Rapid fragmentation during seeded protein aggregation revealed at the single molecule level

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Section 1. Characterisation of lysozyme seed fibrils using AFM

Figure S1. AFM image of unsonicated seeds (a) and sonicated seeds (b). (c) Height (z-dimension) distribution of the sonicated seed sample shown in (a). (d) Contour length distribution of sonicated seeds.
Section 2. Analysis of AFM images in the early stage of seeded lysozyme aggregation (T < 20 min)

Figure S2. Gaussian fitting of the z-profile distribution at T = 5 min sample containing protofibrils and Type 1 fibrils. The fitted diameters of these species are 0.6 ± 0.2 nm and 1.3 ± 0.1 nm. We have deliberately reduced the threshold for segmentation compared to Figure 2a (main text) in order to include protofibrils in the analysis.

Figure S3. Detail of an AFM image of a lysozyme sample at T = 5 min of seeded aggregation, showing the basic rigid units into which seed fibrils disassemble (green arrow) and 2 units that haven’t fully disassembled yet and have a clear junction (red arrow).
Figure S4. Atomic force microscopy images of lysozyme aggregates at T = 0 min (a); T = 5 min (b); T = 15 min (c); T = 60 min (d) and T = 24 h (e) after seeding.

Figure S5. Comparison of the heights (z, nm) of lysozyme aggregates vs. their length (L, μm) collected at T = 5 min and T = 15 min after seeding.
**Figure S6.** The median contour length of lysozyme aggregates in the first hour of sample incubation; the grey shadowed area indicates 25% to 75% confidence zone. The length distribution broadened after T = 15 min.
Section 3. Incubation of sonicated seed fibrils in absence of lysozyme monomers

Figure S7. AFM images of sonicated seed fibrils of lysozyme, added to an incubation solution not containing lysozyme monomers. The solution was prepared at exactly the same composition and concentrations as the solution used during a standard seeding experiment, apart from lysozyme monomer content, which was zero in this experiment. Samples were aliquoted at T = 0 min, T = 25 min and T = 60 min. The AFM images revealed that there was no fragmentation or growth of the seed fibrils over time.
Section 4 Incubation of lysozyme monomers in absence of sonicated seed fibrils

**Figure S8.** Incubation of an unseeded sample of monomeric lysozyme. The incubation was performed at standard composition and conditions, except that this time seed fibrils were not added to the monomeric lysozyme solution. The progression of AFM images of aliquots taken at $T = 0$ min, 15 min, 30 min, 60 min, 16 h and 24 h demonstrate that compared to seeded lysozyme aggregation, the growth rate of unseeded lysozyme is significantly slower. Fibrillar structures were not observed at $T = 60$ min.
Section 5. AFM analysis of the assembly of mature fibrils

**Figure S9.** Detail of an AFM image demonstrating the rigid segments of mature lysozyme fibrils, collected at 24h following seeding.

**Figure S10.** An AFM image of mature lysozyme fibrils collected at 24h following seeding (a, b) and the height profile of a mature fibril (c) showing the periodicity in a height profile caused by twisting. The profile (c) corresponds to the orange section across the fibril shown in (b).
Section 6. Nanopore characterisation

Nanopore, a label-free platform, enables the real-time sensing of heterogeneous samples with broad size distribution at a single molecule level\(^1\). In a typical experiment, biological molecules are electrophoretically or electro-osmotically driven through a nanopore by an externally applied electric field, resulting in a temporal modulation of the measured ionic current\(^2-3\). From these modulations one can extract information on molecular properties such as length and thickness. Previous studies have showed that the cylindrical rod will align the length axis parallel to the applied electric field prior to entering the nanopore\(^1,4\). Nanopore measurements have been used to study the characteristics of different species and kinetic during the aggregation pathway, either with the coated nanopore or alternatively at a low pH = 4\(^1,5-6\). In this work we used quartz capillaries with a nanopore opening (also termed ‘nanopipettes’) to examine the aliquots obtained during the aggregation of lysozyme.

Nanopipettes are a sub-class of solid-state nanopores, exhibiting advantages over solid-state nanopores such as easy fabrication, low noise and good mechanical stability\(^7-8\). The nanopipettes used in the study had a conical geometry at the tip with a nanopore of around 30 nm in diameter, as determined by the SEM images (Figure S11).

