A kinetic model of the aggregation of alpha-synuclein provides insights into prion-like spreading

Short title: Templated seeding of alpha-synuclein aggregation

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Abstract

The protein alpha-synuclein (αS) self-assembles into small oligomeric species and subsequently into amyloid fibrils, that accumulate and proliferate during the development of Parkinson’s disease. However, the quantitative characterisation of the aggregation and spreading of αS remains challenging to achieve. Previously, we identified a conformational conversion step leading from the initially formed oligomers to more compact oligomers preceding fibril formation. Here, by a combination of single-molecule fluorescence measurements and kinetic analysis, we find that the reaction in solution involves two unimolecular structural conversion steps, from the disordered to more compact oligomers, and then to fibrils, which can elongate by further monomer addition. We have obtained individual rate constants for these key microscopic steps by applying a global kinetic analysis to both the decrease in the concentration of monomeric protein molecules and the increase in oligomer concentrations over 0.5-140 μM range of αS. The resulting explicit kinetic model of αS aggregation has been used to quantitatively explore seeding the reaction by either the compact oligomers or fibrils. Our predictions reveal that, although fibrils are more effective at seeding than oligomers, very high numbers of seeds of either type, of the order of $10^4$, are required to achieve efficient seeding and bypass the slow generation of aggregates through primary nucleation. Complementary cellular experiments demonstrated that two orders of magnitude lower numbers of oligomers were sufficient to generate high levels of reactive oxygen species, suggesting that effective templated seeding is likely to require both the presence of template aggregates and conditions of cellular stress.

Significance Statement

Growing experimental evidence suggests that the pathological spreading of alpha-synuclein aggregates in Parkinson’s disease is mediated through a process of templated seeding whereby aggregates catalyse the conversion of soluble protein molecules into their aggregated forms. A molecular level understanding of this process is still lacking. Here, we determine the concentrations and numbers of aggregates necessary for the effective seeding of alpha-synuclein, thus providing a quantitative framework to understand the conditions when its seeded propagation is favourable. We find that high concentrations of aggregates are needed for seeding yet that aggregates cause cytotoxicity at significantly lower concentrations. This suggests that templated seeding is unlikely to be the main mechanism of spreading in Parkinson’s disease but occurs together with oligomer-induced cellular stress.
Introduction

Neurodegenerative disorders such as Parkinson’s (PD) and Alzheimer’s disease are becoming increasingly common as a result of increasing longevity in the population of the modern world, and there are no effective disease-modifying therapies to date (1, 2). The main characteristics of these disorders are the deposition and spreading of aggregated proteins causing neuronal loss that is accompanied with motor and cognitive deficits (3-5).

Alpha-synuclein (αS) is a small (14.5 kDa) intrinsically disordered protein expressed in neurons and presynaptic nerve terminals (6). It is abundant in the neuronal cytosol of a healthy brain, and its function is thought to be associated with axonal transport (7, 8). The assembly of monomeric αS into amyloid fibrils that form Lewy Bodies (LBs) and Lewy neurites is a hallmark of PD (9).

The deposition of αS inclusions in PD follows a common pattern which correlates with clinical symptoms (10). Furthermore, transplanted embryonic neurons in PD patients developed LB deposits, suggesting that these aggregates can spread (11). Experiments in wildtype and transgenic mice showed that injection of fibrils of recombinant αS could lead to the aggregation of the endogenous protein, supporting this concept (12-14), and the selective fate of the aggregate-containing neurons was demonstrated (15). While the pathological spreading of αS has now been reproduced in many laboratories, the molecular mechanism of the observed phenomenon is not fully understood and this is important for rational development of therapies. One emerging explanation is that occurs by prion-like propagation of αS aggregates (16, 17). However, the prion-like role of αS in the process of its pathological spreading is still under debate (18), and alternative hypotheses exist to explain the observations (16). At the molecular level, prion-like propagation is linked to the process of templated seeding, in which the aggregates of αS enter a cell and act as templates to promote the misfolding and aggregation of cellular proteins, resulting in an increased number of aggregates that can then spread to neighbouring cells (19). To date, there is a lack of quantitative investigations of how many aggregates, either oligomers or fibrils, are needed to promote efficiently the aggregation of soluble αS, in order to determine and evaluate the conditions when the templated seeding of αS might be favourable.

To access the quantitative information on the templated seeding requirements for αS, it is important to achieve a comprehensive understanding of its aggregation pathway. A concentration-dependence study can reveal reaction orders of the key steps in a detailed molecular-level mechanism of αS
aggregation, that can be ultimately used to make predictions of αS seeding behaviour. To date, evidence has been obtained about the mechanism of αS aggregation in solution (20-22) and in the presence of lipid membranes that are thought to play an important role in vivo (23-25). Single-molecule studies combined with detailed kinetic analysis have provided with an opportunity of defining the sequence of events during the aggregation process in great detail (26-28). Our related studies using single-molecule fluorescence techniques to follow the aggregation of αS identified a slow conversion step from the initially formed, proteinase K-sensitive oligomers to more compact, proteinase K-resistant oligomers. We determined the apparent rates for this process, and showed that the converted oligomers caused the highest damage to neuronal cells (27) and were stable with respect to the changes in buffer conditions (28).

In the present study, we have extended this approach to analyse the kinetics of aggregation over a 280-fold range of αS concentrations, from 0.5 to 140 µM, and used these data to develop an expanded kinetic model for the aggregation of αS and to determine the rate constants for the individual steps of the reaction. The availability of this model, as well as of data acquired over a wide concentration range, allows us to make quantitative predictions of the aggregation behaviour under a variety of pre-defined conditions, and calculate the numbers of oligomers and fibrils required for the templated seeding of αS. This in vitro analysis allows us to assess specifically the propensity of αS protein to undergo templated seeding process, and reveals that this process will bypass the slow nucleation step only under conditions where a high number of aggregates is present within volumes of the order of the size of a living cell. Additional quantitative cellular assays reveal that cytotoxicity occurs at lower concentrations than that those required for seeding, suggesting that the spreading of αS under more complex in vivo conditions is likely to involve templated seeding in combination with complementary cell-mediated processes.

