurodegenerative disorders such as Parkinson's and Alzheimer's.[1,2] Using electron microscopy, atomic force microscopy (AFM), X-ray diffraction and solid state NMR, the morphology and structures of the amyloid fibrils have been extensively studied.[3–5] These protein fibrils that typically contain a core structure with β-strands are believed to be linked to cellular toxicity, while accumulating evidence recently indicates that intermediate aggregates such as soluble oligomers may be more toxic than fibrils, possibly due to their membrane-perturbing abilities.[5,6]

A better understanding of protein polymerization processes is crucial for unraveling the origins of cellular toxicity of the amyloidal fibrils,[7–9] and for elucidating the detailed molecular mechanisms of peptide self-assembly as well.[10] Great efforts to decipher the mechanism of amyloid conversion have been complicated due to the appearance of intermediates such as oligomers with various morphologies during the aggregation processes.[11–17] These oligomers have been proposed as possible building blocks of the higher-order mature fibrils, and amyloid growth occurs by oligomer addition at an active end,[15,16] while the hierarchical assembly model (HAM) describes that mature fibrils such as β-lactoglobulin are formed by lateral association of long preformed filaments.[13] Although the structures of amyloid fibrils have been well characterized, a comprehensive picture on the aggregation behavior of amyloid fibrils still remains obscure.

In this work, we focus on the self-assembly of the 29-residue peptide glucagon which plays an important role in maintaining normal glucose levels *in vivo*. Glucagon fibrils have been comprehensively studied and it has been found that its fibrosis process could be modulated by ionic strength, peptide concentration, and incubation temperatures.[18–22] Previously, the HAM model was proposed to explain glucagon fibrillation,[21,22] where the filament was regarded as a possible building block of the mature fibrils. Recently, Andersen *et al.* reported a branching mechanism for glucagon fibrils and suggested that secondary nucleation is important for some amyloidogenic proteins.[23,24] Previous studies devoted to the examination of the dynamic aggregation process of amyloid fibrils are often based on solid-state NMR, fluorescence spectroscopy and X-ray scattering. These bulk techniques usually provide an average ensemble picture of the fibrils. Here, we use single-molecule AFM to investigate the evolution of the glucagon aggregation, especially by *in situ* AFM observation during a long aggregation progress (up to eight hours). Our data suggest that it is more likely that (proto)fibrils are formed directly *via* longitudinal growth of oligomers.

Firstly, we studied the aggregates of the peptide glucagon incubated at 37 °C for different time periods, as illustrated in Fig. 1. AFM images of the large area are showed in Fig. S1 (ESI). Initially (1.0 h) many big aggregates with a height of 6–8 nm as well as some small ones with a height of 3–4 nm were found on the mica surface (Fig. 1a). The height distribution of these aggregates is given in Fig. S2 (ESI). When the incubation time became longer, fibrillar structures were observed (Fig. 1b–1d). Closer inspections of these structures revealed two main types of fibril structures: filaments and protofibrils. As indicated with arrows in Fig. 1c, the filaments were thin and smooth, while the protofibrils were thicker and shorter, typically with a periodic height fluctuation along their contours (Fig. 1e and f). To probe the secondary structure of these fibrils, Fourier transform infrared spectroscopy (FTIR) was performed, which showed a major amide I peak located around 1628 cm^{-1}, indicating a β-sheet secondary structure (ESI, Fig. S3).[20] Unlike the growth of insulin fibrils,[25] the height of filaments and protofibrils (N >130) changed very little as a function of time (Fig. 2a), suggesting fibrils grew in a longitudinal direction instead of a radial direction. Surprisingly, it was found that the filaments grew extraordinarily longer than protofibrils for the same incubation time (Fig. 1b–d). The lengths of the two different fibrils are plotted *versus* the incubation time (Fig. 2b). Statistical analysis revealed that protofibrils elongated approximately linearly with the time initially (<10 h) at a rate of 0.9–1.0 nm min^{-1}, then the growth rate gradually decreased with time probably due to the consumption of peptide monomers.[20]

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† Electronic Supplementary Information (ESI) available: experimental details, additional AFM images, height distribution of glucagon aggregates, micro-FTIR spectra and ratio of the numbers of filaments *vs.* protofibrils. See DOI: 10.1039/c1nr10332f/

