



Off-pathway oligomers of α -synuclein and $A\beta$ inhibit secondary nucleation of α -synuclein amyloid fibrils

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Abstract

α -Synuclein (α syn) is a key culprit in the pathogenesis of synucleinopathies such as Parkinson's Disease (PD), in which it forms not only insoluble aggregates called amyloid fibrils but also smaller, likely more detrimental species termed oligomers. This property is shared with other amyloidogenic proteins such as the Alzheimer's Disease-associated amyloid- β ($A\beta$). We previously found an intriguing interplay between off-pathway $A\beta$ oligomers and $A\beta$ fibrils, in which the oligomers interfere with fibril formation via inhibition of secondary nucleation by blocking secondary nucleation sites on the fibril surface. Here, using ThT aggregation kinetics and atomic force microscopy (AFM), we tested if the same interplay applies to α syn fibrils. Both homotypic (i.e. α syn) and heterotypic (i.e. $A\beta$) off-pathway oligomers inhibited α syn aggregation in kinetic assays of secondary nucleation. Initially soluble, kinetically trapped $A\beta$ oligomers co-precipitated with α syn(1–108) fibrils. The resulting co-assemblies were imaged as clusters of curvilinear oligomers by AFM. The results indicate that off-pathway oligomers have a general tendency to bind amyloid fibril surfaces, also in the absence of sequence homology between fibril and oligomer. The interplay between off-pathway oligomers and amyloid fibrils adds another level of complexity to the homo- and hetero-assembly processes of amyloidogenic proteins.

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Introduction

Alpha-synuclein (α syn), a 140 amino acid long protein¹ that makes up 0.5–1% of the total cytosolic protein in the brain,² represents a promising focal point for investigating Parkinson's Disease (PD) and other neurodegenerative diseases due to its involvement in both sporadic and familial forms.^{3,4} It is implicated in multiple detrimental processes to afflicted cells, from oxidative stress stemming from mitochondrial dysfunction to membrane disruption.^{5–7} *In vitro*, α syn presents as an intrinsically disordered protein, but it can assume highly-ordered

β -sheet arrangements upon assembly into amyloid fibrils.^{8–10}

Whereas monomeric α syn is hardly implicated in toxicity, the focus has shifted from amyloid fibrils to smaller species that are now more often implied to have adverse effects.^{11,12} This shift is also true for proteins featured in other amyloidoses, such as amyloid- β ($A\beta$), which is normally associated with Alzheimer's disease (AD).¹³ Distinct amyloidogenic proteins are regarded as a significant factor in the pathogenesis of distinct neurodegenerative diseases, such as α syn in the range of synucleinopathies. However, they often show clinical and

pathological overlap with other amyloidogenic proteins or other neurodegenerative diseases, respectively.^{14,15} For example, >50% of AD patients exhibit not only A β plaques and Tau tangles but also α Syn pathology, and A β plaques are frequently encountered in synucleinopathy cases.^{16,17} The pathological overlap might be a result of a direct interaction between α Syn and A β , which may result in cross-seeding of fibril formation.^{18–23}

Analysis of cross-interactions of different amyloidogenic proteins is hampered by the diversity of aggregate species.^{24,25} Oligomeric species can either appear as on-pathway intermediates of amyloid fibril formation or as metastable off-pathway structures that have to disassemble into monomers before continuing into fibrils (see Fig. 1A).^{26–28} Especially off-pathway oligomers exhibit detrimental activities in line with early pathogenic events in neurodegeneration. For example, α Syn oligomers (α SynOs) impair synaptic function, lead to increased intracellular Ca $^{2+}$ levels, and disturb mitochondria as well as cellular proteostasis.²⁹ Similarly, A β oligomers (A β Os) are synaptotoxic and lead to cognitive decline in mouse and non-human primate models.^{13,30–33} Consequently, they present an interesting target for therapeutic approaches, as in the case of the monoclonal anti-A β antibody lecanemab, which was developed to exhibit particularly high affinity for protofibrillar off-pathway oligomers.³⁴

We have recently characterized a model for protofibrillar A β off-pathway oligomers formed from the dimeric A β variant dimA β .^{27,35} In dimA β ,

two A β 40 units are linked in one polypeptide chain through a flexible glycine–serine-rich linker. DimA β preferentially forms A β O (termed here dimA β O) due to the increased local A β concentration, allowing to study effects of A β O with minimized disturbance by A β monomers or fibrils. Importantly, dimA β O recapitulate the biophysical and functional properties of native A β O.³⁵ Application of dimA β O revealed an intriguing interplay between A β off-pathway oligomers and A β fibrils, beyond the mere competition for A β monomers: Off-pathway oligomers bind to the fibril surfaces, which impedes secondary nucleation, i.e. the auto-catalytic formation of new fibril nuclei on the surface of existing fibrils (Fig. 1).³⁶

