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The properties of α -synuclein secondary nuclei are dominated by the solution conditions rather than the seed fibril strain

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Abstract

α -synuclein (α -syn) is a natively unfolded protein predominantly localized in the presynaptic terminals of neurons. It has been shown that α -syn fibrils are the major component of abnormal neuronal aggregates known as Lewy bodies, the characteristic hallmark of Parkinson's disease. Amyloid fibrils arise through primary nucleation from monomers, which in the case of α -syn is accelerated by suitable surfaces with an affinity for the protein, followed by the elongation of the nuclei by monomer addition. Secondary nucleation, on the other hand, corresponds to the formation of new fibrils when it is facilitated by pre-existing fibrils. While it is well-established that the newly added monomer in the process of fibril elongation adopts

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the conformation of the monomers in the seed, often called templating, it is still unclear under which conditions fibrils formed through secondary nucleation of monomers on the surface of fibrils copy the structure of the parent. Here we show by biochemical and microscopical methods that the secondary nucleation of α -syn, enabled at mildly acidic pH, leads to fibrils that structurally resemble more closely those formed *de novo* under the same conditions, rather than the seeds if these are formed under different solution condition. This result has important implications for the mechanistic understanding of the secondary nucleation of amyloid fibrils and its role in the propagation of aggregate pathology in protein misfolding diseases.

1 Introduction

The formation of ordered fibrillar aggregates of proteins, amyloid fibrils, is the hallmark of a range of human disorders, many of which are neurodegenerative in nature, such as the prion diseases,¹ Alzheimer's (AD) and Parkinson's disease (PD).² One of the defining features of neurodegenerative disorders is their progressive nature, which manifests itself through the spreading of the disease pathology through the central nervous system (CNS) of the affected individual.³

Amyloid fibrils grow through the addition of protein monomers to the fibril ends, a process which keeps the number of fibrils constant but increases their total mass. However, a key question in the context of the spreading of disease pathology is by which molecular mechanism amyloid fibrils proliferate, i.e. increase in number. An increase in the number of fibrils can occur through *de novo* (primary) nucleation, a process which is, by definition, independent of the quantity of already existing aggregates. However, the presence of fibrils enables additional pathways for the formation of more fibrils in what is known as secondary pathways, either through fragmentation^{4,5} or through surface-catalysed secondary nucleation.⁶ In particular the latter process has received increased attention in recent years, because its rate can be significant even in the absence of mechanical shear forces, which strongly favour fragmentation.⁷ Shear forces are likely to be very weak in a physiological context, but some molecular chaperones have been proposed to be able to fragment amyloid fibrils.⁸

Secondary nucleation, the nucleation of new assemblies facilitated by the presence of assemblies of the same kind of monomers, has long been known to play an important role in industrial crystallisation processes.^{9,10} It was also identified to play a key role in the highly cooperative polymerisation dynamics of sickle haemoglobin¹¹ and it was subsequently proposed that secondary nucleation could be responsible for the proliferation of protein aggregates in prion and other neurodegenerative diseases.¹² Indeed, in the last decade, increasing evidence has emerged that secondary nucleation plays an important role in the aggregation kinetics of a range of amyloid proteins, such as IAPP,^{13,14} insulin,¹⁵ the amyloid β peptide⁷ and α -synuclein.^{16,17} Despite its potentially crucial role in disease progression, much remains to be elucidated about the secondary nucleation of amyloid fibrils.⁶ Recent results show that, in the case of the amyloid β peptide, primary and secondary nucleation have distinct thermodynamic profiles,¹⁸ due to the fact that secondary nucleation involves the binding of protein molecules onto the surface of fibrils.¹⁹ It is not known, however, to what extent the actual nucleation reaction on the fibril surface is distinct from the equivalent process in solution or on other types of surfaces. In particular, it is also not known whether structural features of the parent fibril are transmitted onto the fibril generated through secondary nucleation. In the case of secondary nucleation in crystals, addressing a related question has allowed substantial progress in the mechanistic understanding.^{20,21} In particular, it was found that different types of secondary nucleation processes exist for crystals, some of which propagate structural information, in particular chirality, of the parent crystal.^{22,23} Secondary nucleation processes in crystals can be divided into those whereby small pieces of the parent crystal break off and those whereby the secondary nuclei form independently in proximity or in contact with the parent crystal.²⁴ Such a detailed mechanistic understanding has not yet been achieved for the secondary nucleation of amyloid fibrils. In this study we address this question for the specific case of amyloid fibrils formed by the protein α -synuclein (α -syn), which are a major components of Lewy bodies and Lewy neurites, the pathological intracellular deposits found in the CNS of PD patients.²⁵ It has been shown that α -syn can form different amyloid fibril structures, depending on the solution conditions under which the protein is allowed to assemble.^{26,27} Differently assembled structures

adopted by the same protein with the same sequence are known as fibril strains. This phenomenon has first been discovered for the mammalian prion protein, where the difficulty of creating infectious prions *de novo* from recombinant protein has long been known.²⁸ On the other hand, if recombinant prion protein is seeded with *ex vivo* infectious material, infectivity can be maintained over many generations of repeated dilution and seeding.²⁹ The propagation of information is usually explained in terms of amyloid fibril elongation, the addition of monomeric protein molecules onto fibril ends, whereby the fibril end acts as a template and imposes its own structure onto the added molecule. This was found to hold even under conditions where the protein forms a different fibril structure *de novo*.³⁰ The existence of amyloid fibril strains has importance beyond the case of mammalian prions. It has been shown that the structures of fibrils extracted from patients with different AD phenotypes differ.^{31,32} There is evidence that a similar polymorphism exists also in the case of different synucleinopathies,³³ i.e. neurodegenerative diseases characterised by the common feature of formation of deposits of α -synuclein amyloid fibrils. Furthermore, it has been shown that the inoculation of mice with different α -synuclein fibril strains generated *in vitro* led to different disease phenotype and propagation pathways.³⁴ One interpretation of these biological findings is that whichever mechanism is at the origin of fibril amplification and propagation *in vivo* is able to maintain structural information of the fibrils.