**Results**

**Single-molecule FRET measurements show that αS oligomers are formed at 0.5-140 µM concentrations within several hours**

All the single-molecule experiments were performed with full-length (140-residues) αS with an alanine to cysteine mutation at residue 90 for fluorophore incorporation (A90C). The attachment of Alexa Fluor dyes to the cysteine 90 was demonstrated to have no significant effect on the kinetics of fibril formation in our previous studies (27, 28), a result attributable to the fact that this residue is at
the periphery of the non-amyloid component (NAC) region, which is a key constituent of β-sheet fibril core. In addition, attachment at this position results in the fluorophores becoming in close proximity on the formation of β-sheet structure during aggregation, enabling the conversion process to be followed by intermolecular Förster Resonance Energy Transfer (FRET) between dyes located on different monomers. Therefore, as has been shown in our previous work (27, 28), lower FRET efficiencies are observed for initially formed oligomers lacking significant persistent structure, while higher FRET efficiencies are identified for more compact β-sheet-rich oligomers.

We measured αS oligomer formation in solution at physiologically-related pH and 37°C, with initial monomer concentrations of 0.5, 5, 10, 35, 70 and 140 µM using single-molecule FRET (sm-FRET) technique to follow the changes in the numbers of oligomers in the samples within first 54 hours (Fig. 2). In these experiments, using αS labelled with Alexa Fluor 488 (αS-AF488) and Alexa Fluor 594 (αS-AF594), equimolar quantities of αS-AF488 and αS-AF594 were mixed to give the chosen starting concentration, allowed to aggregate (Fig. 1A), and aliquots were withdrawn for the measurements at the times indicated. Upon withdrawal, the solutions were immediately diluted for single-molecule analysis, and continuously passed through a microfluidic channel to reduce the sampling time (28, 29) (Fig. 1B). A 488 nm (blue) laser beam was focussed in the centre of the channel, to excite the AF488 dye directly, and the resulting fluorescence was simultaneously collected in both AF488 (donor) and AF594 (acceptor) channels. AF594 labelled monomers passing through the confocal volume are undetectable, whereas AF488 labelled monomers give rise to single bursts in the donor channel, enabling the level of monomeric αS to be monitored during aggregation reaction. As oligomers typically contain both types of label, they are detected as simultaneous intense bursts in the donor and the acceptor channels, due to the emission from both the directly excited AF488 and from the non-radiatively excited AF594 via FRET. In this manner, oligomers can be distinguished from monomers despite the fact that the latter are found at much higher concentrations; and the oligomer number and fraction in the solution can be quantified as a function of the reaction time to give kinetic profiles of oligomer formation. The analysis of the experimental data is detailed in the Methods section and the results are shown in Figure 2. In addition, the intensities recorded in both donor and acceptor channels for each oligomer were used to determine its FRET efficiency value (eq. 1 in Methods), and the FRET efficiency values of all oligomers detected in each sample are shown as a FRET efficiency histogram (Fig. 2B), which contains information on both the number of oligomers in the sample, and their structural characteristics.
Variations in the number of oligomers and their FRET histograms as a function of the starting protein concentration

Within 54 hours of the initiation of the aggregation reaction, oligomer formation was observed for all concentrations of αS, and the highest concentrations of oligomers were formed in the samples with the highest initial protein concentrations, and varied as a function of the initial monomer concentration (Fig. 2A). We have performed control experiments in order to verify that we monitor the formation of oligomeric aggregates of αS, using TEM imaging and sm-FRET measurements with removal of fibrils at late incubation times, as detailed in SI Appendix, Figures S1-S3. The FRET efficiency histograms of the oligomers probed at various timepoints (Fig. 2B) indicated the existence of two distinct populations, the initially formed, disordered “low-FRET” and the more compact, “high-FRET” oligomers, in agreement with our previous studies that were carried out at the 70 µM concentration of αS (27, 28). In these studies, the high-FRET oligomers were found to be more proteinase K-resistant, more cytotoxic and more stable towards the changes in buffer conditions compared to the low-FRET species, confirming that the changes in the FRET efficiency represent changes in αS oligomer structure. The experimental data, as previously, were divided into groups with different oligomer apparent sizes, based on the number of peaks that were resolvable in the FRET efficiency histograms, as shown in SI Appendix, Figure S4. The groups included “small” oligomers containing 2-5 monomer units (-mers) which showed one peak in the FRET histograms, and “large” oligomers containing 6-150-mers where two peaks could be identified in the FRET histograms. Species composed of more than 150-mers, and species occupying neighbouring time bins, typically observed in the samples past the first day of incubation, were assumed to arise from fibrils and excluded from the measurements (see SI Appendix, Fig. S3). The apparent size estimation was based on oligomer brightness, as discussed in detail in SI Appendix, and the overall distributions are shown in SI Appendix, Figure S8. This shows that the majority of the species detected at all times in the aggregation reaction were smaller than 10-mers. We also performed additional Total Internal Reflection Fluorescence Microscopy (TIRFM) measurements to confirm these results, as detailed in SI Appendix, Figure S9. In the case of the measurements made on samples with the initial concentrations of 35-140 µM, one FRET peak was observed in the histograms of the small oligomers at all aggregation times, which could be fitted to a single Gaussian distribution (SI Appendix, eq. S1) centred at a FRET efficiency value (E) of 0.5 (SI Appendix, section 1.1). For large oligomers, two populations could be distinguished, particularly at late aggregation times, and the histograms were globally fitted to double Gaussian functions (SI Appendix, eq. S1), with the E values of 0.4 and 0.6 (Fig. 2B and SI Appendix, section 1.1). These two populations were assessed to have distinctly different kinetic profiles, with the low-FRET population appearing and increasing within the first 24
hours of the experiment, but the high-FRET population reaching a maximum value at longer times. They were assigned to the two types of αS oligomers reported previously (27), the low-FRET and the high-FRET oligomers, and the time dependence of these two populations was monitored separately. The kinetic traces for these low- and high-FRET species separately are shown in Figure 3B. Examination of the histograms obtained for the lowest concentration samples (0.5 µM) showed that only the low-FRET population could be observed for the studied aggregation period (SI Appendix, Fig. S7B). For the 5 and 10 µM samples, the low-FRET distribution was clearly detectable during the first 9 hours of the measurements, whereas at later times the histograms showed a much broader single distribution, and the overall oligomer concentrations were extracted without separation into the low- and high-FRET sub-populations (Fig. 2B).