Secondary nucleation has been identified as a critical step in amyloid formation of A β , α Syn and many other amyloidogenic proteins, and is considered to contribute to the spreading of amyloid pathology.^{37,38} The identified interplay of off-pathway oligomers and amyloid fibrils entails that the two species mutually influence their time evolution, which affects the extent of their toxic activities.³⁶ For example, amyloid fibril plaques may serve as a reservoir for neurotoxic A β oligomers.³⁹

Here, we investigated if the oligomer-fibril interplay previously observed for A β and lysozyme also applies to α Syn. In particular, we wanted to test if sequence identity of the proteins that constitute the off-pathway oligomers or amyloid fibrils, respectively, is required for this interplay. Therefore, we tested the effects of both

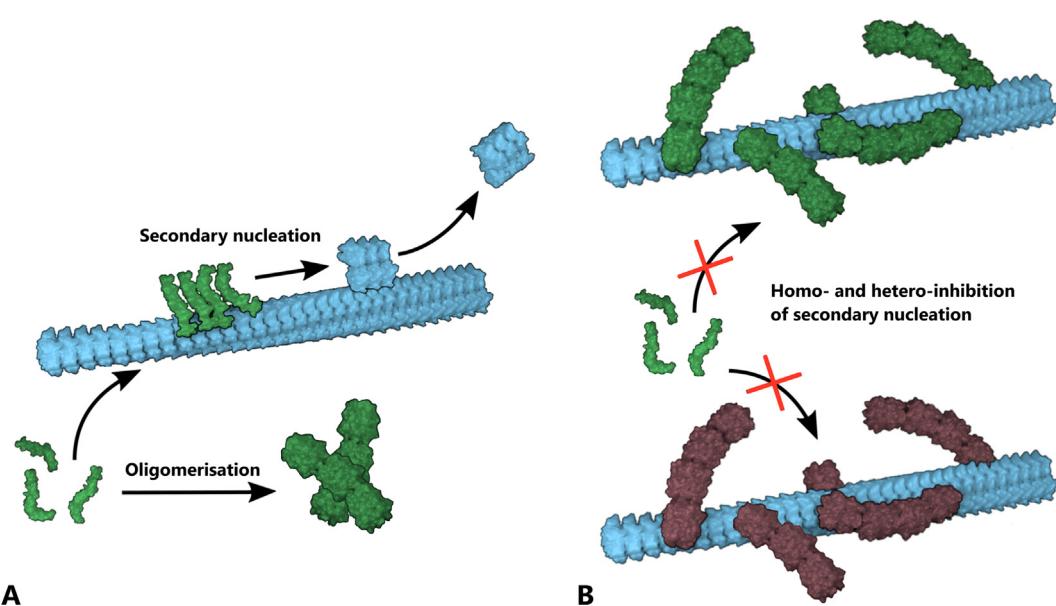


Fig. 1. Suggested mechanism of the inhibition of secondary nucleation by off-pathway oligomers. (A) Scheme of fibril formation by secondary nucleation versus off-pathway oligomer formation. (B) Off-pathway oligomers impede secondary nucleation by binding to fibril surfaces and masking secondary nucleation sites.

homo-oligomers (i.e. α SynO) and hetero-oligomers (dimA β O) on α Syn fibril formation.

Results

Both homo- and hetero-amyloid oligomers inhibit α Syn secondary nucleation

Secondary nucleation drives aggregation of full-length α Syn in particular under slightly acidic conditions, with C-terminal truncation shifting the pH window of dominant secondary nucleation into the neutral pH range.^{40,41} To investigate the effect of homo- and hetero-oligomers on secondary nucleation of full-length α Syn we therefore performed aggregation assays at a pH of 5.0. We have previously shown that at this pH, in the presence of low concentrations of pre-formed fibril seeds, the monomer concentration dependence of the aggregation kinetics is in agreement with a driving role of secondary nucleation.⁴² In the aggregation assays an α Syn monomer concentration of 70 μ M and quiescent conditions were chosen in order to ensure the critical role of secondary nucleation. Test reactions in the absence of pre-formed fibril seeds or in the presence of the nucleation-specific inhibitor AS69fus⁴² confirmed the critical role of secondary nucleation under the chosen experimental conditions. (Fig. 2).

α SynO (homo-oligomers) were prepared according to the protocol for off-pathway α Syn oligomer preparation by dialysis against water, lyophilisation, subsequent resuspension and separation by SEC.⁴³⁻⁴⁵ In atomic force microscopy (AFM) α SynO are imaged as spherical structures

that persist under the experimental conditions of the secondary nucleation assay (Fig. 3, top row).

