Extrinsic Amyloid-Binding Dyes for the Detection of Individual Protein Aggregates in Solution

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Abstract

Protein aggregation is a key molecular feature underlying a wide array of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases. To understand protein aggregation in molecular detail it is crucial to be able to characterise the array of heterogeneous aggregates that are formed during the aggregation process. We present here a high-throughput method to detect single protein aggregates, in solution, from a label-free aggregation reaction and demonstrate the approach with the protein associated with Parkinson's disease, alpha synuclein. The method combines single-molecule confocal microscopy with a range of amyloid-binding extrinsic dyes, including thioflavin T and pentameric formyl thiophene acetic acid, and we show that we can observe aggregates at low picomolar concentrations. The detection of individual aggregates allows us to quantify their numbers. Furthermore, we show that this approach also allows us to gain structural insights from the emission intensity of the extrinsic dyes that are bound to aggregates. By analysing the time evolution of the aggregate populations on a single-molecule level, we then estimate the fragmentation rate of aggregates, a key process which underlies the multiplication of pathological aggregates. We additionally demonstrate that the method permits the detection of these aggregates in biological samples. The capability to detect individual protein aggregates in solution opens up a range of new applications, including exploiting the potential of this method for high-throughput screening of human biofluids for disease diagnosis and early detection.

Confocal probe volume

The dimensions of the confocal volume were obtained from the autocorrelation function, of a stationary sample of AF488 (triplicate 100 pM), as measured using fluorescence correlation spectroscopy (FCS). The autocorrelation function was fitted using a 3D diffusion model; Eq. 1[1,2].

$$G(\tau) \approx \frac{1}{N} \cdot \frac{1}{1+\frac{4D}{\omega^2}\cdot\tau} \cdot \frac{1}{\sqrt{1+\frac{4D}{{z_0}^2}\cdot\tau}} \quad (1)$$

where N is the number of molecules in the confocal volume, ω is the laser beam waist, z_0 is half the depth of focus in the z direction and D is the diffusion coefficient ($4.35 \cdot 10^{-10}$ $m^2 \cdot s^{-1}$ for AF488)[3].

The confocal volume is modelled as a Gaussian and hence the volume can be calculated using Eq. 2.

$$V_{eff} \approx \pi^{\frac{3}{2}} \cdot w^2 \cdot z_0 \quad (2)$$

For the 488 nm laser, ω was found to be 0.48 ± 0.04 μm, and z_0 to be 1.38 ± 0.16 μm; therefore the confocal volume, V_{eff} = 1.80 ± 0.39 fL. For the 445 nm laser, ω was found to be 0.62 ± 0.08 μm, and z_0 to be 0.91 ± 0.07 μm; therefore the confocal volume, V_{eff} = 1.94 ± 0.67 fL.