**ROS measurements**

We have previously reported that oligomers can be taken up rapidly by neurons and astrocytes and that the high-FRET oligomers promote the production of reactive oxygen species (ROS) when applied to primary neuronal cultures (27). In order to check whether the oligomers of αS generated in these experiments were able to cause cellular damage, we performed ROS assays and found that the oligomers prepared at either 70 or 5 µM initial protein concentration induced the production of ROS, as detailed in SI Appendix, section 1.8. and Figure S15. Subsequently, we performed a series of ROS measurements to determine the lowest concentration of aggregates needed to be added in order to produce detectable ROS (SI Appendix), and this concentration was around 50 pM, which corresponds to 30 oligomers per volume approximately corresponding to a single cell (10 µm)³.

**The critical aggregation concentration of αS is sub-micromolar**

We measured the critical aggregation concentration (C_{αS}) of αS under the conditions used in this study, by estimating the total concentration of both monomers and oligomers that were released from αS fibrils after prolonged incubation of the fibrils in pure buffer solution. TEM images of the samples of fibrils after the incubation in buffer are in SI Appendix, Figure S10, and confirm oligomer release form the fibrils, in agreement with the observations in our previous work (27). The C_{αS} value corresponds to the total concentration of monomer present in equilibrium with fibrillar aggregates and was measured in supernatants after removal of fibrillar pellets by ultracentrifugation. This approach yielded a value of 0.7±0.2 µM. The method used for the C_{αS} measurement is fully described in SI Appendix, section 1.7. This C_{αS} value is lower than the result in an earlier report, 28 µM, which
was measured using quantitative amino acid analysis (20). It is closer to a more recently reported value of ca. 2.7 µM, which was obtained from absorption measurements of denatured supernatants and subsequent extrapolation to the situation exploring the absence of denaturant (30).

**Kinetic analysis**

**Two simple models are consistent with the kinetic data**

In our previous study of the aggregation of αS (27), we identified a conversion from relatively disordered low-FRET oligomers to more compact high-FRET oligomers, and were able to perform the kinetic analysis of these data to provide the rate of this conversion. In light of the previously unidentified experimental information on the different protein species formed during the aggregation of the protein at different concentrations, we have been able to expand the model obtained in the previous study. This yielded an explicit and, importantly, predictive aggregation model. Similarly to the earlier analysis, we replace the simplified nucleated polymerization models (31-33), in which monomeric units are in direct equilibrium with fibrillar structures, by nucleation-conversion-polymerization models (34), which introduce a series of conformational conversion steps prior to fibril formation. Such a class of models can be solved analytically for early reaction times, allowing a global fit to both monomeric and oligomeric data. The simplest such model considers a general mechanism whereby low-FRET oligomers are formed from monomeric units in solution. These low-FRET oligomers then convert to high-FRET oligomers, which in turn convert into fibrils (Fig. 3A). Fibrils then grow by monomer addition as has been inferred from the observation that the elongation of the fibrils is initially linear in monomer concentration (22). The assumption of the on-pathway nature of the oligomers is supported by multiple observations. The presence of the lag phase in the formation of high-FRET oligomers relative to that of the low-FRET oligomers suggests that these species originate from the rearrangement of their preceding low-FRET species, as an off-pathway relationship cannot generate such an effect. This is further confirmed by the oligomer release upon fibril disaggregation (SI Appendix, Fig. S10), which was previously demonstrated to occur with the high-FRET oligomers being released at early times, followed by the low-FRET species upon longer incubations (27). By the principle of microscopic reversibility this shows that both high- and low-FRET species are on the pathway to form fibrils. In addition, we have recently demonstrated a correlation between the rates of the oligomer and fibril formation for αS and its pathological mutants, particularly the inhibition of both of these processes for A30P variant, further supporting the oligomer on-pathway nature (35). Additionally, from the TEM imaging experiments of aggregation and disaggregation (SI Appendix, Fig. S2 and S10), the diameters of oligomers and fibrils generated in these experiments appear comparable. Lastly, we can directly observe that oligomers
can elongate in the presence of freshly added αS monomer using TIRFM imaging (SI Appendix, Fig. S11). Since sm-FRET data suggest that oligomers account for a very small percentage of total system mass throughout the aggregation reaction, this allowed a coarse grained treatment of each oligomer population (as described in Methods). The dependence of these conversion processes on oligomer size has only a very minor effect on the overall conversion kinetics and thus does not enter the analysis and we only consider an average overall flux between populations. These considerations do not, however, discriminate between different possible reaction orders of the conversion reactions, and so both a monomer independent unimolecular conversion model (Fig. 3A) and a monomer dependent bimolecular conversion model (SI Appendix, Fig. S12A) were considered. Lastly, both models were simplified by setting the conversion rate constant for the low-FRET to high-FRET oligomer conversion step equal to that for the high-FRET oligomer to fibril conversion, since allowing these rate constants to differ introduces an extra free parameter to the fitting procedure in each case, yet makes no significant difference to the quality of the fits. The kinetic equations and method used are discussed in further detail in the Methods section.

