Off-pathway oligomers of α-synuclein and Aβ inhibit secondary nucleation of α-synuclein amyloid fibrils

Marie P. Schützmann, Wolfgang Hoyer

Suggested Citation:
Schützmann, M., & Hoyer, W. (2025). Off-pathway oligomers of α-synuclein and Aβ inhibit secondary nucleation of α-synuclein amyloid fibrils. Journal of Molecular Biology, 437(10), Article 169048. https://doi.org/10.1016/j.jmb.2025.169048

Research Article

Off-pathway oligomers of α-synuclein and Aβ inhibit secondary nucleation of α-synuclein amyloid fibrils

Marie P. Schützmann [1], **and Wolfgang Hoyer** [1,2,*]

*1 - **Institut für Physikalische Biologie,** Faculty of Mathematics and Natural Sciences, Heinrich Heine University Düsseldorf, Düsseldorf, 40204 Germany*

*2 - **Institute of Biological Information Processing (IBI-7: Structural Biochemistry),** Forschungszentrum Jülich, Jülich, 52428 Germany*

Correspondence to Wolfgang Hoyer:*Institut für Physikalische Biologie, Faculty of Mathematics and Natural Sciences, Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany. *wolfgang.hoyer@hhu.de (W. Hoyer)*

https://doi.org/10.1016/j.jmb.2025.169048

Edited by: Sheena Radford

Abstract

α-Synuclein (αSyn) 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 (Aβ). We previously found an intriguing interplay between off-pathway Aβ oligomers and Aβ 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 αSyn fibrils. Both homotypic (i.e. αSyn) and heterotypic (i.e. Aβ) off-pathway oligomers inhibited αSyn aggregation in kinetic assays of secondary nucleation. Initially soluble, kinetically trapped Aβ oligomers co-precipitated with αSyn(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.

Introduction

Alpha-synuclein (αSyn), a 140 amino acid long protein[1] 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*, αSyn presents as an intrinsically disordered protein, but it can assume highly-ordered β-sheet arrangements upon assembly into amyloid fibrils.[8–10]

Whereas monomeric αSyn 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 (Aβ), 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 αSyn 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 Aβ plaques and Tau tangles but also αSyn pathology, and Aβ plaques are frequently encountered in synucleinopathy cases.[16,17] The pathological overlap might be a result of a direct interaction between αSyn and Aβ, 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, αSyn oligomers (αSynOs) impair synaptic function, lead to increased intracellular Ca^{2+} levels, and disturb mitochondria as well as cellular proteostasis.[29] Similarly, Aβ oligomers (AβOs) 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-Aβ antibody lecanemab, which was developed to exhibit particularly high affinity for protofibrillar off-pathway oligomers.[34]

We have recently characterized a model for protofibrillar Aβ off-pathway oligomers formed from the dimeric Aβ variant dimAβ.[27,35] In dimAβ, two Aβ40 units are linked in one polypeptide chain through a flexible glycine–serine-rich linker. DimAβ preferentially forms AβO (termed here dimAβO) due to the increased local Aβ concentration, allowing to study effects of AβO with minimized disturbance by Aβ monomers or fibrils. Importantly, dimAβO recapitulate the biophysical and functional properties of native AβO.[35] Application of dimAβO revealed an intriguing interplay between Aβ off-pathway oligomers and Aβ fibrils, beyond the mere competition for Aβ 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 Aβ, αSyn 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 Aβ oligomers.[39]

Here, we investigated if the oligomer-fibril interplay previously observed for Aβ and lysozyme also applies to αSyn. 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