Here we show that under conditions where secondary nucleation of α -syn is observed, the structural information of seed fibrils formed under different conditions is only preserved at high seed concentration, i.e. where elongation of the initially added seeds dominates the generation of fibril mass. At low seed concentrations, where most of the fibrils form through secondary nucleation, the end product of the reaction closely resembles the fibrils that form *de novo* under these solution conditions. This result establishes that secondary nucleation is clearly distinct from classical seeding and has implications for the interpretation of biological and clinical data on the propagation of fibril strains.

2 Results

2.1 Solution conditions determine the structure and proteolytic susceptibility of α -syn fibrils

We started by generating α -syn amyloid fibrils under three different solution conditions, at neutral pH at low and high ionic strength (I) and mildly acidic pH at low I, according to published protocols.^{17,26,34} These reactions were carried out in polystyrene plates, the surface of which triggers heterogeneous primary nucleation, in order to obtain seed fibrils within a reasonable time frame.¹⁷ It was demonstrated previously that at neutral pH, α -syn can adopt either a twisted fibril structure (twists) at higher I (50 mM Tris-HCl pH 7.5, 150 mM KCl, 0.02% NaN₃) or a ribbon-like fibril structure (ribbons) at very low I and in absence of salt (5 mM Tris-HCl pH 7.5, 0.02% NaN₃). We followed the *de novo* formation of fibrils under these two sets of solution conditions by Thioflavin-T (ThT) fluorescence and found very distinct assembly kinetics and ThT fluorescence intensities. Indeed, the formation of the twists is complete already after approximately 20 h, whereas formation of the ribbons needs approximately 80 h before the plateau is reached (Figure 1 A). This difference in kinetics can be explained through the unfavourable electrostatic interactions between individual α -synuclein molecules, which are negatively charged at neutral pH. A higher I leads to more effective screening of this repulsion¹⁶ and hence to accelerated aggregation. There is also approximately an order of magnitude less ThT fluorescence emitted by the sample with the ribbon fibrils, suggesting a difference in binding affinity, stoichiometry and/or fluorescence quantum yield of the bound ThT between the different types of fibrils made at pH 7.5. We also made fibrils in 10 mM MES buffer at pH 5.5, where α -synuclein is closer to its isoelectric point and where aggregation is therefore very rapid even at low I (Figure 1 A). We have shown previously by applying a variety of experimental and theoretical methods that under these conditions, the mechanism of amyloid fibril formation of α -synuclein is qualitatively different compared to neutral pH, due to the contribution of secondary nucleation at this mildly acidic pH.^{16,17} The role of secondary nucleation is most clearly illustrated by pH-dependent, very weakly seeded experiments (see Supplementary Figure

3) under conditions (low binding plate, quiescent) where no fibril formation is observed without added seeds (Figures 2 A,B and 3 A,B).

Under conditions where *de novo* formation of α -synuclein does occur (high binding plates, shaking), secondary nucleation manifests itself through the very rapid completion (compared to the reactions at higher pH) of the reaction after only approximately 5 hours (Figures 1 A,B). The subsequent decrease in fluorescence intensity in the sample at acidic pH could be caused by the very pronounced higher order assembly of the fibrils at this pH,¹⁶ a process which can be expected to influence the accessibility of the fibril surface for ThT as well as the fluorescence quantum yield of the bound dye.

We imaged the samples after the plateau phase was reached by atomic force microscopy (AFM, Figure 1 C-E) and cryo-electron microscopy (cryo-EM, Figure 1 F-H) and found the previously reported twisted and ribbon-like morphology for the fibrils formed at neutral pH at high and low I, respectively. The twists present a height of circa 8 nm (Figure 1 C) with a repetitive height variation due to the twist of ca. 4 nm and a twist pitch of ca. 200 nm. The ribbons, instead, present a height of ca. 5 ± 2 nm and no twist (Figure 1 D) and the fibrils made at pH 5.5, which we call needles, are twisted, but with a shorter node-to-node distance (ca. 100 nm) than those made at neutral pH. We also performed proteinase K (pK) digestion and found that both fibril types made at neutral pH showed a very similar susceptibility to proteolytic degradation (Figure 1 G and H). In contrast, the fibrils formed at pH 5.5 show a much greater degree of degradation, as after 5 min of incubation with pK, the fibrils are fully digested. In these experiments, we took great care to eliminate any potential direct influence of the difference in formation conditions on the efficiency of the pK enzyme (see Methods section for details), by performing all digestions under the same conditions, regardless of the conditions under which the respective fibrils had been made. We therefore also checked whether the digestion buffer at neutral pH led to a dissolution of the fibrils made at mildly acidic pH. We found that the fibrils remained intact even after incubation for several days in the digestion buffer (see Supplementary Figure 5) and we therefore conclude that the fibrils made at pH 5.5 possess a much higher intrinsic proteolytic susceptibility than those prepared at neutral pH.

Figure 1: Formation and microscopic characterization of three α -syn fibril strains (A) *De novo* aggregation kinetics of 100 μ M α -syn monomer in polystyrene plates at pH 7.5 and high I (blue), pH 7.5 and low I (red) at pH 5.5 and low I (green). (B) The same aggregation kinetics as in (A), but with a zoom into the lower fluorescence values. AFM (C-E) and cryo-EM (F-H) images of the twists, ribbons and low pH fibrils ("needles"), respectively, with AFM height profiles of the different fibril types. (I) SDS-PAGE gel of the proteinase K digestion of the twists (dark cyan), ribbons (red) and low pH fibrils (green). (J) Quantification of the digested bands from the SDS-PAGE gel shown in (I).