As hetero-oligomers, A β oligomers were chosen due to the possibly disease-relevant interaction of A β and α Syn.¹⁴⁻²³ Oligomers formed by the synthetic A β dimer dimA β , which consists of two A β 40 units linked in one polypeptide chain, were chosen as oligomer model. DimA β forms kinetically stable oligomers that reliably mimick off-pathway A β oligomers.^{27,35} Application of dimA β enables facilitated control over the multimerisation state, as it assembles into oligomers at low micromolar concentrations and does not convert into fibrils in the absence of agitation.^{27,35} At neutral pH, dimA β O are spherical and curvilinear species^{27,35} (Fig. 3, bottom row). When the pH is decreased to the pH of the secondary nucleation assay of full-length α Syn, these species cluster into large aggregates (Fig. 3, middle row). We have previously shown that these dense aggregates are indeed assemblies of small A β O.³⁵

We first tested if α SynO inhibit the proliferation of α Syn fibrils. 70 μ M α Syn monomer was incubated in the presence of 100 nM preformed α Syn fibril seeds and increasing concentrations of α SynOs. The aggregation time courses showed a prolongation of the lag-time in dependence of the α SynO concentration (Fig. 4A), indicating that α SynOs inhibit secondary nucleation of α SynO amyloid fibrils. This is in agreement with Yang et al., who observed that α Syn off-pathway oligomers inhibited seeded α Syn aggregation.⁴⁶ Similarly, Lorenzen et al. showed that certain α Syn off-pathway oligomers inhibit α Syn amyloid formation.⁴⁴

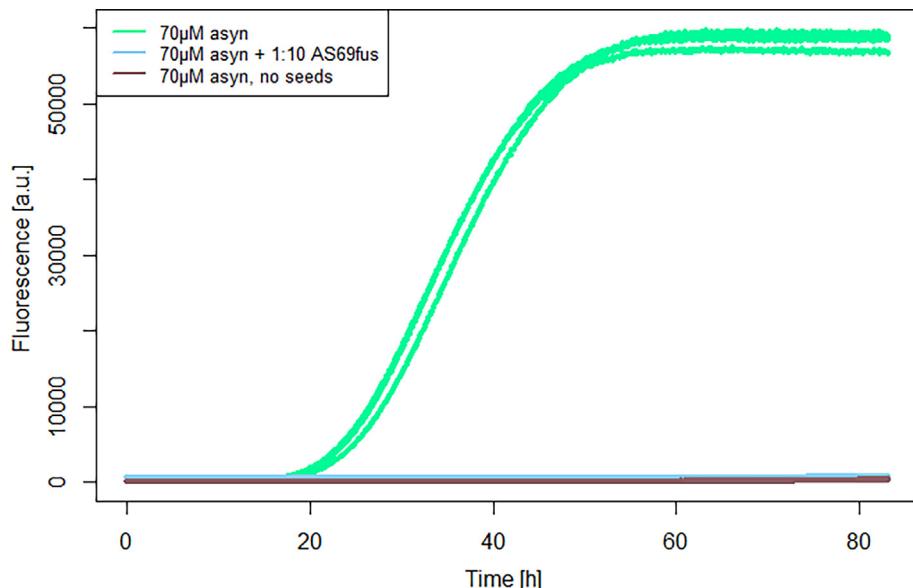


Fig. 2. Quiescent α Syn secondary nucleation assay at pH 5.0. 70 μ M α Syn was incubated in the presence of 100 nM preformed α Syn fibril seeds. No aggregation was detectable in the absence of seeds or in the presence of the inhibitor AS69fus, which inhibits secondary nucleation under these conditions.⁴²