We were thus able to use both the unimolecular and the bimolecular conversion models to fit the kinetic data over the entire concentration range, particularly the oligomer concentrations (Fig. 3B and SI Appendix, Fig. S12B), to extract rate constants and a nucleation reaction order. A summary of the results is given in Figure 3, and the obtained values are in agreement with our earlier studies (27, 28). In both cases we obtain a nucleation reaction order $n$ close to one, suggesting that the initial nucleation step governed by $k_n$ is a non-elementary step, for example a change in conformation, which can be resolved in principle into a series of steps by more detailed modelling.

**A unimolecular conversion model explains the observed intermediate FRET histograms at low concentrations**

In order to distinguish between a unimolecular conversion mechanism and a bimolecular conversion mechanism, both models were used with their fitted rate constants to predict the numbers of low-FRET and high-FRET oligomers with reaction time at concentrations below 10 µM, and these predictions are shown in Figure 4. A unimolecular conversion model predicts similar and stable relative abundance of low-FRET and high-FRET oligomers up to 54 hours during aggregation reactions under these conditions, whereas a bimolecular model predicts much larger variations with time between and within each population. The former finding is consistent with the observation of blurred single FRET peaks at these concentrations, shown in Figure 2B; the observed FRET behaviour is intermediate between the low-FRET and high-FRET extremes, whereas a bimolecular model would
predict significant dominance of the low-FRET population’s contribution to the overall FRET behaviour. A unimolecular conversion model is thus preferred and retained for further study below. The unimolecular conversion model gives a rate constant for each conversion step of $9.5 \times 10^{-2}$ h$^{-1}$ giving a half-life of each of the conversion steps of the aggregation reaction, outlined in Figure 3A, of about 7 hours. The overall characteristic reaction time, as measured by an inflection in the production of fibril mass with time, is given by this model as 34 hours for 140 μM, and 183 hours for 0.5 μM. The value of $k_+$ obtained here, $25 \text{ M}^{-1} \text{s}^{-1}$, for the addition of monomer to a short fibril is slower than that obtained for the addition of monomer to large fibrils under similar but not identical conditions (22). This suggests that the short fibrils formed early in the aggregation process still differ in structure from mature fibrils.

**Seeding predictions from the unimolecular conversion model**

In templated seeding, the addition of pre-formed aggregates to the protein solution causes the acceleration of the aggregation reaction (36). Seeding by αS fibrils has been efficient *in vitro* (22, 37). However, determining whether oligomers of αS can also take part in the process of seeding has been difficult experimentally, since they are present in low concentrations during the aggregation process, their different types are hard to isolate and stabilize, and the precise quantification of these seeds prior to their addition remains challenging. Experimental evidence exists for both the promotion (38) and the inhibition (39) of aggregation reaction induced by various αS oligomers.

Having established the extended kinetic model that is consistent with all the experimental data we used this model to explore the impact of seeding with either high-FRET oligomers or small fibrils. Low-FRET oligomers were not considered in this analysis owing to their demonstrated lower stability (27, 28). We determined the concentrations of either high-FRET oligomers or small fibrils that are required to double the initial aggregation rate of αS over a $10^{10}$-fold range of the different initial concentrations of αS (see SI Appendix, Fig. S13). According to the predictions, lower concentrations of fibrils than of oligomers are required in order to double the aggregation rate of αS, meaning that fibrils will be more prone to seed this reaction if present at equal concentrations. In addition, we assessed how sensitive the system is to seeding over the explored αS monomer concentration range, by looking at the ratio of the monomer concentration to seed concentration, termed “effectiveness” (SI Appendix, Fig. S13). The results reveal that the impact of seeding will vary not only depending on the nature of the seeds, but also on the initial αS monomer concentration (34); below 10 nM of
monomeric αS seeding by either types of aggregates will be relatively ineffective, whereas will become effective between 10 nM-1 μM, and particularly prominent at the concentrations above 1 μM.

To assess the role of seeds in αS aggregation at more physiologically-related conditions, we estimated the numbers of oligomers or fibrils that would need to be introduced to a volume approximately corresponding to a single cell (10 μm)$^3$ in order to double the aggregation rate in the presence of a set of chosen initial concentrations of αS, which include the range of reported in vivo concentrations of αS (40, 41). According to the kinetic model, above the C$_{αS}$ the seeding will result in the formation of fibrils, while below the C$_{αS}$ it will lead to faster production of oligomers. Table 1 summarises these predictions, and the first column lists the initial concentrations of αS, followed by columns containing the numbers of either high-FRET oligomers or fibrils that are required to be added to these initial concentrations to double the aggregation rate. In addition, Table 1 includes the concentrations of αS monomer that would be needed to prepare the required oligomer seed numbers during the aggregation process. Following these predictions, if the monomer concentration is 2 μM, 10,000 fibrils are needed, or 16,000 high-FRET oligomers, and an approximately five times higher (9.4 μM) concentration of αS monomer would be required for the production of this number of oligomers. We have investigated how the resulting seed numbers vary upon either lowering or increasing the initial concentration of αS, as illustrated in Table 1, and the required seed numbers remained in the order of thousands of species. For example, at 2 nM concentration, which is the lowest αS concentration considered in this analysis, 2,700 high-FRET oligomers or 2,400 fibrils are needed to double the aggregation rate. The seed numbers at 3 μM and 4 μM of initial αS, mimicking the conditions of the protein overproduction (42, 43), are also included (Table 1).