2.2 Fibril elongation preserves the structural information of α -syn fibril strains

We performed seeded experiments under conditions where fibril elongation dominates the aggregation process in PEGylated plates to minimize heterogeneous primary nucleation at the plate surface. As mentioned above and demonstrated previously,^{16,17} secondary nucleation of α -synuclein amyloid fibrils is undetectable at neutral pH, and under quiescent conditions fragmentation can also be neglected.¹⁶ Therefore, in seeded experiments at pH values above 6, fibril elongation will be the only process with an appreciable rate. However, even under conditions where secondary nucleation is observed, i.e. pH below 6, the reaction can be strongly biased towards elongation by adding a high proportion of seed fibrils at the beginning of the experiment. Fibril elongation is usually several orders of magnitude faster than any nucleation process under any given set of conditions; if this were not the case, then fibrillar structures of high aspect ratio would not form. Therefore it is always possible to seed an aggregation reaction strongly enough such that fibril elongation is by far the dominant process.

We performed seeded experiments at various concentrations of twist (Figure 2 A) and ribbon seeds (Figure 2 B), at pH values above (6.5) and below (5.5) the threshold for secondary nucleation previously identified for full length α -syn ($\sim$ pH 5.8¹⁶ and see Supplementary Figure 3 A). We find that the two types of fibrils act as efficient seeds under all conditions, and most of the curves show the typical concave shape of a reaction dominated by fibril elongation.¹⁶ Only the reactions seeded with ribbons at pH 5.5 do not display the typical single exponential behavior, however, the absence of a lag time suggests that even these reactions are dominated by elongation. The main result of these experiments is that under both sets of solution conditions, the fibrils at the end of the experiment, when the ThT fluorescence has reached a plateau value, have a very similar susceptibility to pK digestion (Figure 2 E-H) and appearance by AFM imaging (Figure 2 C-D) as the parent fibrils. This is in particular also true under the more acidic pH conditions (pH 5.5), where fibrils formed *de novo* have a much higher susceptibility to pK digestion than the twist and ribbon fibrils (Figure 1 I-J). Therefore, these results show that under conditions where the overall aggregation process is dominated by fibril growth, the structural properties of the seed fibrils are transmitted onto the

newly added monomers. This finding is in agreement with previous reports that show that the so-called templating effect in fibril elongation is able to overcome the structural preferences induced by the solution conditions.³⁰ The reactive flux of monomers is determined by the lowest accessible free energy barrier, corresponding to the addition onto the end of the templating seed. This process can lead to the adoption of a fibril structure which is not necessarily the thermodynamically most stable state under a given set of solution conditions.

2.3 Secondary nuclei are structurally determined by solution conditions rather than the strain of α -syn seed fibrils

Having established that fibril elongation preserves the fibril structure, we next probed whether this was also the case under conditions where most of the fibril mass at the end of the experiment stems from the growth of fibrils that have formed through secondary nucleation, i.e. weakly seeded aggregation experiments at mildly acidic pH (5.5). A simple argument illustrates that under such weakly seeded conditions the growth of the initially added seed fibrils indeed contributes only little to the total generation of fibril mass. If a reaction is seeded with 1% or less (monomer equivalents) of pre-formed seeds, then at the end of the experiment, where a large fraction of the monomer has been converted into fibrils, the seed fibrils should be on average up to two orders of magnitudes longer than at the beginning, if no new fibrils have formed and if the existing fibrils have not amplified through secondary processes. However, a comparison between AFM images of the initial seeds (Supplementary Figure 1) and the fibrils at the end of the experiment (Figure 3 C,D) reveals that the average increase in length of the fibrils is at most one order of magnitude and not two, suggesting that the majority of the fibrils have been formed through secondary nucleation. We find that seeded experiments with both twists (pH 7.5 and high I) and ribbons (pH 7.5 and low I) show clear signatures of a process dominated by secondary nucleation (i.e. sigmoidal traces with sharp transition), at low seed concentrations (Figure 3). The time at which half of the monomers have converted to fibrils, t_{half} , is estimated as ca. 8 h for 50 μ M monomers with 1% seed formed at pH 5.5 low I¹⁷ whereas with the same amount of seeds formed at pH 7.5 high I or pH 7.5 low I

Figure 2: The structural characteristics of fibril elongation Seeded Kinetics of 50 μ M α -syn monomers in 10 mM MES pH 6.5 and 10 mM MES pH 5.5 with 0%, 5% and 10% twists (pH 7.5 high I, **A**) and ribbons (pH 7.5 low I, **B**). AFM images of the aggregated solutions in 10 mM MES pH 6.5 seeded with 8% twists (**C**) or 8% ribbons (**D**). SDS-PAGE gels of the proteinase K digestion at 0 and 5 minutes of the fibrils from experiments at pH 5.5 or 6.5 seeded with 10% twists (**E**) or ribbons (**G**). Quantification of the digested bands for the fibrils formed in the presence of the twists (**F**) and the ribbons (**H**) in comparison with the respective parent fibrils.

we find t-half values of ca. 30 and 60 h, respectively.

Interestingly, the seed concentration at which sigmoidal behaviour is observed (Figure 3 A, B) differs between twists and ribbons. While experiments seeded with twists only show sigmoidal behaviour when the seed concentration is below 0.8% by mass, such behaviour is observed with the ribbons for all employed seed concentrations (up to 4%). This finding can be partly explained by a difference in length distribution of the twists and ribbons, as suggested by cryo-EM (Figure 1 F-H and Supplementary Figure 1) leading to a difference in relative contribution of fibril elongation and secondary nucleation. In addition, it is possible that an intrinsic difference exists in the ratio between the rate constants of fibril growth and surface nucleation.