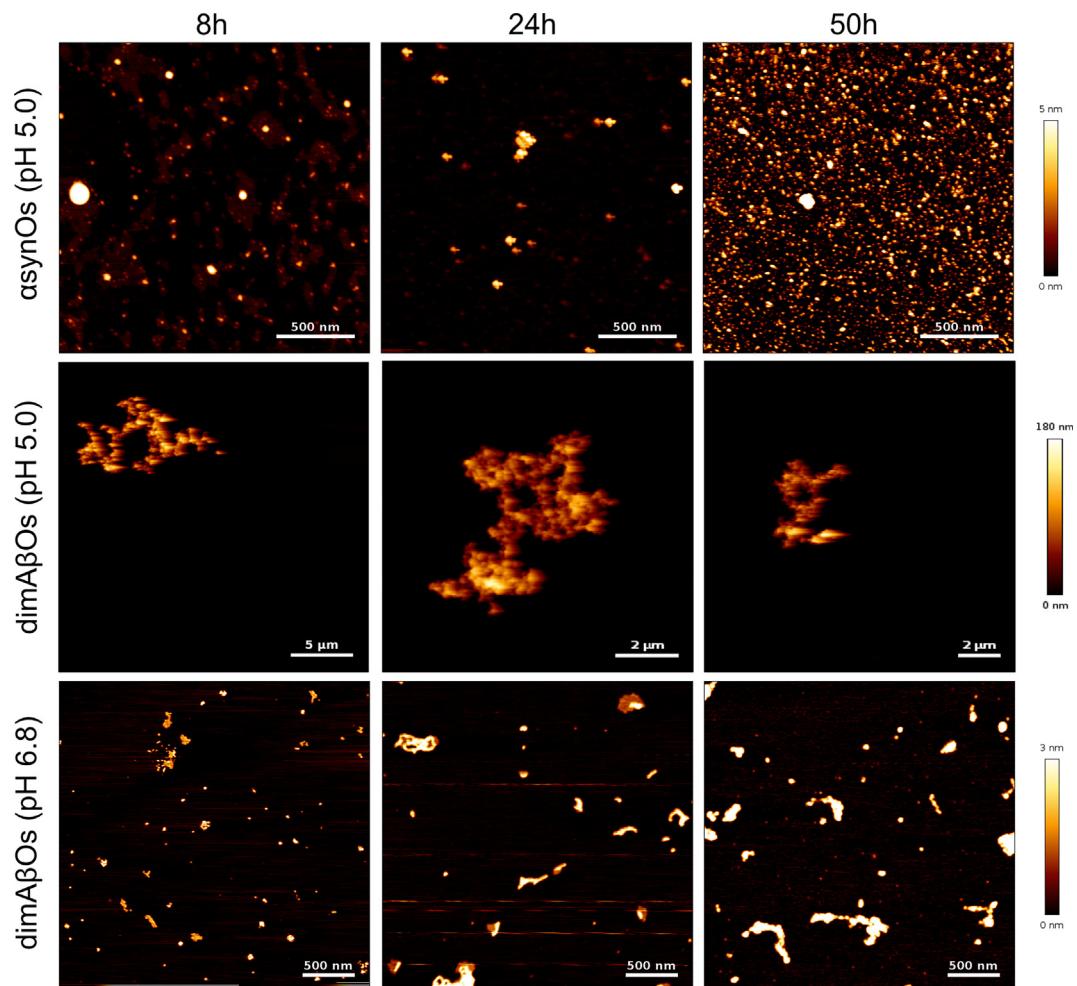


Fig. 3. AFM of oligomers applied in this study. Oligomer morphology and stability over time were investigated under the solution conditions of the secondary nucleation assays. Top row, α SynOs at pH 5.0; middle row, dimA β Os at pH 5.0; bottom row, dimA β Os at pH 6.8. Note that the clusters of dimA β O at pH 5.0 are of far greater height than the other oligomer species.

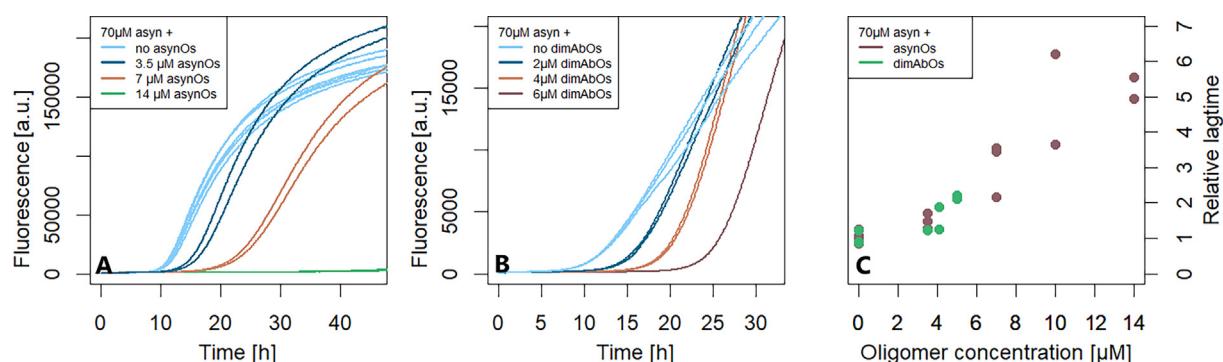


Fig. 4. Off-pathway oligomers increase the lag-time of α Syn secondary nucleation kinetics. Kinetics of secondary nucleation assays in presence of α SynO (A) or dimA β O (B). Both assays were done under quiescent conditions at pH 5, incorporating 70 μ M of monomer and 100 nM of seeding fibrils, which were bath-sonicated before. (C) Dependence of lag-times on the concentration of off-pathway oligomers. The lag-times were extracted from the linear part of all ThT curves recorded at pH 5 and then normalized on the lag-time of the samples containing no added oligomeric species.

