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Analysis of the length distribution of amyloid fibrils by centrifugal sedimentation

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ABSTRACT

The aggregation of normally soluble peptides and proteins into amyloid fibrils is a process associated with a wide range of pathological conditions, including Alzheimer's and Parkinson's diseases. It has become apparent that aggregates of different sizes possess markedly different biological effects, with aggregates of lower relative molecular weight being associated with stronger neurotoxicity. Yet, although many approaches exist to measure the total mass concentration of aggregates, the ability to probe the length distribution of growing aggregates in solution has remained more elusive. In this work, we applied a differential centrifugation technique to measure the sedimentation coefficients of amyloid fibrils produced during the aggregation process of the amyloid β (M1–42) peptide (Aβ42). The centrifugal method has the advantage of providing structural information on the fibril distribution directly in solution and affording a short analysis time with respect to alternative imaging and analytical centrifugation approaches. We show that under quiescent conditions interactions between Aβ42 fibrils lead to lateral association and to the formation of entangled clusters. By contrast, aggregation under shaking generates a population of filaments characterized by shorter lengths. The results, which have been validated by cryogenic transmission electron microscopy (cryo-TEM) analysis, highlight the important role that fibril–fibril assembly can play in the deposition of aggregation-prone peptides.

Amyloid fibrils represent a general class of protein aggregates associated with a rich variety of functional and pathological cellular behavior [1,2], including the onset and progress of a wide range of human neurodegenerative disorders [3–5]. In addition to their role in many biological systems, amyloid fibrils are increasingly finding applications in biomaterials science thanks to their robust material properties such as high mechanical resistance and biocompatibility [6–8].

In many questions of fundamental and practical interest focusing on the behavior of amyloid fibrils, it is crucial to characterize the length distribution of the fibril population. For instance, the physical size of amyloid species has emerged as a key property that affects their interactions with living cells [9,10]. In particular, smaller aggregates possess a significantly enhanced neurotoxic potential and are thought to be central components in the amyloid cascade underlying the development and onset of Alzheimer's disease [5,11]. Furthermore, the length distribution of a growing ensemble of fibrils contains high-level information about the self-assembly mechanism that underlies the formation of the fibrillar aggregates from soluble monomeric units.

Indeed, amyloid fibrils are the result of a complex aggregation process that involves several microscopic nucleation and growth events [12–15]. An understanding of the microscopic aggregation mechanisms is fundamental in situations where fibril formation must be avoided and is a prerequisite for drug discovery against amyloid fibrils in medical sciences [12]. Moreover, control of the microscopic aggregation pathways is crucial in the applications where fibrils are used as biomaterials. Indeed, fibrils produced within an initially homogeneous sample can exhibit very different morphologies depending on the aggregation conditions [16–18]. This feature opens up possibilities to tune the material properties by modulating the conditions during aggregate formation provided that the fundamental mechanisms underlying the self-assembly

Abbreviations used: AFM, atomic force microscopy; Aβ42, amyloid β (M1–42) peptide; EDTA, ethylenediaminetetraacetic acid; ThT, thioflavin T; cryo-TEM, cryogenic transmission electron microscopy; DLS, dynamic light scattering.

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are known. Finally, kinetic analysis based on chemical rate equations is emerging as a powerful tool to identify microscopic mechanisms underlying macroscopic aggregation processes [13,19]. This kinetic strategy involves validating the proposed microscopic mechanisms by comparing time-resolved experimental data with integrated rate laws [12]. In this approach, the level of detail of the mechanistic information achieved depends on the amount of experimental information content because more refined models can be discriminated by increasing the number of constraints. Therefore, determination of the full-length fibril distribution represents valuable experimental information for model validation [20,21].

Current techniques to measure the length of amyloid fibrils involve mainly single-molecule imaging techniques such as atomic force microscopy (AFM) [22–28], electron microscopy [29], and super-resolution fluorescence microscopy [30,31]. In these approaches, the length distribution is reconstructed by measuring the length of a large number of individual fibrils. The application of this technique has enabled quantitative analysis of the fibril distribution of different proteins under specific conditions [25–28]. In addition, AFM techniques represent very suitable methods to investigate the morphology of amyloid fibrils at nanometer resolution [22–24,32,33], and quantitative methods to reconstruct the length distribution by microscopy techniques have been continuously developed [34]. However, these methods often require drying of the sample on a grid, an operation that can potentially alter the fibril length distribution and induce fragmentation. Moreover, the large amount of data required to provide sufficient statistics typically makes the procedure time-consuming.