We then proceeded to investigate the fibrils formed under these weakly seeded conditions, by AFM and cryo-EM imaging as well as by pK digestion. We investigated in particular the end product of the reaction seeded with 0.4% twists (pH 7.5 high I) and with 1.2% ribbons (pH 7.5 low I), both of which showed a clear signature of secondary nucleation. AFM (Figure 3 C-D, left) and cryo-EM (Figure 3 C-D, right) revealed in all cases fibrils more similar to those formed *de novo* at pH 5.5 than any of the parent seeds, in particular also in the reactions seeded with ribbons. Furthermore, we performed pK digestions of these samples (Figure 3 E and F), as well as of samples without the fluorescent dye ThT that had been otherwise prepared identically to the ones which were used to follow the aggregation kinetics (Figure 3 A,B). The rationale behind this control is that ThT, which binds to the surfaces of fibrils, possibly might influence the structural characteristics of the nuclei that form on the surface. Our results show that the presence of ThT does not modify the overall fibril morphology of the end product (as judged by AFM imaging, see supplementary Figure 4) and the resistance to enzymatic digestion of the resulting nucleated fibrils (Figure 3 E-F).

As additional controls, we performed pK digestion on monomer incubated without seeds (and which did not aggregate under these conditions) and on fibrils formed *de novo* at pH 5.5. We find in all cases that the end products of seeded reactions at pH 5.5 low I resemble much more the *de novo* formed fibrils, rather than the parent seeds formed at pH 7.5. In particular, the pK experiments reveal a digestibility of the end products of reaction conditions with seeds formed at

pH 7.5 which is almost as high as that of the *de novo* formed fibrils. The latter, in turn appear as completely digested as the intrinsically disordered monomer (Figure 3 E-F).

While most of the fibrils at the end of the weakly seeded aggregation reaction stem from secondary nucleation, as in the un-seeded reaction at pH 5.5, the growth of the initially added seeds made under other conditions may lead to a potentially non-negligible contribution of the initial seeds to the final mixture of fibrils. We therefore also performed experiments in which fibrils are trapped inside a filter membrane in a multiwell plate,¹⁷ incubated for a certain period of time with a solution of monomer under conditions that are conducive to secondary nucleation, followed by pushing the solution out of the membrane by applying a vacuum. The evolution of fibril mass is then followed over time (Figure 4 A) by ThT fluorescence. We observed a sigmoidal aggregation curve for the filtrate, indicative of a weakly seeded aggregation reaction, suggesting that some of the secondary nuclei/oligomers have detached during the incubation period in the filter and after the mixture has been washed through the filter grow to fibrils that act as seeds. Indeed, in analogy to the secondary nucleation in crystals, the detachment of the product of secondary nucleation and its evolution into an independent aggregate is a defining feature of the overall process of secondary nucleation and underlines the autocatalytic amplification of fibril mass.

We also performed cryo-EM images of the reaction mixture where $\sim 2.8 \mu\text{g}$ fibrils had been trapped in the membrane and incubated with $50 \mu\text{M}$ monomer (Figure 4 B) and we found that the fibrils do not resemble the ribbons which acted as parent seeds, but rather the fibrils formed *de novo* at acidic pH.

3 Discussion

Our results, summarised in Figure 5, establish that a fundamental difference exists between fibril elongation and secondary nucleation. In the former the templating effect of the seed fibril dominates over the solution conditions, this situation is reversed in the latter process. Therefore, first and foremost, our study provides additional strong evidence that the amplification of α -synuclein

Figure 3: The structural characteristics of secondary nucleation. Weakly seeded aggregation kinetics of 50 μ M α -syn monomers at mildly acidic pH with different concentrations of twists (formed at pH 7.5 high I, **A**) and ribbons (formed at pH 7.5 low I, **B**). (**C**, **D**) AFM (left) and cryo-EM (right) images of aggregated solutions at the end of the reaction seeded with 0.4% twists (**C**) and with 1.2% ribbons (**D**). (**E**, **F**) SDS-PAGE gels of the pK digested twist- (**E**) and ribbon-seeded (**F**) reactions, as well as of monomer and fibrils formed *de novo* at pH 5.5 as controls. (**G**, **H**) Quantification of the bands of the gels shown in **E** and **F**.

Figure 4: **Trap and seed experiments.** (A) Secondary nucleation kinetics followed by ThT seeded with 3 μM ribbons and 15 μM (dark blue) or 50 μM (dark cyan) monomer added. (B) Cryo-EM of the fibrils resulting from the weakly (with ribbons) seeded experiment at 50 μM monomer shown in (A).

amyloid fibrils at mildly acidic pH stems from the nucleation of new fibrils, rather than from fragmentation of existing ones.¹⁷

The dominance of the solution conditions over the seed structure in secondary nucleation can be understood as follows. In general, the high efficiency of seeding can be explained by a much lower free energy barrier of fibril growth compared to nucleation, as has for example recently been quantified for the amyloid β -peptide.¹⁸ Therefore, even if the template does not correspond to the most favorable structure under a given set of conditions, the free energy barrier of monomer addition to this template is still likely to be lower than that of the *de novo* nucleation of fibrils. However, the difference in free energy barrier between the nucleation of different fibril strains is likely to be smaller than between any given nucleation process and elongation. Therefore, the solution conditions are able to exert a stronger influence in the case of nucleation, and are apparently even able to overcome any potential templating effect that the underlying seed fibril might exert in the process of secondary nucleation. We can at present not exclude that some nuclei form that are imprinted with the structural information of the parents, but that convert later due to their being unstable under the solution conditions of secondary nucleation. On the other hand, it is also important to emphasize that the formation of very similar types of fibrils under mildly acidic solution condition, both by primary and secondary nucleation, does not necessarily imply that these are the most stable fibrils under these conditions. It merely implies that these fibrils present the lowest free energy

barrier for nucleation.