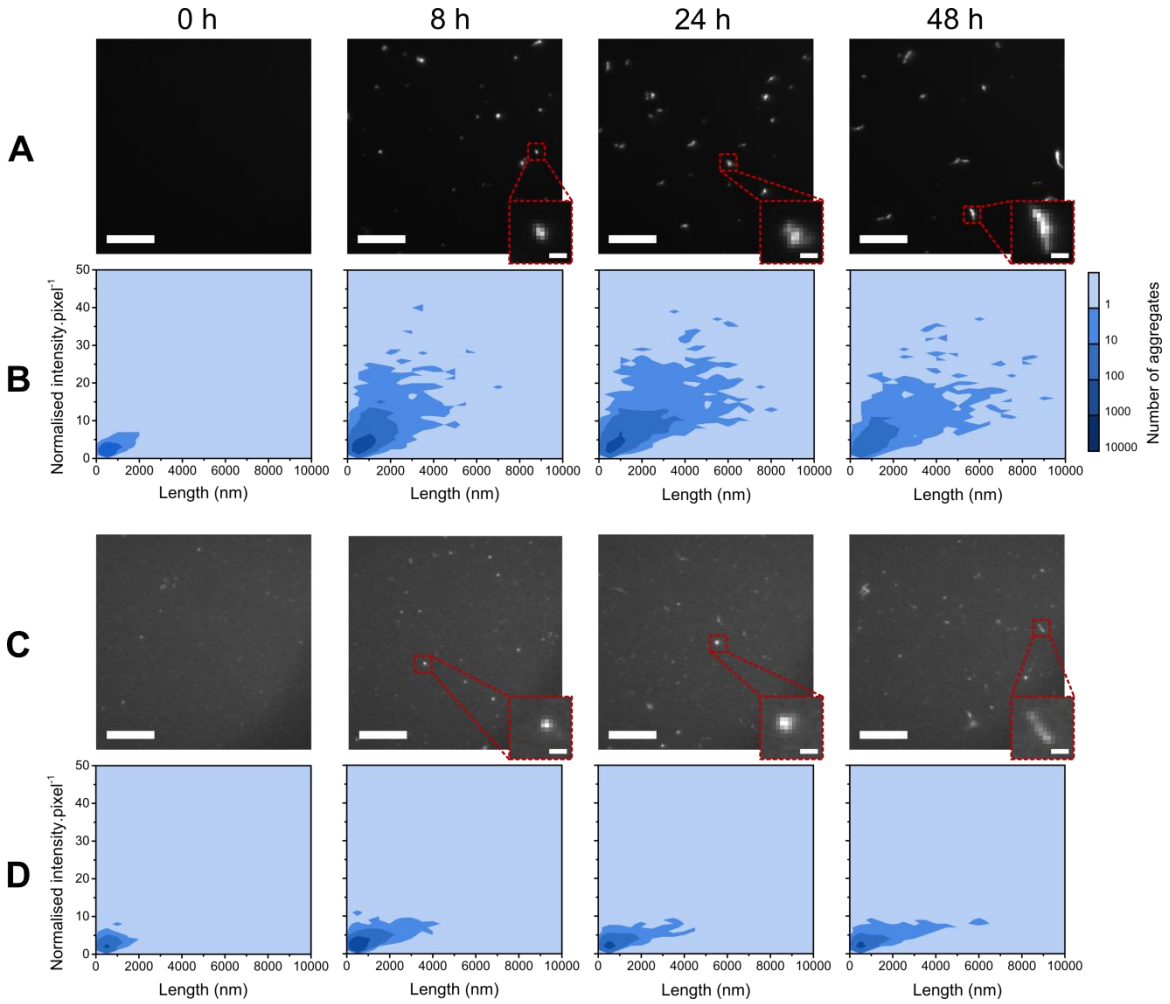

Figure S1: Two-dimensional contour plots for the aggregation of αS monitored by diThT (**B**) and PicoGreen (**D**) as a function of normalised intensity per pixel and length (n=3), with corresponding representative TIRFM images (**A** and **C**, respectively). The contrast was optimised and fixed for each dye and the scale bar is 10 μm for all images and 1 μm for all zoomed insets.

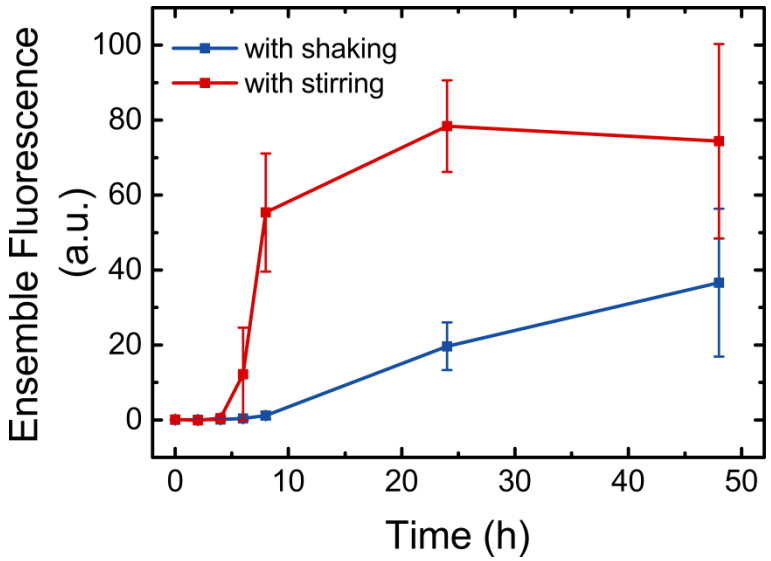

Figure S2: Ensemble ThT fluorescence measurements monitoring the aggregation of αS with shaking and with stirring. The ensemble fluorescence tracked correlates well with the single-molecule confocal measurements for both aggregation in the shaking and the stirring regimes.

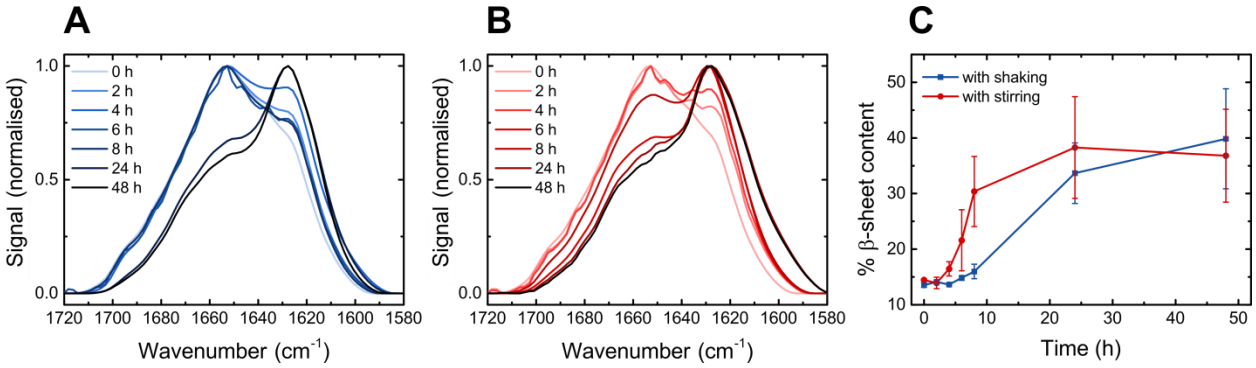

Figure S3: FT-IR spectra were taken for time-points acquired during the aggregation of αS with shaking **(A)** and with stirring **(B)**. Curve fitting of the amide region (1720-1580 cm^{-1}) and baseline subtraction were carried out and all absorbance spectra were normalised for comparison. For both regimes a gradual decrease in the random coil content was observed, indicated by the diminishing peak at 1655 cm^{-1} and a concomitant increase in β-sheet secondary structure, indicated by the emergence of a peak at 1626cm^{-1}.[4] Deconvolution of the spectra with Gaussian curves was performed using a Levenberg-Marquardt algorithm. All plots were fitted with gaussians centred at 1626 cm^{-1}, 1640 cm^{-1}, 1655 cm^{-1}, 1680 cm^{-1} and 1695 cm^{-1}. We calculated the percentage of β-sheet content present by dividing the integral of the gaussian centred at 1626 cm^{-1} by the total integral for each plot **(C)** (n=2). The increase in β-sheet content occurs markedly earlier for the regime with stirring (6-8 h), compared to that with shaking, which corresponds well to both the ensemble ThT fluorescence measurements and the single-molecule confocal measurements.

References

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