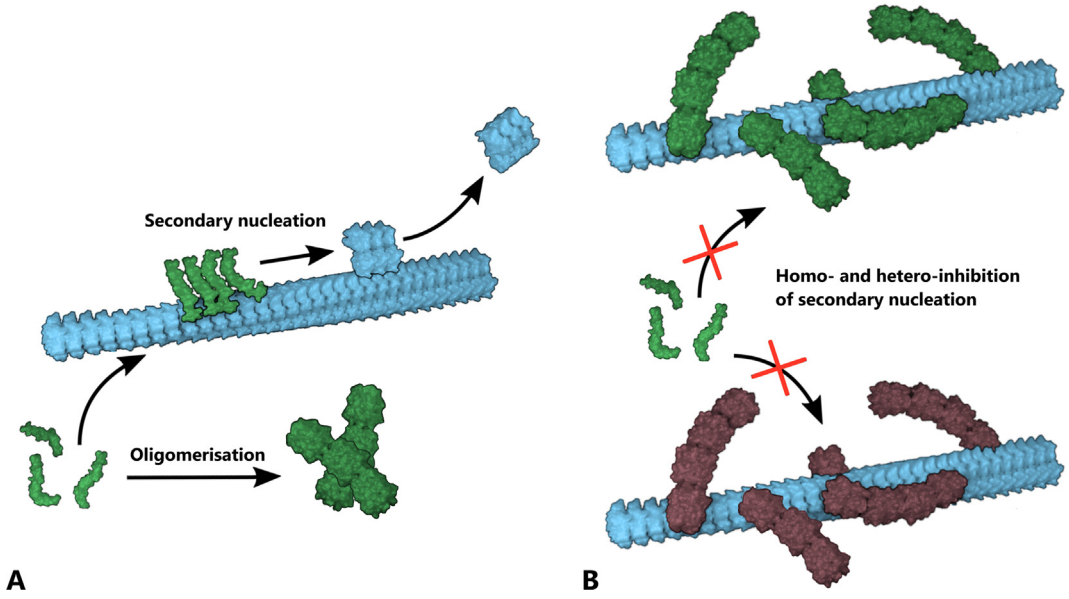

Fig. 1. Suggested mechanism of the inhibition of secondary nucleation by off-pathway oligomers. (A) Scheme of fibril formation by secondary nucleation versus off-pathway oligomer formation. (B) Off-pathway oligomers impede secondary nucleation by binding to fibril surfaces and masking secondary nucleation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

homo-oligomers (i.e. αSynO) and hetero-oligomers (dimAβO) on αSyn fibril formation.

Results

Both homo- and hetero-amyloid oligomers inhibit αSyn secondary nucleation

Secondary nucleation drives aggregation of full-length αSyn 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 αSyn 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 αSyn monomer concentration of 70 μM 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 AS69fus[42] confirmed the critical role of secondary nucleation under the chosen experimental conditions. (Fig. 2).

αSynO (homo-oligomers) were prepared according to the protocol for off-pathway αSyn oligomer preparation by dialysis against water, lyophilisation, subsequent resuspension and separation by SEC.[43–45] In atomic force microscopy (AFM) αSynO are imaged as spherical structures that persist under the experimental conditions of the secondary nucleation assay (Fig. 3, top row).

As hetero-oligomers, Aβ oligomers were chosen due to the possibly disease-relevant interaction of Aβ and αSyn.[14–23] Oligomers formed by the synthetic Aβ dimer dimAβ, which consists of two Aβ40 units linked in one polypeptide chain, were chosen as oligomer model. DimAβ forms kinetically stable oligomers that reliably mimick off-pathway Aβ oligomers.[27,35] Application of dimAβ 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, dimAβO 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 αSyn, these species cluster into large aggregates (Fig. 3, middle row). We have previously shown that these dense aggregates are indeed assemblies of small AβO.[35]

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

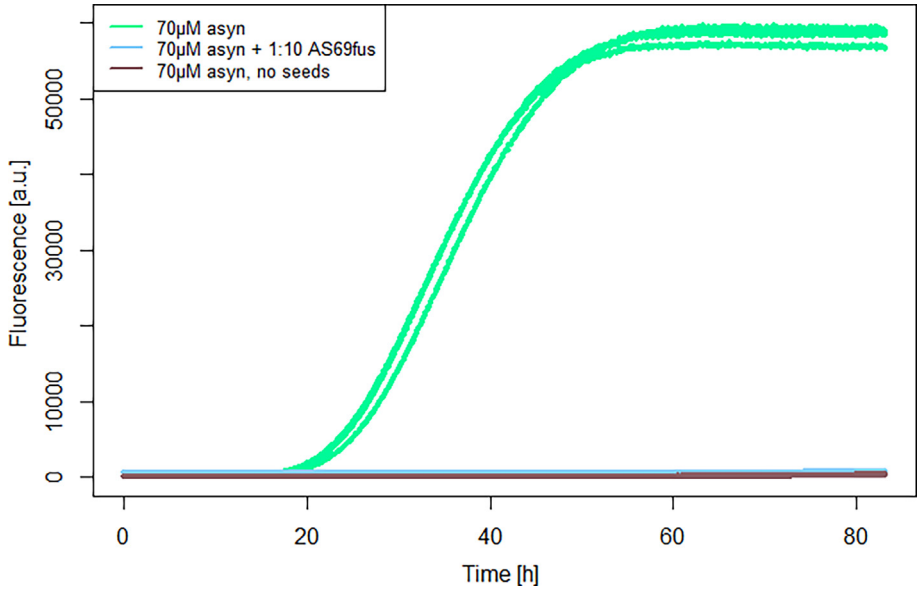

Fig. 2. Quiescent αSyn secondary nucleation assay at pH 5.0. 70 μM αSyn was incubated in the presence of 100 nM preformed αSyn fibril seeds. No aggregation was detectable in the absence of seeds or in the presence of the inhibitor AS69fus, which inhibits secondary nucleation under these conditions.[42]