Figure 5: **Summary of the results of this study** (A) Formation of amyloid fibrils *de novo* under different solution conditions (blue and green boxes) can lead to different fibril structures (strains). (B) Fibril elongation is able to propagate the structural characteristics of the seed, even under conditions where a different type of fibril would form *de novo*. (C) Secondary nucleation is not able to propagate the structural characteristics of the seed under conditions where a different type of fibril forms *de novo*. The fibrils formed through secondary nucleation resemble those formed *de novo*. Therefore secondary nucleation is only autocatalytic for fibrils formed under these conditions.

Similar effects to the ones described in this study have been reported for a fragment of the mammalian prion protein³⁵ and the amyloid β -peptide ($A\beta$).³⁶ However, even though the amyloid fibril formation of the prion fragment appears to feature surface-catalysed secondary nucleation,³⁵ the same has not yet been established for the full length mammalian prion protein. For $A\beta$, it was shown that $A\beta(1-40)$ and $A\beta(1-42)$ interact at the level of primary nucleation, but that fibrils of $A\beta(1-40)$ are very inefficient in nucleating $A\beta(1-42)$ fibrils and *vice versa*.³⁷ Here, the difference in fibril structure was found not to propagate through surface nucleation in cross-seeding experiments.³⁶ However, in this case two different sequences are considered and the lack of structural propagation is probably due to the inability of the $A\beta(1-42)$ to adopt the fibril fold of $A\beta(1-40)$.

Indeed, it has been shown that these two sequences adopt quite distinct fibril folds.^{38,39}

The current work is to our knowledge the first study addressing the ability of surface-catalysed secondary nucleation to propagate the structural information of α -syn parent seeds when these do not represent the most favourable (either kinetically or thermodynamically) form under the solution conditions of the seeding experiment.

For α -syn, our findings are interesting in the light of previous studies that showed that α -syn is highly metastable in its disordered monomeric form, even inside live cells.^{40,41} Suitable interfaces, such as polymer-water,⁴² air-water⁴³ or lipid-water,⁴⁴ are required to enable the heterogeneous primary nucleation of α -syn amyloid fibrils. The strong pH-dependence of its secondary nucleation could solely stem from a change in the charge of the fibril surface and monomers, induced by lowering the pH, which renders the fibril surface more conducive for catalysing the nucleation reaction. This hypothesis is supported by recent results that show that truncation of the acidic C-terminus of α -syn leads to a strong up-shift of the pH range within which secondary nucleation is observed.⁴⁵

Secondary nucleation has received much attention in recent years due to its suspected role in the spreading of amyloid pathology^{6,12} as well as in the generation of toxic oligomers.⁷ Indeed, Parkinson's disease is a particularly striking example of the importance of aggregate spreading, as it has been hypothesized that α -syn aggregate pathology might originate from the digestive tract and then spread towards the central nervous system.^{46,47} Moreover, this hypothesis has been then supported by experiments, whereby model animals were injected into the gut with either PD brain lysate or various pure monomeric and aggregated forms of α -syn, and where subsequently α -syn reached the brain, supposedly via the vagal nerve.⁴⁸

Our results indicate that secondary nucleation on fibril surfaces is not able to faithfully propagate structural (strain) information of seeds, if the structure of the seeds represents one of the less favourable polymorphs under the solution conditions of the seeding reaction. Given the clinical and biological observations on differences in fibril strain compositions between patients,^{31,32} as well as the differences in propagation and disease phenotype when animal models are inoculated

with different types of aggregates,³⁴ it is therefore possible that fibril elongation and fragmentation, processes that are able to preserve the fibril strain²⁹ (Figure 2), play important roles in aggregate proliferation *in vivo* even if the seeds move to a compartment in which their strain is not the most stable one. The current experiments can not yet answer whether strain information is propagated by secondary nucleation on seeds having a more stable structure under the solution condition of the reaction. If the results of the present study are to be interpreted in the context of the spreading of aggregate pathology in animal models after inoculation with aggregates produced from recombinant protein³⁴ an additional aspect needs to be considered. We show here that a given concentration of seed fibrils (in monomer equivalents) of twist and ribbon fibrils can have very different seeding efficiencies (e.g. Figure 3 A,B). While this difference may be caused partly by intrinsic differences in the properties of the fibril strains, it will also contain contributions from the difference in seed particle concentration caused by the different length distribution of the twists and ribbons. Therefore, some part of the differential spreading and toxicity behaviour of these types of fibrils in mouse models³⁴ could be caused simply by the fact that different numbers of seed fibrils are injected. Therefore, a role of secondary nucleation in the spreading of pathology cannot be ruled out based on our current findings. In addition, it is increasingly recognised that secondary nucleation plays an important role in the generation of oligomeric species of amyloid proteins⁷ and that in general such oligomers are more cytotoxic than the final fibrillar states.⁴⁹ It is conceivable that the phenomena of aggregate spreading and generation of toxicity are decoupled, in the sense that only a small fraction of the cytotoxic oligomers generated through secondary nucleation is able to evolve into fully formed fibrils and hence propagate the pathology.

In conclusion, our study establishes that the surface-catalysed secondary nucleation of α -syn does not propagate the structural characteristics of the seed fibrils when these are moved to solution conditions that (kinetically or thermodynamically) favour the nucleation of a different fibril strain. Under such conditions, nucleation of α -syn monomer on the fibril surface is more reminiscent of heterogeneous primary nucleation on foreign surfaces.