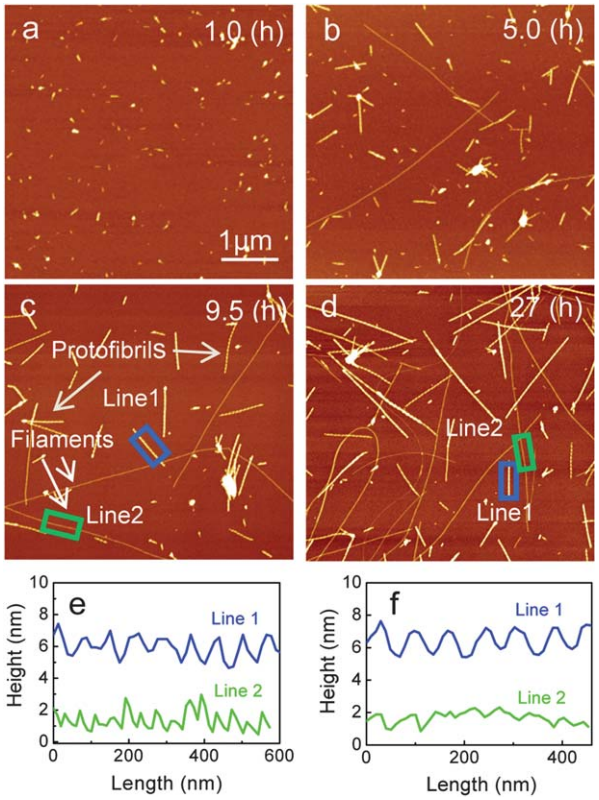

Fig. 1 *Ex situ* AFM images of glucagon after incubating for four different time periods: a) 1.0 h, b) 5.0 h, c) 9.5 h, d) 27 h, respectively. Scale size: 5.0 × 5.0 μm^2; e) and f): cross-sectional analysis of the fibrils marked with colored rectangles and colored lines in c and d).

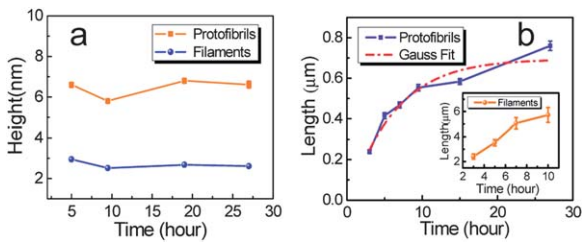

Fig. 2 a) The heights of protofibrils (orange rectangles) and filaments (blue circles) as a function of incubation time, b) growth rate of protofibrils and filaments (inset).

Interestingly, the growth rate for filaments was about 11.7 nm min^{-1}, about one order of magnitude faster than the protofibrils.

The gradual elongation of protofibrils with the incubation time (Fig. 1b–d) and especially the discrepancy in growth rate of the two types of fibrils are difficult to explain by the traditional HAM model in which a protofibril is proposed to form by the twisting of two or more pre-formed long filaments around each other. Our data suggest that it is more likely that filaments and protofibrils are elongated by directly adding oligomers to the ends of the extending fibrils.

To reinforce the picture of the glucagon aggregation, *in situ* AFM measurements were performed to observe the aggregation progress directly. Fig. 3a–c show a sequence of AFM images of glucagon aggregated on a mica surface in the early stages. It can be seen that the vertical height of these fibrils is about 6–7 nm (Fig. 3d),

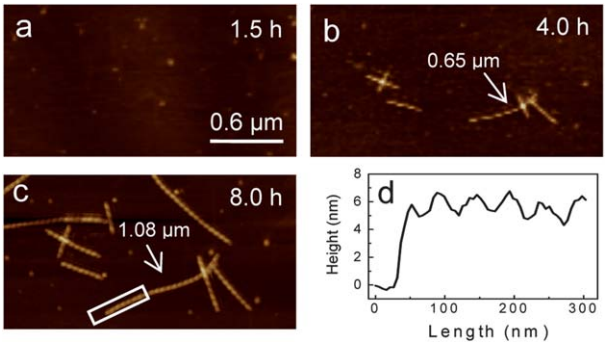

Fig. 3 *In situ* AFM images of glucagon fibrils extending on mica substrate with a monomer concentration of 0.5–1.0 mg ml^{-1}; a–c) images obtained after 1.5, 4.0 and 8.0 h, respectively; d) cross sectional profile along the contour of a fibril as indicated in c). Scan size: 2.4 × 1.2 μm^2.