In order to investigate the effects of the elevated concentration of the initial monomeric αS on the aggregate production, we used the kinetic model to estimate the number of oligomers that would be formed in a 48-hour period at the initial αS monomer concentration of 2 μM in the (10 μm)$^3$ volume, and determined how this number changes if the concentration is 3 μM instead (SI Appendix, Fig. S14). This analysis shows that the simulated cell-like volume would contain about $10^4$ oligomers in a period of 48 hours, if there was no inhibition of the aggregation reaction. This number is again large, of the same order of magnitude as the numbers of species required for seeding, although, for example, the number of proteasomes available for the degradation in a biological cell was estimated to be about 1 million (44). However, if the oligomers were degraded significantly slower than monomers then oligomer formation could reduce the overall rate of proteasomal degradation in a cell. Interestingly, the consequence of the increased initial monomer concentration of αS from 2 μM
to 3 μM in this simulation is that higher concentrations of both oligomers and fibrils are produced at all times up to 48 hours (SI Appendix, Fig. S14). Considering the ability of oligomers to cause cellular damage, demonstrated in this and other studies (45), this observation is consistent with the established correlation between an increase in the concentration of αS and pathology.

Discussion

Here, we have exploited single-molecule FRET microscopy to study the initial steps of αS aggregation, and explored systematically the formation of oligomers over a range of the starting protein concentrations from 0.5 to 140 μM. We then developed an extended kinetic model to analyse the experimental data over the full range of the examined αS concentrations, which allowed an estimation to be made of the rate constants of the main microscopic steps of the reaction shown in Figure 3A, and making predictions of number of αS seeds required to increase the aggregation rate, providing an insight into the conditions when the process of templated seeding might be favourable.

Following our predictions, templated seeding by fibrils will enable the aggregation reaction to proceed to growth of more fibrils by monomer addition, without the steps dependent on the formation and conversion of oligomers. Seeding is expected to be particularly effective at minimum concentrations of αS above 1 μM (SI Appendix, Fig. S13), supporting the importance of αS concentration elevation in the disease. To quantify the number of seeds required to impact on the aggregation rate of αS in a volume approximately corresponding to a single cell (10 μm)^3, we estimated how many oligomers or fibrils would need to be introduced to this volume in order to double the aggregation rate of αS (Table 1). Clearly, these calculations do not aim to address various complex factors of the cellular environment, among which are, for example, the presence of cellular organelles and lipid surfaces, or altered salt content and pH that could increase the number of aggregates (22, 25), or molecular chaperones (46) and protein degradation systems (47), which prevent aggregate formation and remove aggregates once formed. Nevertheless, the predicted seed numbers based on our analysis provide a quantitatively insight into the requirements to observe effective templated seeding of αS in vitro, which is significant because quantification of this process is currently lacking due to experimental difficulties in its elucidation. The predicted numbers of aggregates are large, in the order of 10^4 species per cell-like volume, values which corresponds to micromolar concentrations of the aggregates, requiring multiple species to enter this volume at the same time. These requirements for the templated seeding of αS are relatively high in comparison to,
for example, recently reported results for tau K18 using a similar approach (26). It is interesting to note that our kinetic analysis can be used to predict some of the conditions when a small number of oligomers or fibrils may be effective at seeding the aggregation reaction, and hence templated seeding might readily occur. Two factors appear to be important for this process to be favourable. The first determining factor is the nucleation rate, and if the nucleation rate is slow then a small number of oligomers or fibrils are most effective. For example, if the nucleation rate of αS is reduced in our model by a factor of $10^3$, which is close to the published nucleation rate for the tetrapeptide repeat of tau (26), but all the other rates remain the same, then the number of high-FRET oligomers required to double the initial aggregation rate at 2 μM in the (10 μm)$^3$ volume would be reduced approximately 39-fold, from 16,000 to 410. In order for a single high-FRET oligomer to be required for templated seeding, the nucleation rate constant of αS must be reduced by a markedly larger factor of $10^8$. The other determining factor is the initial protein concentration, and whether it is above or below the critical aggregation concentration, which at our conditions was found to be 0.7 μM for αS. At 2 μM, above the critical aggregation concentration, 16,000 oligomers are needed while below it, at 0.2 μM, only 5,900 oligomers will be sufficient. These oligomers, if added to a hypothetical cell, will lead to the faster formation of more oligomers, but not fibrils. Therefore, templated seeding will be more effective at low concentrations of free monomer.