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Supporting Information Available

Details of experimental procedures can be found in the Supplementary Materials. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Supplementary materials: The structure of α -synuclein secondary nuclei is dominated by the solution conditions rather than the seed fibril strain

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1 Experimental methods

1.1 Expression and purification of α -syn

The human codon-optimized wild type α -syn was expressed in *Escherichia coli* BL21 (DE3) using a synthetic gene with *E. coli* optimized codons cloned into the pT7-7 vector. α -syn was expressed for 24 h at 37°C in auto-induction 2YT-medium (16 g/L Trypton, 10 g/L Yeast extract and 5 g/L NaCl) with 50 mM sodium phosphate pH 7.2 (PB), 2 mM MgCl₂, 1.8% glycerol (v/v), 0.05% glucose (w/v), 0.2% lactose (w/v). The cell pellet was washed in a saline solution (0.8 % NaCl) after being centrifuged at 5000 g for 15 minutes at 4°C. Cell lysis was carried out by heat treatment (at

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95°C for 15 minutes) with 25 mM Tris-HCl pH 8, 1 mM EDTA and by sonication using a VS70/T tip with a Bandelin Sonopuls sonicator (BANDELIN electronic) at 60% power (15 minutes, 15 seconds pulses spaced out by 1 minute) on ice. Streptomycin sulphate was added to a final concentration of 10 mg/ml per litre of initial culture and the mixture was stirred at 4°C for 15 minutes before centrifuging the lysed cells and the DNA at 20000 g for 30 minutes at 4°C. The supernatant was retained and ammonium sulphate was added to a final concentration of 1.75 M and stirred at 4°C for 15 minutes. The precipitated α -syn was collected by centrifuging at 20000 g for 30 minutes. The protein pellet was re-suspended in 25 mM Tris-HCl pH 8, 1 mM EDTA and dialysed at room temperature (at circa 25°C) for 2 hours and then the protein was filtered (0.42 μ m) before loading onto a HiTrap Q HP anion exchange (AEC) column (GE Healthcare). Before applying the salt concentration gradient in order to elute α -syn, the column was washed for 10 minutes in 25 mM Tris-HCl pH 8, 8 M urea. The gradient was run in two steps, 0-35 % (in 100 ml), 35-45 % (in 50 ml) and 45-90 % (in 50 ml) of 25 mM Tris-HCl pH 8, 800 mM NaCl. The protein concentration was calculated by absorbance measurement at 276 nm using a Jasco V-650 UV-VIS Spectrophotometer and a molar extinction coefficient of 5600 M⁻¹ cm⁻¹. The protein-containing fractions were combined and the protein precipitated again with ammonium sulphate as described above and the pellet stored at -20°C.

1.2 Preparation of α -syn polymorphic fibrils

The α -syn protein pellet from the AEC chromatography run was resuspended in circa 1 ml of experimental buffer (5mM Tris-HCl pH 7.5, 0.02 % NaN₃; 50 mM Tris-HCl pH 7.5, 150 mM KCl, 0.02 % NaN₃ or 10 mM MES pH 5.5) and loaded onto a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated with the respective buffer. The monomeric fractions were collected in low-bind Eppendorf tubes (Eppendorf, Germany) and the first generation of the fibrils was prepared in 96-well high binding plates (Corning 3601) in a Fluorostar Omega (BMG Labtech, Offenburg, Germany) plate-reader following the ThT fluorescence (excitation with a 448-10 nm filter, emission with a 482-10 nm filter, bottom optics) at 37°C under constant shaking (700 rpm)

and in the presence of a glass bead (2.85-3.45 mm). The formed fibrils were then used to seed (using 2 % seed by mass) fresh monomer solutions in the corresponding buffers in the absence of ThT under quiescent conditions at 37°C in a Thermomixer (Eppendorf, Germany).

1.3 Determination of the concentration of α -syn fibrils

In order to quantitatively compare the different α -syn fibril morphologies and their seeding efficiencies, it was necessary to determine the fibril mass concentration at the plateau of the reaction. The fibrils were centrifuged in a bench top centrifuge 5415R (Eppendorf, Germany) at 16000 g and 25°C for 30 minutes. The pelleted fibrils were resuspended in 8 M Urea and were then mixed 4:1 with sample buffer (5X) and analyzed by electrophoresis using a 15% SDS-PAGE gel, where the supernatant of the pelleted fibrils as well as α -syn monomers were included as quantification standard. Furthermore, the protein concentration in the supernatant after the centrifugation of the fibrils was measured by UV absorbance in order to quantify the monomers in equilibrium with the fibrils.

1.4 ThT kinetic assay

For the kinetic experiments probing secondary nucleation, α -syn monomers were freshly purified by SEC in the appropriate buffer (10 mM MES buffer pH 5.5, 0.02 % NaN₃) just prior its use for the kinetic measurements. This measure avoids the presence of pre-formed aggregates that could alter the results. The protein aliquots were kept on ice for up to several hours, until use. The seed fibrils were sonicated for 5 min in an ultrasonic bath before use, in order to homogenize the seed preparation (Supplementary Figure X). The aggregation kinetics were followed by ThT fluorescence, with filter and optics settings as described above, but in low binding and clear-bottom half-area 96 well plates (Corning n° 3881). The experiments were performed at 37°C under quiescent conditions.

Figure 1: **AFM images of the twists and ribbons after sonication** AFM images of the twist (A) and ribbon (B) fibrils after 5 minutes of treatment in an ultrasonic bath. The fibrils were always treated in this way before use as seeds.