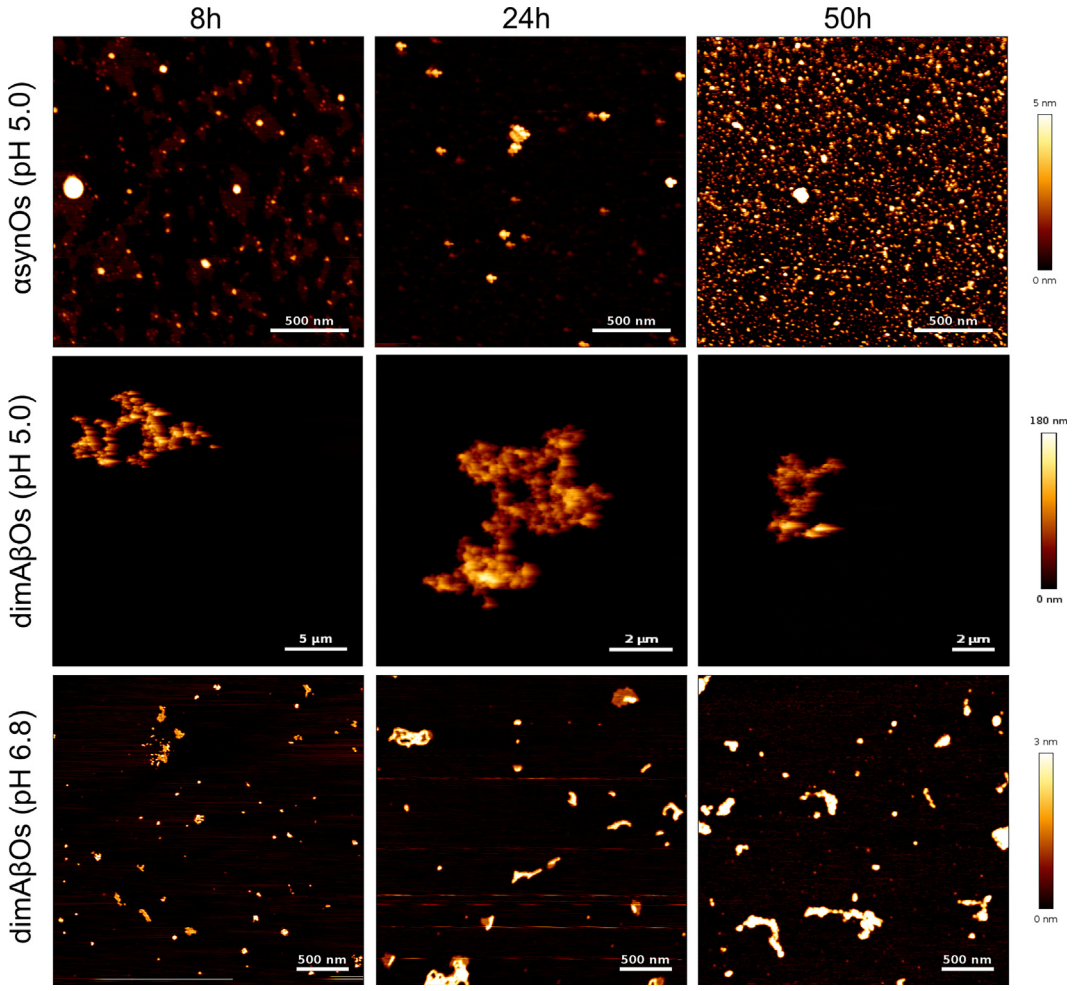

Fig. 3. AFM of oligomers applied in this study. Oligomer morphology and stability over time were investigated under the solution conditions of the secondary nucleation assays. Top row, αSynOs at pH 5.0; middle row, dimAβOs at pH 5.0; bottom row, dimAβOs at pH 6.8. Note that the clusters of dimAβO at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