comparable to the height of protofibrils measured by *ex situ* AFM in air. Fig. 3 also shows that the fibrils increased in length with time. After 4 and 8 h incubation, the length of the fibril marked in Fig. 3 increased to 0.65 μm and 1.08 μm, respectively. Although the growth rates varied from one fibril to another, the fibrils elongated a little faster on the mica surface than in the liquid environment. This is consistent with previous reports that a surface can facilitate the fibrils' growth[26,27] in spite of the fact that the lateral diffusion of protein molecules on surfaces could be slowed down.[28,29] It is most likely that the adsorption of molecules on the surface increases its local concentration, which normally results in a higher rate of fibrillation. It is also possible that the adsorption of protein on the surface induces its conformational changes, which is critical for protein association.[26] Notably, a periodic height fluctuation along the contour of the fibril can be clearly observed (Fig. 3c–3d), further indicating that these fibrillar structures are protofibrils rather than filaments. The above observations strongly argue in favor of our hypothesis that protofibrils are formed by adding oligomers directly into the active ends.

Taken together, our experimental data suggest possible aggregation pathways of glucagon as schemed in Fig. 4. At the first step of this scenario, soluble monomers assemble into small spherical oligomers. Then, there are mainly two competing aggregation pathways: (1) the small oligomers join together to form filaments, and it is likely that the filaments would undergo an additional structural rearrangement after oligomers assembly, resulting in smooth features on its surface;[22] (2) two (or more) small oligomers self-associate to form large oligomers, which then elongate further into (proto)fibrils by coalescing large oligomers in an axial direction. The resulting fibrils can be composed of two or more filaments, depending on the extent

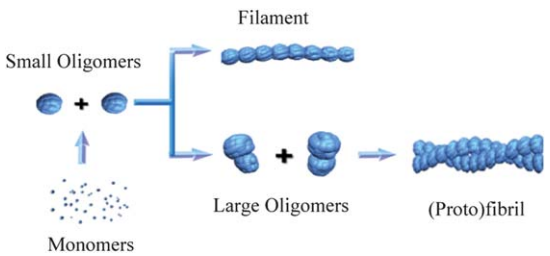

Fig. 4 Schematic drawing illustrating the glucagon aggregation and fibrillation process.

the large oligomers initially grew in height.[7] The above proposed picture for assembly of glucagon (proto)fibril is similar to a linear colloidal aggregation model suggested for other amyloid fibrils based on transmission electron microscopy and dynamic light scattering data.[30]

De Jong *et al.* and Dong *et al.* used the HAS model to explain the process of glucagon fibrillation by visualizing the nanostructures of fibrils formed at different stages of the incubation.[21,22] They found disc-shaped oligomers form at an early stage. These nanostructures then rearrange to assemble into filaments, which probably subsequently associate to form several different classes of higher-order fibrils. Unlike their observations, our data showed that there existed a remarkable discrepancy in growth rate for filaments and protofibrils in our experiment. It is worthwhile to stress that an important piece of evidence supporting the HAS model is that filaments predominated over protofibrils and higher-order fibrils at an early stage, and then decrease in the following days.[14] However, contrary to their experimental observation, our *ex situ* AFM data (Fig. 1 and Fig. S1) showed that protofibrils were dominated initially especially within 7 h (Fig. S4, ESI). After this stage, a few filaments appeared with a faster growth rate, and the surface coverage of filaments over protofibrils increased with time. Recent fluorescence emission spectra and NMR experiments provide additional evidence supporting our model.[31,32] It showed that there existed oligomers during glucagon fibrillation and the oligomers interacted directly with the growing fibrils. In addition, amyloid fibrils from human amylin do show that mature fibrils were unlikely to be assembled by the lateral association of filaments (called protofibrils for amylin).[7]

In summary, both *ex-situ* and *in situ* AFM investigations on the assembly process of glucagon indicated that the filaments and protofibrils grew longitudinally, which allowed us to establish a putative model to describe the pathways of glucagon assembly. This model might be applied to account for the amyloidosis process of other amyloid proteins.[15,16,33] Our results would become important when we consider the recent study that showed neuronal cell death mediated by β-amyloid was critically dependent on ongoing nucleation-dependent polymerization,[34] rather than the fibril itself.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (No. 11074137, 10874198, and 10975175), the National Basic Research Program of China (No. 2007CB936000), and the K. C. Wong Magna Fund in Ningbo University

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