1.5 Trap and seed experiments

In order to remove the contribution of the seed fibrils to the final population of fibrils stemming from secondary nucleation, we also performed trap and seed experiments. After saturating the membrane of the AcroPrep 96 well filter plate (Pall Life Sciences, Ann Arbor, MI) with ca. 20 μM α -syn monomer solution, $\sim 2.8 \mu\text{g}$ fibrils were trapped in the membrane by vacuum filtration of a fibril suspension using a MultiScreenHTS (Millipore) manifold for 2 s/pulse until the liquid had been drawn off. The trapped fibrils were washed with the buffer used for secondary nucleation experiments (10 mM MES pH 5.5, 0.02 % NaN_3) before incubation with 50 μM α -syn monomers under the same buffer conditions. The plate was incubated at 37°C in the plate reader for 2 h. After the incubation was finished, the flow through was collected into a standard 96-well non-binding plate (Corning 3881) and 20 μM ThT was added before sealing the plate and recording the time course of the fluorescence intensity in a Fluorostar Omega (BMG Labtech, Offenburg, Germany) plate reader at 37°C under quiescent condition, with filter and optics settings as stated above.

1.6 Proteolytic digestion

In order to be able to directly compare the sensitivity of the α -syn fibrils made under different conditions towards proteolytic digestion with proteinase K (pK), all fibrils were digested under the same buffer conditions. The activity of pK strongly depends on the solution pH,¹ and probably also to some extent on the ionic strength. The latter effect could be direct, through a modification of intrinsic enzyme activity, or indirect, through a change in accessibility of the cleavage sites due to higher order association of the fibrils, a process that is itself strongly influenced by the solution ionic strength.² Therefore, the fibrils were pelleted, the supernatant removed and the fibril pellet was resuspended in the digestion buffer (50 mM Tris-HCl pH 7.5, 0.02 % NaN₃) and sonicated in a bath for 5 min (Bandelin Sonorex, RK100H). The mix was prepared with a final fibril concentration of 0.34 mg/ml and 0.020 mg/ml of pK with a molar ratio enzyme: protein of 1:17 (w/w). The samples were incubated at 37°C in a Thermomixer (Eppendorf, Germany) and then mixed 4:1 with the sample buffer (5X) for SDS-PAGE and heated for 5 minutes to 95°C in a Thermo-block (Techne Ori-Block) in order to dissolve the digested fibrils. The samples were loaded onto a 15% SDS-PAGE gel (Bio-rad, USA) and separated by electrophoresis using 1x Tris Glycine SDS (TGS) as running buffer (Bio-rad, USA). The bands of the gel were quantified with the Fiji software package (ImageJ, USA). The integrated intensities of each band were expressed relative to the monomeric band for time zero, i.e. the dissolved, undigested fibrils.

1.7 Atomic Force Microscopy

α -syn fibrils were diluted in their corresponding buffer or in PBS (10 mM Phosphate Buffer pH 7.4, 137 mM NaCl, 3 mM KCl), to improve the fibril attachment on the mica, to a final concentration of 10 μ M. Five μ l were added onto freshly cleaved mica for 5 minutes before rinsing gently with distilled water to remove the excess of salt and they were dried under a gentle N₂ flow. AFM images were taken in air using a Nanowizard III atomic force microscope (JPK, Germany) in tapping mode in air with a silicon cantilever with silicon tip (OMCL-AC160TS, Olympus) with a radius of 7 ± 2 nm and a typical force constant of 26 N/m or with a super sharp silicon tip (SSS-

NCH-10, Nanosensors) with a radius of 2 nm and a typical force constant of 42 N/m.

1.8 Cryo-electron Microscopy

α -syn fibrils formed *de novo* or in the light seeding experiments were diluted in their corresponding buffer to a final concentration of 20 μ M and sonicated in a ultrasonic bath for 1 minute just before blotting of the carbon grids. The sonication homogenizes the suspension and disperses the bigger fibril assemblies that form especially at mildly acidic pH conditions.² The samples were prepared as thin liquid films, < 300 nm in thickness, on lacey carbon filmed copper grids and plunged into liquid ethane at -184°C using a Leica EM GP (Leica, Germany). The images were taken with a JEM 2200FS (Jeol, Japan) microscope equipped with an in-column energy filter (Omega filter), which allows zero loss imaging. The acceleration voltage was 200kV and zero-loss images were recorded with a digital TVIPS F416 camera using SerialEM under low dose conditions with a 30 eV energy selecting slit in place. The images were acquired using different magnification objectives from 12k to 80k. The images were scaled using the Fiji software package.

2 Supplementary data

2.1 Quantification of the degree of conversion of α -syn into fibrils

After preparation and centrifugation of the α -syn fibrils (see description above), the supernatant was collected in order to determine the concentration of free monomer. The concentration of α -syn monomers was measured by applying the supernatant of fibrils formed in the presence and in the absence of ThT (See Fig. 1A, B of the main paper) into a 15% SDS-PAGE gel (Figure 2 A), in parallel with a known concentration of α -syn monomers for subsequent semi-quantitative analysis. The band at 15 kDa of the samples was analysed, and its intensity was integrated with the help of the Fiji software (ImageJ, USA) and then the integrated intensities were normalised with respect

to those of the monomer standards (Figure 2 B). The concentration of the monomers left in the supernatant of the fibrils prepared in the presence or absence of ThT was found to be comparable, suggesting that the dye does not significantly influence the equilibrium of *de novo* fibril formation.

Figure 2: Quantification of the free monomer concentration after the *de novo* formation of different α -syn fibrils (A) 15% SDS-PAGE gel of the supernatant at the end of the *de novo* formation of α -syn fibrils in 10 mM MES pH 5.5, and of the twists and the ribbons (See Figure 1 A and B of the main paper). (B) Quantification of the free monomer concentration from SDS-PAGE gels of the fibrils formed in MES pH 5.5, and of twists and ribbons made in the presence or in the absence of the fluorescent reporter dye ThT (see Figure 1 A and B of the main paper, total concentration of α -syn in the samples was 100 μ M).

The free monomer concentration was also quantified at the end of the secondary nucleation reactions (Figure 3). This quantification enabled us to establish a more reliable ratio α -syn : pK in the subsequent digestion experiments. Moreover, it confirmed that most of the monomers were used to form new fibrils despite the fact that only a low percentage of seed fibrils was used in these experiments, suggesting that the large majority of the newly formed fibril mass was the result of secondary nucleation, rather than mere elongation of the seeds.