Having confirmed the inhibitory effect of off-pathway oligomers of one protein on amyloid fibrils of the same protein (i.e., homo-inhibition) we went on to investigate if cross-protein inhibition (i.e., hetero-inhibition) also occurs. When different concentrations of dimA β Os were added to 70 μ M α Syn monomer seeded with 100 nM α Syn fibrils, a prolongation of the lag-time to an extent similar to the one caused by α SynOs was observed (Fig. 4B and C). This indicates that sequence identity is not required for amyloid oligomers to inhibit amyloid fibril formation. The data furthermore suggest that the clustering of dimA β Os at pH 5.0 does not abrogate their potential to inhibit secondary nucleation of fibrils.

DimA β Os inhibit α Syn1–108 secondary nucleation by binding to fibrils

Previously, we reported a direct interaction between dimA β O and A β fibrils.³⁶ This direct interaction could explain the inhibitory effect of A β oligomers on A β fibril formation, as the oligomers may block the sites of secondary nucleation on the amyloid fibril surface (Fig. 1B). Here, we aimed to investigate if the same mechanism could apply to the cross-inhibition of α Syn secondary nucleation by dimA β O. However, detection of the oligomer-fibril interaction was dependent on the separation of fibril-bound and free oligomers by way of faster sedimentation of the fibrils in comparison to free oligomers. This previously allowed us to pull down oligomers with fibrils and then image the complex by atomic force microscopy (AFM).³⁶ Here, however, at the acidic pH used in the α Syn secondary nucleation experiment dimA β Os assemble into larger clusters and therefore display similar sedimentation behavior to α Syn fibrils, prohibiting separation (Fig. 3, middle row).³⁵ Therefore, the assay was adapted by employing instead of wild-type α Syn a variant lacking the C-terminus (α Syn1–108). This variant shows secondary nucle-

ation at a higher pH,⁴¹ at which dimA β Os do not clump and hence do not sediment by themselves.

To confirm that secondary nucleation is indeed active and inhibited by α SynO under these conditions, we first repeated the ThT aggregation assays with α Syn1–108 at pH 6.8, both under quiescent (Fig. 5A) and shaking (Fig. 5B) conditions, both with and without addition of dimA β Os. The data displayed an oligomer concentration-dependent increase of lag-time (Fig. 5A,B) similar to the experiments with wt α Syn done at pH 5.0, confirming that α Syn1–108 is a suitable model for investigating effects on secondary nucleation at an increased pH of 6.8.

Since monitoring of secondary nucleation in the kinetic assay strictly requires fibril elongation to occur in addition to secondary nucleation, we next ruled out that dimA β oligomers affected fibril elongation. A setup was chosen that enforces specifically this process: By utilizing quiescent conditions and relatively high concentration of 10% seeding fibrils (in monomer equivalents), which were sonicated to increase the number of fibril ends, the elongation process was dominant, as shown in Fig. 5C. The addition of 2 and 5 μ M dimA β Os, which respectively constitutes 8 and 20% of the added monomer (comparable to the addition of 5 and 14 μ M dimA β Os in the secondary nucleation assays) did not show an effect on the initial increase in ThT fluorescence, which reflects the elongation rate (Fig. 5C). The data support that dimA β Os inhibit α Syn1–108 fibril formation by interfering with secondary nucleation but not with fibril elongation.

After showing that α Syn1–108 is a suitable α Syn variant to observe the inhibitory effect of dimA β Os on α Syn secondary nucleation at neutral pH, a potential direct interaction of dimA β Os with α Syn1–108 fibrils was investigated by AFM. Preformed α Syn1–108 fibrils were coincubated with dimA β Os at pH 6.8, followed by centrifugation and subsequent imaging of both supernatant and

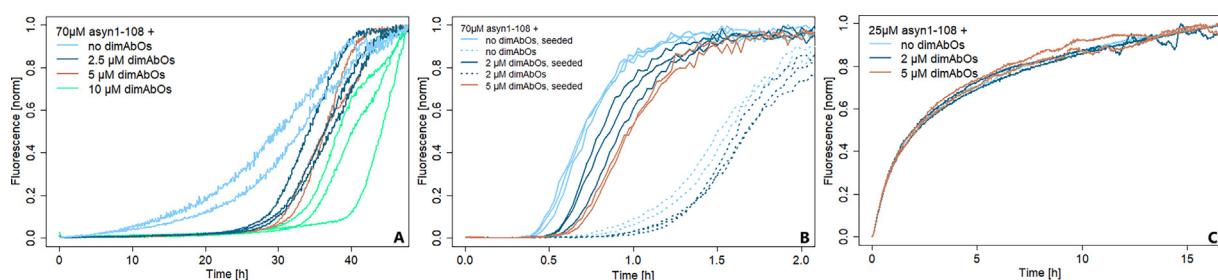


Fig. 5. DimA β oligomers interfere with secondary nucleation but not with elongation of α Syn1–108 fibrils. (A,B) Kinetics of secondary nucleation assays of α Syn1–108 with 100 nM seeds in the presence of varying concentrations of dimA β O under quiescent (A) or under shaking (B) conditions. (C) Kinetics of fibril elongation of 25 μ M α Syn1–108 with 2.5 μ M seeds under quiescent conditions in the presence of varying concentrations of dimA β O. All assays were performed at pH 6.8.