It is interesting to discuss our seeding predictions in the context of prion-like propagation of αS, since the processes of templated seeding and prion-like spreading have been frequently linked in literature (16). However despite occurring at the same time, the processes can be distinguished at the molecular level, as is schematically illustrated in Figure 5, and the templated seeding mechanism only constitutes a part of the spreading process. Templated seeding results in an accelerated production of protein aggregates. In isolation, this would lead to the formation of aggregates and followed by their passive diffusion (Fig. 5B). In order to achieve sustainable aggregate-driven spreading and prevent dilution of aggregates as they propagate from cell to cell, a process of aggregate amplification is essential in addition to templated seeding (Fig. 5C). In combination, these two processes would create a positive feedback loop, involving aggregate production and multiplication, resulting in the aggregate-driven (prion-like) spreading of αS, schematically shown in Figure 6A. Therefore effective prion-like spreading of αS in vivo requires a combination of conditions favouring both the templated seeding and the aggregate amplification processes. However, although αS aggregate amplification was demonstrated in vitro at low pH (22), we detected no amplification at neutral pH in these experiments and thus additional cellular based processes are needed, not included in the kinetic model, to achieve the aggregate amplification required for sustained spreading.
We have found that the oligomers of αS produced during the aggregation process are neurotoxic, which is consistent with our previous work (27). Using ROS measurements, we estimated that the numbers of oligomers required to promote the production of ROS in neuronal cells under our experimental conditions are in the order of tens of oligomers, which is similar to earlier results reported for amyloid-beta peptide, where cellular damage was found to occur when a single oligomer entered a recipient cell (48). The numbers of aggregates required for ROS production are therefore two orders of magnitude lower than what is required for the templated seeding, and such difference suggests that the templated seeding by the oligomers occurs less readily than the cellular damage caused by the aggregates themselves. This idea is consistent with the long-established link between the oxidative stress and neurodegeneration (49), and the fact that the oxidative reactions can promote the aggregation of αS (50), and is corroborated by experimentally observed correlation between αS seeding and cellular toxicity (15). Based on our findings, we hypothesize that for small aggregates in vivo, if templated seeding occurs it is under conditions of raised levels of ROS, which in turn may promote αS aggregation, resulting in the aggregate spreading in a cell-driven way that does not strongly depend on the seeding effectiveness, as illustrated in Figure 6B. Since our results suggest that cellular stress is required for sustained spreading, and larger αS fibrils were previously found less effective at exerting it (27), this implies that a larger number of fibrils than of oligomers will be required for the spreading to occur. Taken together, our quantitative analysis suggests that the mechanism of templated seeding by oligomers or fibrils is unlikely to solely drive the spreading of αS aggregates in PD, since this will always occur under conditions of cellular stress. Ultimately, this suggests that reducing cellular stress may be a possible therapeutic strategy to prevent the spread of disease through the brain.

**Summary**

In summary, we have characterised the early stages of αS aggregation using in vitro single-molecule experiments and kinetic analysis. Our proposed model treats the initially formed low-FRET oligomers, the more compact high-FRET oligomers and the fibrils of αS as distinct species, requiring successive conversion steps. The combination of single-molecule measurements and kinetic analysis used in this study has provided a quantification of αS aggregates in seeding the aggregation process, which is important in the context of PD, and the approach is applicable to other peptides and proteins that are likely to be involved in neurodegenerative diseases.
Methods

αS Sample Preparation for sm-FRET

Monomeric full-length A90C and wild-type αS were expressed and purified according to previously described protocol (51). A90C was labelled with maleimide-linked Alexa Fluor 488 (AF488) or Alexa Fluor 594 (AF594) (Life Technologies) and separated from the free dyes according to the previously reported protocol (27, 28, 52). Aliquots were flash-frozen, stored at −80°C and thawed once before use. For sm-FRET aggregation experiments, a 1:1 molar ratio of AF488 and AF594-labelled monomeric αS were combined in Tris buffer (25 mM Tris, 0.1 M NaCl, pH 7.4) with 0.01% NaN₃, up to a starting protein concentration of either 0.5, 5, 10, 35, 70 or 140 µM and a sample volume of 300 µL. It is established that αS aggregation in vitro can be promoted by constant agitation (53), therefore the solutions were incubated in the dark at 37°C with constant agitation at 200 rpm (New Brunswick Scientific Innova 43), and aliquots were withdrawn at regular intervals for sm-FRET experiments. The purity of the starting material was confirmed by sm-FRET measurements of the samples prior to the incubation.

sm-FRET Data Acquisition

Aliquots from the dual-labelled aggregating samples were diluted by a serial dilution 10³-10⁵-fold in Tris buffer at room temperature immediately before the measurement, a concentration suitable for single-molecule analysis, keeping the multiple occupancy events negligible. The analysed solution was introduced into an inlet of a straight-channelled microfluidic device (PDMS, 25 µm height, 100 µm length) via a gel-loading tip, and passed through the channel at a constant rate of 2 cm/s by a syringe pump (PHD2000, Harvard Apparatus), according to the previously reported method (29).

The setup used for single-molecule measurements (Fig. 1B) was analogous to previously described (54). For the FRET measurement, a collimated 488 nm laser beam (Spectra Physics NewPort Cyan) was directed through a back port of an inverted microscope (Nikon Eclipse Ti-U) at 2 mW power (measured at the back port of the microscope), where it was reflected by a dichroic mirror (Semrock Di01 R405/488/594) and sent through an oil immersion objective (Plan Apo VC 60 x, NA 1.40, Nikon) to be focused 10 µm into the centre of the microfluidic channel. Fluorescence signal was collected by the same objective, imaged onto a 100 µm pinhole (Thorlabs) and separated into two channels by a dichroic mirror (Horiba 585DRLP). Donor fluorescence was filtered by a long-pass (Edge Basic 514) and a band-pass filter (535AF45 Omega Filters) before being focused onto an avalanche photodiode, APD (Perkin Elmer). Acceptor fluorescence was directed through a long-pass filter (610ALP Horiba) and a band-pass filter (BrightLine 629/53) before being focused onto a second APD. Synchronous
output from the APDs was collected by custom-implemented field-programmable gate array, FPGA (Celoxica RC10). Data were acquired for 400 s (80 frames, 100,000 bins per frame, 50 µs bin-width) per aliquot, and consisted of time-binned photon bursts in the donor and the acceptor channel (SI Appendix, Fig. S5). It was verified that the chosen experimental conditions and the detection time lead to the stable rate of coincident events (SI Appendix, Fig. S6), suggesting the absence of oligomer dissociation during the measurements.