Thus, analyzing the properties of the fibrils directly in solution avoids many complications associated with surface-based microscopy techniques. In this respect, the measurement of rotational or translational diffusion motion in solution represents an attractive path to probe the properties of asymmetrical objects in solution [35,36]. However, access to direct information on diffusional processes commonly implies the use of light scattering techniques [32,37,38], which can be challenging to apply to polydisperse samples. An attractive alternative approach is to exploit the differences in the sedimentation coefficients of fibrils with different lengths to separate the fibrils directly in solution in the presence of a centrifugal field [39]. An example of this method is analytical centrifugation [40–45], which has been proven to be a powerful tool in measuring the distribution of amyloid fibrils with high resolution [46]. This technique, however, typically requires analysis times on the order of several hours or days, and controls need to be performed to verify the absence of possible modifications of the length distribution during this time period.

In this work, we applied centrifugal sedimentation on a disc centrifuge photosedimentometer, which enables samples to be analyzed on a time scale of a few minutes [47]. This approach has been traditionally applied to characterize several colloid systems [48–50] and has been extensively used for preparative fractionation of amyloid samples [51]. Here we demonstrate how this technique allows the analytical characterization of structural features of the length distributions of amyloid fibrils produced under different aggregation conditions. We focus on the analysis of fibrils generated by the amyloid β (M1–42) peptide (Aβ42) associated with Alzheimer's disease [4,19,52]. We show that under quiescent conditions interactions between Aβ42 fibrils lead to lateral association between filaments and to the formation of entangled clusters. In contrast, aggregation under shaking conditions generates a population of individual fibrils characterized by shorter lengths.

Materials and methods

Materials

The Aβ(M1–42) peptide (MDAEFRHDSGYEVHHQKLVFFAEDVGS NKGAIIGLMVGGVVIA), here called Aβ42, was expressed in *Escherichia coli* from a synthetic gene and purified as described by Walsh and coworkers [53] except that size exclusion with spin filters was replaced by gel filtration. Samples were doped with approximately 5% of Aβ(MC1–42) labeled with the dye Alexa 488 (attached to the N-terminal cysteine using maleimide chemistry [54]) to increase the light scattering cross section of the fibrils and, therefore, the detection sensitivity of the analysis.

Amyloid fibril preparation and aggregation kinetics

Aggregation under quiescent conditions of 7 μM Aβ42 in 20 mM phosphate buffer (pH 8.0) with 200 μM ethylenediaminetetraacetic acid (EDTA) and 20 μM thioflavin T (ThT) was induced by incubating samples in a low-binding polyethylene glycol (PEG)-coated polystyrene 96-well plate (Corning 3881) at 37 °C in a plate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany). Aggregation kinetics was monitored by recording ThT fluorescence through the bottom of the plate at 480 nm after excitation at 440 nm. Aliquots of 100 μl for the centrifugal analysis were taken at selected time points during the reaction.

Aggregation under shaking conditions was induced by incubating a solution of 7 μM Aβ42 in 20 mM phosphate buffer (pH 8.0) with 200 μM EDTA for 1 h at 37 °C in a shaker (Vibrax-VXQ, IKA, Staufen, Germany) operating at 2000 rpm. The presence of fibrils after the incubation period was verified by ThT fluorescence.

Short fibrils were also prepared by sonicating 300 μl of 7 μM freshly prepared Aβ42 fibrils for 10 min with a tip sonicator (3 mm exponential microprobe, Soniprep 150 Plus, MSE, London, UK) working in pulse mode with 1-s pulses and 1-s waiting time (i.e., 50% duty cycle). The length of the fibrils before and after sonication was checked by dynamic light scattering analysis using a Wyatt DynaPro Plate Reader instrument (Wyatt Technology, Dernbach, Germany) operating in the backscattering mode at an angle of 158° with a laser wavelength of 830 nm.

Differential centrifugal analysis

Sedimentation coefficients were determined using a CPS disc centrifuge (DC24000UHR, CPS Instruments Europe, Oosterhout, Netherlands). Under the application of a centrifugal force, fibrils with different lengths migrate with different velocities according to their sedimentation coefficient, *s*. In this system, fibrils move from the injection point to the edge of the rotating disc, where particles are detected by light scattering. Application of Mie theory, together with calibration with known standards prior to each analysis, allows the estimation of the concentration of the sedimenting objects. Fibrils migrate in gradients of either 2–12% or 4–24% sucrose in MQ water, which avoids the rise of streaming instability during operation. In this work, samples of 100 μl were injected and rotated at 16,000 or 21,000 rpm for 5 min for fibrils with *s* in the range from 10^3 to 5×10^5 S and for 40 min for fibrils with *s* in the range from 10^2 to 5×10^5 S.

The software uses Stokes' law to evaluate the equivalent spherical size of the sample particles according to the equation