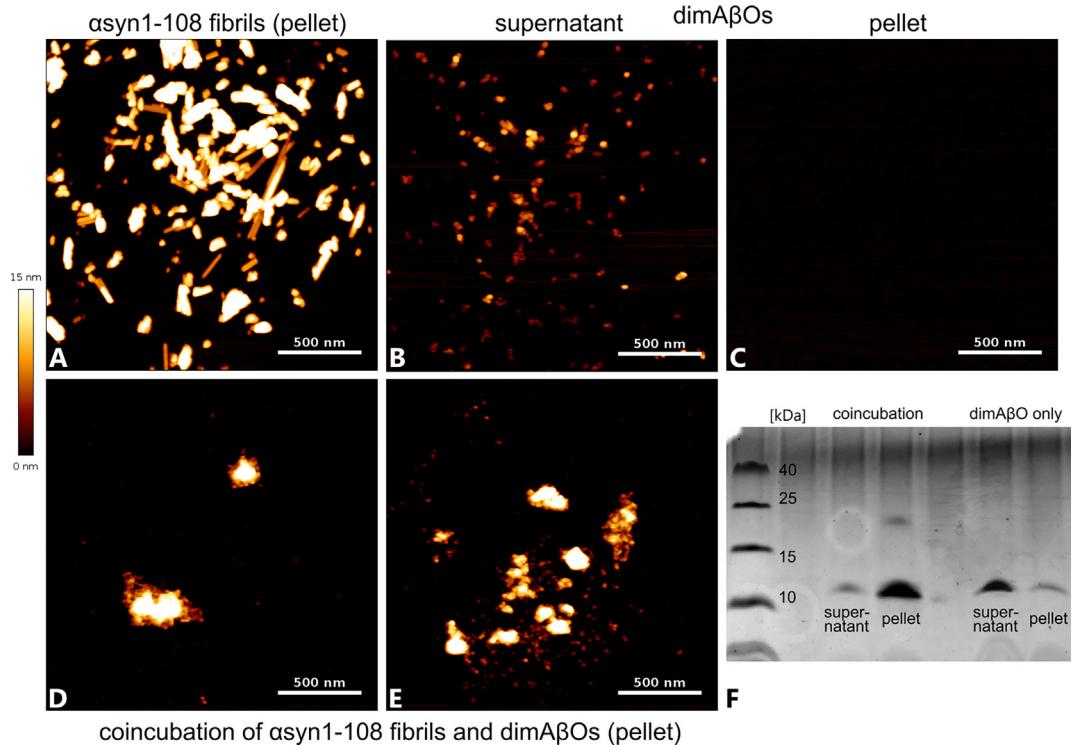


Fig. 6. DimA β Os bind to α Syn1–108 fibrils. Fibrillar α Syn1–108 aggregates and dimA β O were prepared separately and analyzed either individually (A-C,F) or after coincubation (D-F). Upon centrifugation, pellets and supernatants were analyzed by AFM (A-E) and SDS-PAGE (F). DimA β is visible as a band at 11 kDa in SDS-PAGE. In SDS-PAGE of the coincubation sample, α Syn1–108 fibrils did not enter the gel; α Syn1–108 is therefore not detectable.

resuspended pellet. Both α Syn1–108 fibrils and dimA β Os were imaged separately as controls.

As reported before, α Syn1–108 formed short fibrils with a tendency to cluster into bundles that were found in the pellet (Fig. 6A).⁴⁷ In contrast, dimA β Os remained in the supernatant as curvilinear assemblies (Fig. 6B-C). The height of dimA β Os was lower than that of α Syn1–108 fibrils and fibril bundles. Upon coincubation, spherical and curvilinear assemblies of the height of dimA β Os were found in the pellet, suggesting that dimA β Os were co-precipitated by α Syn1–108 aggregates (Fig. 6D-E). This finding is supported by SDS-PAGE analysis of the partitioning of dimA β into the supernatant and the pellet fractions. Upon coincubation with α Syn1–108 aggregates, the largest part of dimA β shifted into the pellet fraction (Fig. 6F). In the AFM images of coincubation samples, the spherical and curvilinear assemblies were associated with aggregates of larger height, which likely correspond to α Syn1–108 fibril clusters (Fig. 6D-E). In contrast to α Syn1–108 aggregates imaged in absence of dimA β Os, individual fibrils were not detectable in the coincubation samples. This suggests that dimA β Os promote further clustering of α Syn1–108 fibrils into dense aggregates. Taken together, AFM and SDS-PAGE of the coincubation samples provides evidence of a direct interaction of α Syn1–108 fibrils with dimA β Os, as observed

before in the homotypic case of A β fibrils and dimA β Os.