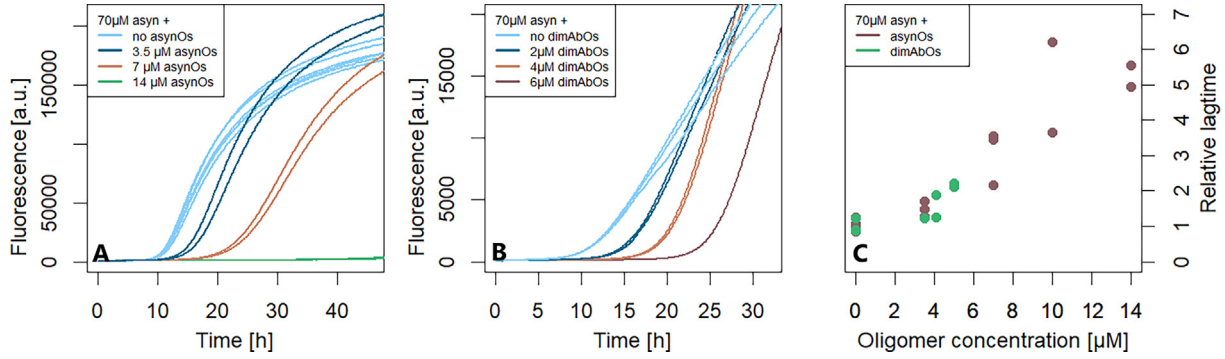

Fig. 4. Off-pathway oligomers increase the lag-time of αSyn secondary nucleation kinetics. Kinetics of secondary nucleation assays in presence of αSynO (A) or dimAβO (B). Both assays were done under quiescent conditions at pH 5, incorporating 70 µM of monomer and 100 nM of seeding fibrils, which were 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 dimAβOs were added to 70 μM αSyn monomer seeded with 100 nM αSyn fibrils, a prolongation of the lag-time to an extent similar to the one caused by αSynOs 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 dimAβOs at pH 5.0 does not abrogate their potential to inhibit secondary nucleation of fibrils.

DimAβOs inhibit αSyn1–108 secondary nucleation by binding to fibrils

Previously, we reported a direct interaction between dimAβO and Aβ fibrils.[36] This direct interaction could explain the inhibitory effect of Aβ oligomers on Aβ 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 αSyn secondary nucleation by dimAβO. 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 αSyn secondary nucleation experiment dimAβOs assemble into larger clusters and therefore display similar sedimentation behavior to αSyn fibrils, prohibiting separation (Fig. 3, middle row).[35] Therefore, the assay was adapted by employing instead of wild-type αSyn a variant lacking the C-terminus (αSyn1–108). This variant shows secondary nucleation at a higher pH,[41] at which dimAβOs do not clump and hence do not sediment by themselves.

To confirm that secondary nucleation is indeed active and inhibited by αSynO under these conditions, we first repeated the ThT aggregation assays with αSyn1–108 at pH 6.8, both under quiescent (Fig. 5A) and shaking (Fig. 5B) conditions, both with and without addition of dimAβOs. The data displayed an oligomer concentration-dependent increase of lag-time (Fig. 5A,B) similar to the experiments with wt αSyn done at pH 5.0, confirming that αSyn1–108 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 dimAβ 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 μM dimAβOs, which respectively constitutes 8 and 20% of the added monomer (comparable to the addition of 5 and 14 μM dimAβOs 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 dimAβOs inhibit αSyn1–108 fibril formation by interfering with secondary nucleation but not with fibril elongation.

After showing that αSyn1–108 is a suitable αSyn variant to observe the inhibitory effect of dimAβOs on αSyn secondary nucleation at neutral pH, a potential direct interaction of dimAβOs with αSyn1–108 fibrils was investigated by AFM. Preformed αSyn1–108 fibrils were coincubated with dimAβOs at pH 6.8, followed by centrifugation and subsequent imaging of both supernatant and