In addition to SDS-PAGE gel-based analysis, we also used UV-Vis absorption spectroscopy to quantify the monomer concentration. For the monomer quantification by UV absorption, it is necessary to determine the contribution of the ThT to the absorbance at 275 nm. For this purpose, a calibration curve of the ThT in 10 mM MES pH 5.5 was prepared (Figure 3), whereby the absorption at 275 nm is plotted against the absorption at 412 nm, the absorption maximum of ThT, and the slope was found to be 0.161. This curve allows to determine the contribution of the ThT at 275 nm based on its absorbance at 412 nm and hence to isolate the contribution to the absorbance

Figure 3: **Quantification of the concentration of free monomer after secondary nucleation kinetics** (A) SDS-PAGE gel of the supernatant of the end products of the aggregation assays seeded with 0.4 % and 0.8 % twists and 1.2 % and 2% ribbons (see Figure 3 A in the main paper). (B) Quantification of the free monomer concentration in the supernatant of the SDS-PAGE gel shown in A. (C) Calibration curve used to calculate the absorbance contribution of the ThT at 275 nm. (D) Quantification by UV-Vis spectroscopy of the free monomers (blue) and free ThT molecules (orange) in the supernatant at the end of aggregation reactions seeded with twists and ribbons which were formed in the presence of ThT.

stemming from the protein.³

2.2 Aggregation kinetics of α -syn at different pH values

α -syn aggregation is strongly dependent on the pH value of the solution.² While for pH values above approximately pH 6, no evidence for secondary nucleation has been found, the latter process does contribute to the aggregation at slightly more acidic pH values. This qualitative change in behaviour can be clearly seen in Figure 4 A, where a decrease in pH in increments of 0.2 pH units, starting from pH 6, leads to an ever shorter lag time. At pH 6, no convex behaviour is observed, suggesting the complete absence of any process that increases the number of growing fibrils.² When monomeric α -syn is seeded with either twists or ribbons in 10 mM MES pH 6.5, above the threshold pH value below which secondary nucleation is detectable, fibril elongation is the only molecular process that contributes to the aggregation process (Figure 4 B). Seeding under these conditions leads to the formation of mature fibrils with the same characteristics as the parent seeds (Figure 2 in the main manuscript).

Figure 4: **pH dependence of seeded α -syn aggregation** (A) Weakly seeded α -syn aggregation experiments display a strong pH dependence, with only reactions at pH values below pH 6 displaying the typical convex shape indicative of the presence of secondary pathways. (B) Seeded experiments at pH 6.5 display the typical concave shape indicative of a reaction strongly dominated by fibril elongation.

2.3 Thioflavin-T does not influence the morphology of the secondary nucleated fibrils

We also investigated whether the presence of ThT during the fibril formation had any visible effect on the morphology of the fibrils formed through secondary nucleation. It has been reported that very high concentrations of ThT can alter the polymorphism of α -synuclein fibrils.⁴ The ThT concentrations used in this previous study are more than one order of magnitude higher than those usually used for amyloid detection. However, as both ThT binding and secondary nucleation occur at the surface of the fibril and it has not yet been established whether or not the ThT and the monomeric protein occupy different binding sites, we imaged the end products of low seed reaction in the presence and absence of ThT. We found that, as judged by AFM imaging, the morphology of the secondary nucleated fibrils formed in the presence of 20 μ M ThT did not appreciably differ from those formed in the absence of ThT, independently of whether they were seeded with twists or ribbons (Figure 5).

Figure 5: AFM images and height profiles of the fibrils formed through secondary nucleation AFM images of the samples seeded with twists in the presence of ThT (A) and in its absence (C) with height profiles along the fibril axes (B and D, respectively). AFM images of the samples seeded with ribbons in the presence of ThT (E) and in its absence (G) with height profiles along the fibril axes (F and H, respectively).

Figure 6: **Stability of the MES fibrils in the digestion buffer** AFM images of MES pH 5.5 fibrils, after 5 min of ultrasonic bath, in MES pH 5.5 buffer (A) and in the digestion buffer (DB) after 5 min (B). (C) ThT fluorescence of the MES fibrils and monomers in their buffer (blue) and in the DB after 5 minutes (orange) and 6 days (green).

2.4 Proteolytic digestion of α -syn fibrils

As discussed in the main manuscript, we found that the fibrils formed *de novo*, under pH conditions where secondary nucleation contributes significantly to the aggregation process displayed a markedly greater susceptibility to digestion by pK compared to the twists and ribbons formed at neutral pH. In order to ensure that this difference in behaviour is due to intrinsic differences in fibril structure, rather than a dissolution of the low pH fibrils in the proteolysis buffer, we tested the stability of these fibrils in proteolysis buffer. We measured the ThT fluorescence intensity of the MES pH 5.5 fibrils and monomers in this buffer, before performing a pH jump to pH 7.4, as in the digestion buffer. We found that the fluorescence intensity of the fibrils after 5 minutes of incubation at pH 7.4 (corresponding to the time scale involved in the pK digestion experiments) was slightly decreased, but nevertheless still 1-2 orders of magnitude more intense than for pure monomer (Figure 6 C). The same holds even after incubation for 6 days. Moreover, AFM images taken after 5 min of incubation in the proteolysis buffer still was found to contain fibrils (Figure 6 B), which show no significant difference to those directly imaged from the buffer in which they were formed (Figure 6 A). Taken together, these findings rule out the possibility that the low resistance of the MES pH 5.5 fibrils towards digestion by pK is due to a dissociation of the fibrils caused by the change in pH value from 5.5 to 7.4.

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