Discussion

Here, we find that off-pathway α Syn oligomers interfere with secondary nucleation of α Syn fibrils. This interference with secondary nucleation can explain the previously observed inhibitory effect of α SynOs on fibril formation.^{46,44} In addition, by combining a well-established α Syn secondary nucleation assay with the well-characterized A β off-pathway oligomer model dimA β , we were able to observe that heterotypic (i.e., A β) oligomers inhibit α Syn secondary nucleation to a similar extent as homotypic (i.e., α Syn) oligomers. With regard to the mechanism underlying inhibition of secondary nucleation, pull-down and AFM experiments show that dimA β O binds to fibril surfaces (Fig. 6). The resulting obstruction of secondary nucleation sites on fibril surfaces can well explain the inhibition of secondary nucleation (Fig. 1). This entails that off-pathway oligomer surfaces are not active in secondary nucleation, in contrast to the obstructed fibril surfaces, suggesting that the surface features of the highly ordered cross- β architecture of amyloid fibrils are essential for secondary nucleation.⁴⁸

We have previously reported inhibition of secondary nucleation by binding of off-pathway

oligomers to amyloid fibril surfaces for $\text{A}\beta$ and for lysozyme (ref ²⁷). The finding that the same applies to αSyn indicates that the interaction between these two distinct protein assembly types might be more the rule than exception. In this context it is interesting to note that there is evidence for delayed fibril formation under conditions of increased off-pathway oligomer formation for further amyloid proteins.²⁸

The fact that both homo- and hetero-oligomers inhibit secondary nucleation demonstrates that sequence identity between the fibril-forming and the oligomer-forming protein is not required. This is in contrast to the highly sequence-specific self-assembly at the fibril end during fibril elongation.⁴⁹ The oligomers' interaction with fibril surfaces apparently depends on more universal properties of amyloid oligomers. One likely factor is the presence of hydrophobic patches on oligomer surfaces which may interact with ladders of hydrophobic amino acid residues along amyloid fibril surfaces.^{50,51} In general, both oligomers and fibrils consist of multiple copies of identical molecules, enabling multivalent interactions of increased stability, potentially involving the rigid cross- β core of amyloid fibrils as well as the less ordered segments that constitute the fuzzy coats of fibrils and oligomers.

The interaction of off-pathway oligomers with fibrils has implications for the distribution and time evolution of the different aggregated species. By inhibiting the growth of fibrils which compete for monomers, off-pathway oligomers promote their own formation and slow down their replacement by amyloid fibrils. In addition, plaques of amyloid fibrils might accumulate off-pathway oligomers and serve as a reservoir of these particularly detrimental species.³⁹ The observation that hetero-oligomers are amenable to this type of interaction is interesting in the light of reports on interactions and pathological overlap of different amyloid proteins, including αSyn and $\text{A}\beta$.^{14–23,48} The data in this study demonstrates that such cross-interactions may also involve the interplay between amyloid fibrils of one protein with off-pathway oligomers of another protein. The interplay between off-pathway oligomers and amyloid fibrils adds another level of complexity to the homo- and hetero-assembly processes of amyloidogenic proteins.

Materials and methods

Protein expression and purification

Expression of αSyn and AS69fus, co-expression of dim $\text{A}\beta$ and their respective purification was carried out as described previously.^{52,27,42} The gene encoding dim $\text{A}\beta$ included an N-terminal methionine, a first $\text{A}\beta 40$ unit, a $(\text{G}_4\text{S})_4$ linker, and a second $\text{A}\beta 40$ unit.

Preparation of αSyn fibrils

αSyn wildtype fibrils were prepared in 25 mM sodium acetate buffer, 50 mM NaCl, pH 5, while $\alpha\text{Syn}1–108$ fibrils were prepared in 25 mM MOPS, 50 mM NaCl, pH 6.8, both at a protein concentration of 25 μM and in a 1.5 ml reaction vial. 0.03% NaN_3 was added to prevent protein decomposition. To induce fibril formation, the samples were incubated at 37°C over night under shaking conditions at 800 rpm after adding a glass bead.