**sm-FRET Data Analysis**

Data were analysed using custom-written Igor Pro (Wavemetrics) code, according to a previously reported method (28). Time-bins with intensities greater than 15 photons/bin in the donor (emission from AF488) and the acceptor (AF594) channel simultaneously (the AND criterion) (55) were assigned to be due to oligomeric events and selected for the analysis. The donor counts which did not fit the criterion but were above the applied threshold were saved separately and assigned as monomeric αs bursts. The threshold of 15 photons/bin for both channels was determined using the previously established optimised threshold selection method (56). The values of the photon bursts were corrected for the cross-talk and the autofluorescence from the donor to acceptor channels according to:

\[ I_D' = (D - A_D) \]

\[ I_A' = (A - A_A - C \times D) \]

where \( I_D' \) is the modified intensity in the donor channel, \( D \) is the original intensity in the donor channel, \( A_D \) is the autofluorescence in the donor channel (1.6 photons/bin, the average signal from buffer only), and \( I_A' \) is the modified intensity in the acceptor channel, \( A \) is the original intensity in the acceptor channel, \( A_A \) is the autofluorescence in the acceptor channel (1.3 photons/bin), \( C \) is the cross-talk from donor to acceptor channel (13%). The crosstalk was negligible from the acceptor to donor channel.

For every simultaneous oligomeric burst, the FRET efficiency was calculated:

\[
E = \frac{I_A'}{(I_A' + \gamma I_D')}
\]

where \( I_D' \) is the donor intensity in the presence of an acceptor, \( I_A' \) is the acceptor intensity and \( \gamma \) is the gamma factor specific to the instrument (0.99), which accounts for the relative detection efficiencies of the dyes and their quantum yield.

The FRET efficiency values were binned into histograms with bin width of 0.05 (Fig.2).

Subsequently, the data were split into two size groups: small (2-5-mers) and large (6-150-mers). Large species, either occupying consecutive time-bins or greater than 150-mers, were excluded from the analysis as detailed in Horrocks et al. (29). At small sizes, one peak was observed in the FRET
efficiency histograms in all measured samples (SI Appendix, Fig. S4). At large sizes, either one (at 0.5-10 µM), or two (at 35-140 µM) FRET efficiency peaks could be distinguished. The two distinguishable peaks were assigned to be due to low-FRET oligomers and high-FRET oligomers. The FRET efficiency histograms were integrated to give oligomer kinetic traces, as detailed in SI Appendix, section 1.1. For 0.5-10 µM samples, the overall change in oligomer populations was obtained. For 35-140 µM samples, the separate kinetic traces for low-FRET and high-FRET oligomers were resolved.

**Kinetic Analysis**

The following kinetic moment equations were used to model the aggregation of αS, neglecting the relatively slow reverse conversions and depolymerization reactions:

**Monomer independent unimolecular conversion model:**

\[
\begin{align*}
\dot{M}_1(t) &= n k_m(t)^n - k_1^+ P_1(t), \\
\dot{M}_2(t) &= k_1^+ P_1(t) - k_2^- P_2(t), \\
\dot{M}_3(t) &= k_2^- P_2(t), \\
m(t) &= m_{tot} - M_1(t) - M_2(t) - M_3(t)
\end{align*}
\]

**Monomer dependent bimolecular conversion model:**

\[
\begin{align*}
\dot{P}_1(t) &= k_m(t)^n - m(t) k_1^+ P_1(t), \\
\dot{P}_2(t) &= m(t) k_1^+ P_1(t) - m(t) k_2^- P_2(t), \\
\dot{P}_3(t) &= m(t) k_2^- P_2(t), \\
\dot{M}_1(t) &= n k_m(t)^n - m(t) k_1^+ M_1(t) + 2k_1^+ m(t)P_1(t), \\
\dot{M}_2(t) &= k_1^+ M_1(t) - k_2^- M_2(t) + 2k_2^- m(t)P_2(t), \\
\dot{M}_3(t) &= k_2^- M_2(t) + 2k_4 m(t)P_3(t), \\
m(t) &= m_{tot} - M_1(t) - M_2(t) - M_3(t)
\end{align*}
\]

where:

- \( P_1 \) = number concentration of low-FRET oligomers,
- \( P_2 \) = number concentration of high-FRET oligomers,
$P_3$ = number concentration of fibrils,
$M_1$ = mass concentration of low-FRET oligomers (concentration of monomer residues involved),
$M_2$ = mass concentration of high-FRET oligomers (concentration of monomer residues involved),
$M_3$ = mass concentration of fibrils (concentration of monomer residues involved),
$m(t)$ = concentration of free monomer units,
$m_{tot}$ = total concentration of monomer in the system,
$k_1^+$ = rate constant governing growth of low-FRET oligomers,
$k_2^+$ = rate constant governing growth of high-FRET oligomers.

All other symbols are defined in Figure 3 and SI Appendix, Figure S12.

These moment equations are derived by taking sums of the system’s kinetic master equations over the length distribution of each species, as previously described (31-34). Both models are first linearized for early times by taking $m(t) = m_{tot}$ (discarding the last equation in each case) and then solved analytically using the Mathematica 10.0 software package, to obtain closed-form expressions for each population. Initial conditions were chosen according to the seeding scenario explored (for data fitting, unseeded conditions are represented by $P_1(0) = P_2(0) = P_3(0) = M_1(0) = M_2(0) = M_3(0) = 0$ and $m(0) = m_{tot}$). The resulting expressions were then globally fitted to experimental data up to 33 h for a range of values for $m_{tot}$ via a weighted least-squares Levenberg-Marquardt algorithm, leaving all rate constants and the nucleation reaction order free (see Fig. 3 and SI Appendix, Fig. S12). The negligible mass of oligomers observed throughout the aggregation reaction suggests that the consumption of monomer mass by growth of oligomers can be neglected; terms involving $k_1^+$ and $k_2^+$ were thus neglected in both models, with no adverse effect observed on the quality of the resulting fits. Furthermore, $k_1^C$ and $k_2^C$ were equated into a single parameter $k_c$ in each model, as described in the main text in the Kinetic Analysis section.