Figure S11. (a) SEM images of one of the quartz capillaries (nanopipettes) used in this study. (b) The nanopore at the tip of the capillary.
Section 7. Nanopore functionalisation

Single molecule translocation of lysozyme fibrils with unfuctionalised nanopore showed complete blockage after a period of time, with the current dropping to around 0 nA (Figure S12a). This was due to the strong interaction between positively charged lysozyme fibrils and negatively charged walls of the quartz capillary. Thus, to minimise the pore-protein interaction, the nanopores were functionalised with 7.5% polybrene, according to the previously published procedure\(^9\), which made the quartz surface positively charged and thus reduced the lysozyme-pore absorption.

The modification was achieved by immersing the capillaries in the 7.5% polybrene solution, as well as adding polybrene solution into the capillaries for 15 min, followed by rinsing with translocation buffer.

The current-voltage curves were recorded before and after the functionalization (Figure S12b). After functionalisation, the nanopore showed positive rectification with the ratio \(I_{-500\text{mV}}/I_{500\text{mV}}\) of 1.25. This was consistent with the rectification behaviour observed in positively charged conical geometries, verifying the successful functionalisation of the positively charged polybrene to the surface of the nanopore. After the functionalisation, dramatic decrease of the conductance with time was no longer observed, indicating that the nanopore was not blocked by the lysozyme fibrils.

Previously, Erik C. Yusko et al.\(^1\) utilized the lipid biolayer-coated nanopore to avoid the clogging and successfully analysed the aggregation of amyloid beta peptide. Nikolay Martyushenko et al. proposed the nanopore-assisted analysis of lysozyme aggregation without a surface modification\(^6\), however, low pH was required in this study, which may have caused the evaporation of the solution and thus the change in the current baseline\(^6\). Although surface functionalization was required in our experiment, it was fast (15 min), facile (simple immersion) and overall considerably more convenient as compared to the modification with the fluid lipid bilayer.

![Figure S12. (a) Current-time trace of lysozyme aggregate translocation through a unfuctionalised nanopore; the absence of functionalisation led to a significant blockage (zero current) after recording for a period of time. (b) Current-voltage curve of the nanopore before and after surface functionalisation with polybrene.](image-url)
Section 8. Zeta potential measurements

The nanopore current enhancement events were observed in previous studies of DNA translocation and were documented to be related to the salt concentration and the counterions on the surface of the translocating macromolecules\textsuperscript{10-11}. With the concentration of KCl between 10 mM ~ 300 mM, translocation of DNA resulted in current enhancement due to the positive counterions loosely attached\textsuperscript{11}. During the translocation of DNA, although the occupation of DNA inside the nanopore reduced the number of charge carriers available for ionic transport, the counterions shielding DNA served as ion carriers, giving rise to an increased overall conductance.

To further investigate the nature of current enhancement (upwards translocation events) as compared to current blockades (downwards events) for lysozyme aggregation in 100 mM KCl, zeta potential measurements of the aggregating mixtures were carried out. The charge calculated from nanopore sensing (the integrated current area over time, Figure S14a) and zeta potential (Figure S13) of the lysozyme aggregation showed the same trend, decreasing to a dip at 10 min after seeding and increasing from 15 min onwards. Electrophoretic mobility was obtained from the raw data in zeta potential measurement, showing a decreasing trend from 1.0 ± 0.2 μm$^2$/cm/(V*s) at 0 min to 0.7 ± 0.1 μm$^2$/cm/(V*s) at 10 min, followed by an increase after 20 min onwards to 3.5 ± 0.2 μm$^2$/cm/(V*s) at 20 h, Figure S14b.

![Figure S13. The zeta potential at various aggregation time points. The inset shows the zoom into the first 60 minutes of sample incubation.](image)
Figure S14. (a) The charge calculated from nanopore sensing and (b) the mobility obtained from zeta potential measurements for lysozyme aggregation at various sampling time points. The grey shadowing in (a) indicates 25% to 75% confidence interval; the error bars in (b) represent the standard deviation.
References