Preparation of αSyn - and dim $\text{A}\beta$ oligomers

Off-pathway oligomers were prepared following previously published protocols^{35,43,44}. Dim $\text{A}\beta$ oligomers were prepared by dissolving HFIP-treated and lyophilised dim $\text{A}\beta$ in a small volume of 50 mM NaOH, if necessary facilitated by 1 min of bath sonification. Afterwards, 25 mM MOPS, 50 mM NaCl, pH 6.8, was added before the pH was adjusted with 50 mM HCl, obtaining a final dim $\text{A}\beta$ concentration of either 10 or 20 μM , depending on the experiment. 0.03% NaN_3 was added to prevent protein decomposition. To induce dim $\text{A}\beta$ oligomer formation, the samples were incubated at 37°C for about 20 h under quiescent conditions, checked for oligomer formation by AFM and if necessary, left for longer.

αSyn was dialysed over night against water, lyophilized and then resuspended in 25 mM MOPS, 50 mM NaCl, pH 7.4 (since αSyn off-pathway oligomers do not form at pH 5 using the protocol applied here) at a concentration of 12 mg/ml before incubation for 4 h. After centrifugation for 10 min at 16000 rpm, the sample was loaded onto a Superdex 200 increase 10/300 GL SEC column and the oligomer peak was separated from the monomer peak. The oligomers were then concentrated using a spin-concentrator with a cut-off of 3 kDa.

ThT aggregation kinetics

For secondary nucleation assays for wildtype αSyn , αSyn fibrils were sonicated for 3 min in a sonication bath before adding them to a concentration of 100 nM (all concentrations in monomer equivalents) into the wells of a 96-well low-binding plate (Greiner) with 25 mM ThT, 0.03% NaN_3 , off-pathway oligomers in varying concentrations, 70 μM freshly thawed αSyn monomer and 50 mM sodium acetate buffer, 50 mM NaCl, pH 5. The plate was put in a BMG FluoStar Omega and data points were collected at 37°C every 5 min using the BMG Reader Control software (version 5.40).

For $\alpha\text{Syn}1–108$, in addition to the above-mentioned assay, a shaking assay was employed.

For this, 100 nM fibrils were added into the wells of a 96-well low-binding plate (Greiner) with 25 mM ThT, 0.03% NaN_3 , off-pathway oligomers, 70 μM freshly thawed α Syn monomer, 50 mM MOPS, 50 mM NaCl, pH 6.8, and a glass bead. The plate was put in a BMG FluoStar Omega and data points were collected at 37°C every 100 s while shaking at 600 rpm (double-orbital) using the BMG Reader Control software (version 5.40).

For the elongation assay, 2.5 μM fibrils were added after 1 s probe sonification at an amplitude of 10% into the wells of a 96-well low-binding plate (Greiner) with 25 mM ThT, 0.03% NaN_3 , off-pathway oligomers in varying concentrations, 25 μM freshly thawed α Syn monomer, 50 mM MOPS, 50 mM NaCl, pH 6.8. The plate was put in a BMG FluoStar Omega and data points were collected at 37°C every 100 s under quiescent conditions.

To determine the lag-times, the linear part of the sigmoidal curves was fitted through the point of steepest ascend and extrapolated to the baseline.

Fibril-oligomer coincubation

5 μM α syn1–108 fibrils and 5 μM preformed dim $\text{A}\beta$ oligomers were combined in 50 μL 25 mM MOPS, 50 mM NaCl, pH 6.8, and agitated at 25°C for 10 min before centrifugation at 8000xg for 5 min. The supernatant was then transferred into another vial and the translucent pellet was resuspended in 20 μL buffer, yielding two samples. For the control samples, only the dim $\text{A}\beta$ oligomers were agitated and centrifuged in parallel, yielding two samples as well.

SDS–polyacrylamide gel electrophoresis

The coincubation samples were analyzed qualitatively by SDS–PAGE and colloidal Coomassie staining. For this purpose, 16 μL of each sample was mixed with 4 μL of 5x sample buffer yielding a concentration of 1x buffer (15% glycerol, 4% SDS, 50 mM Tris–HCl, pH 7.4, 2% β -mercaptoethanol) and 15 μL of each sample was applied onto a 20% Tris/Tricine gel containing a 5.6% stacking gel prepared according to standard protocols. Electrophoresis was performed at a constant current of 40 mA per gel.

Atomic force microscopy

5 μl of the samples were taken, applied onto freshly cleaved muscovite mica, and left to incubate under high humidity for 15 min before carefully washing with 200 μl MQ water and drying under a stream of filtered N_2 gas. Imaging was performed in intermittent contact mode (AC mode) in a JPK Nano Wizard 3 atomic force microscope (JPK, Berlin) with a silicon cantilever and silicon tip (OMCL-AC160TS-R3, Olympus) with a typical tip radius of 9 ± 2 nm, a force constant of 26 N/m,

and resonance frequency around 280 kHz. Image processing was done using the JPK DP Data Processing Software (version spm-5.0.84). For the presented height profiles, a polynomial fit was subtracted from each scan line first independently and then using limited data range.

DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Wolfgang Hoyer reports financial support was provided by European Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.].

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