The overall characteristic reaction time $t_a$ in the unimolecular model, as measured by the time at which an inflection is observed in the fibril mass concentration $M_3(t)$ without linearization of the equations, is given to a good approximation by $t_a = (2k_4k_5m_{tot}^n)^{-1/2} + 2k_c^{-1}$ as described previously (34). For seeding simulations, a doubling in the initial reaction rate was quantified by considering a halving in the tenth time of the reaction upon seeding; that is, the time taken for the fibril mass concentration $M_3(t)$ to reach $m_{tot} / 10$, which lies within the range of validity of the linearized early time solution described above.
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References:


Figure Legends:

Fig. 1: Schematic representation of sm-FRET experiment to probe the aggregation of alpha-synuclein (αS). (A) 1:1 stoichiometric ratio of αS monomer labelled with Alexa Fluor 488 and Alexa Fluor 594, shown as blue and orange spheres, was combined at a given initial concentration and allowed to aggregate. During the process, the monomeric protein assembles into oligomers, the main focus of the present experiments, and then amyloid fibrils. Aliquots were withdrawn, diluted and analysed using a single-molecule microscope. (B) Schematic of the setup used for the single-molecule FRET experiments.

Fig. 2: Results of the sm-FRET experiment. (A) Kinetic profiles of oligomer formation and monomer depletion, plotted against the incubation time (SEM, N=6, N is a separate sample). To note, the increase in the oligomer concentrations for 0.5 μM solutions is present and the resulting species are in the low-nanomolar range, which is not readily visible on the scale in A. Magnification is shown in SI Appendix, Figure S7A. (B) Representative FRET efficiency histograms, resulting from sm-FRET aggregation experiments with initial protein concentrations of 5, 10, 70 and 140 μM, detected over 400 s. The data were split into two apparent size groups: small (2-5 monomer units) and large (6-150 monomer units) oligomers. For the large oligomers, illustrative fits to Gaussian functions are shown in blue (SI Appendix, eq. S1), and the resulting mean FRET efficiency values, E, were: for 5 μM, 9 h E=0.34, 27 and 51 h global E=0.49; for 10 μM, 9 h E=0.33, 27 and 51 h global E=0.52; for 70 μM, global E(low-FRET)=0.37 and global E(high-FRET)=0.71; for 140 μM, global E(low-FRET)=0.44 and global E(high-FRET)=0.67. Further details of the fitting functions and the resulting average parameters (N=6) are given in SI Appendix, section 1.1.

Fig. 3. Modelling the kinetics of αS aggregation. (A) The model considers coarse-grained conversion reaction between oligomeric populations as a whole, with no size dependence, and allows fibrils to grow once formed. Here, unimolecular conversions with no monomer dependence are assumed between populations (an alternative model with bimolecular conversions is presented in SI Appendix, Figure S12A). (B) The resulting nucleation-conversion-polymerization model is used to describe the observed populations, whereby monomer units form low-FRET efficiency oligomers.
with rate constant $k_n$ and an average reaction order of $n$. These oligomers can then convert to ordered high-FRET efficiency oligomers via a first-order reaction with rate constant $k_f$, with a subsequent final first-order conversion to fibrils with rate constant $k_f'$. Fibrils can then recruit single monomer units to grow in a succession of elongation steps, with a length-independent rate constant $k_e$. At early reaction times, reverse reactions can be neglected, and conversion constants were fixed as equal such that $k_f' = k_f' = k_e$. The resulting simplified model, with four free parameters, was fitted globally to early-time (up to 33 hours) kinetic data showing changes with time in monomeric and oligomeric populations for a range of initial monomer concentrations. The resulting nucleation reaction order was found to be $n = 0.90 \pm 0.1$ with rate constants $k_n = (4.0 \pm 2.0) \times 10^{-4} \mu M^{-1} h^{-1}$, $k_c = (9.5 \pm 5.0) \times 10^{-2} h^{-1}$, and $k_e = (9.0 \pm 7.0) \times 10^{-2} \mu M^{-1} h^{-1}$.

**Fig. 4.** Predictions of $\alpha$S aggregation kinetics at low concentrations by nucleation-conversion-polymerization models. After fitting to the available kinetic data, both unimolecular (Fig. 3) and bimolecular (SI Appendix, Fig. S12) conversion models were used to predict how the concentrations of low-FRET efficiency oligomers and high-FRET efficiency oligomers vary over time at low initial monomer concentrations; the fitting was carried out by considering only the total concentration of oligomers at these low monomer concentrations. As the fitting was carried out over a reaction time of 33 hours and overlaid over 54 hours, a similar time range was used for the predictions. From the third column, it is clear that a unimolecular conversion model alone predicts similar concentrations of both types of oligomers, and furthermore predicts a ratio of concentrations that is very stable with changing initial monomer concentration. These predictions are consistent with the single-peak FRET histograms at low concentrations shown in Figure 2B.

**Fig. 5.** Schematic representation of the processes required for $\alpha$S spreading. (A) Seeding results in the formation of aggregates from monomeric protein, and amplification involves the multiplication of existing aggregates. (B) Seeding process alone would not lead to efficient spreading due to the possibility that the formed aggregates would be diluted out. (C) The combination of seeding and amplification can lead to continuous aggregate spreading.

**Fig. 6.** Schematic outline of two simplified models of $\alpha$S aggregate spreading in cellular environment. (A) Aggregate-driven, or prion-like, propagation of aggregated species. The primary role of the aggregate upon entering a cell is to induce the aggregation of monomeric protein by the mechanism
of templated seeding. (B) Cell-driven model. The initial role of the aggregate is to induce cellular stress, which disrupts the homeostasis and creates conditions where protein aggregation becomes favourable.