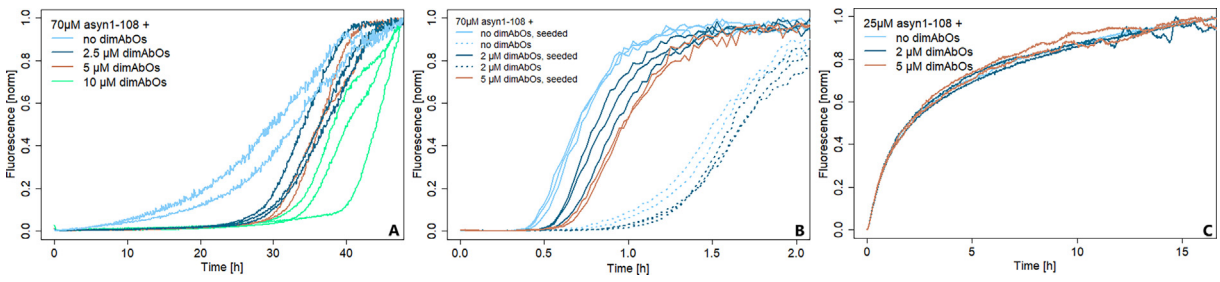

Fig. 5. DimAβ oligomers interfere with secondary nucleation but not with elongation of αSyn1–108 fibrils. (A,B) Kinetics of secondary nucleation assays of αSyn1–108 with 100 nM seeds in the presence of varying concentrations of dimAβO under quiescent (A) or under shaking (B) conditions. (C) Kinetics of fibril elongation of 25 μM αSyn1–108 with 2.5 μM seeds under quiescent conditions in the presence of varying concentrations of dimAβO. All assays were performed at pH 6.8.

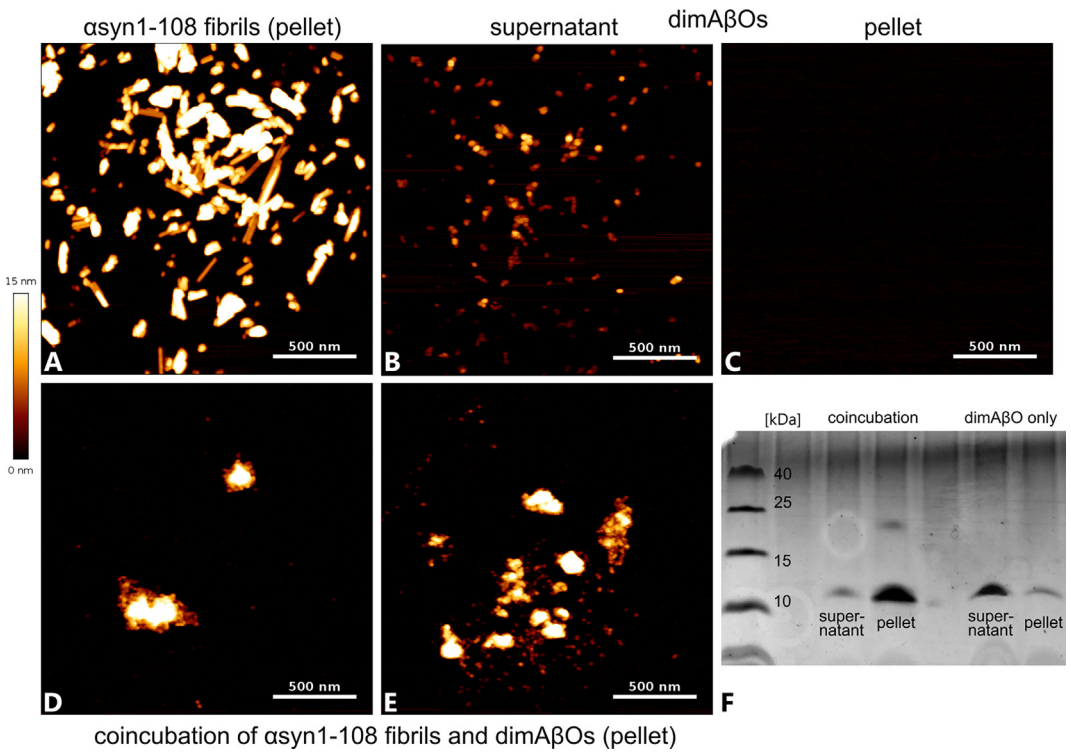

Fig. 6. DimAβOs bind to αSyn1–108 fibrils. Fibrillar αSyn1–108 aggregates and dimAβO were prepared separately and analyzed either individually (A-C,F) or after conincubation (D-F). Upon centrifugation, pellets and supernatants were analyzed by AFM (A-E) and SDS–PAGE (F). DimAβ is visible as a band at 11 kDa in SDS–PAGE. In SDS–PAGE of the coincubation sample, αSyn1–108 fibrils did not enter the gel; αSyn1–108 is therefore not detectable.

resuspended pellet. Both αSyn1–108 fibrils and dimAβOs were imaged separately as controls.

As reported before, αSyn1–108 formed short fibrils with a tendency to cluster into bundles that were found in the pellet (Fig. 6A).[47] In contrast, dimAβOs remained in the supernatant as curvilinear assemblies (Fig. 6B-C). The height of dimAβOs was lower than that of αSyn1–108 fibrils and fibril bundles. Upon coincubation, spherical and curvilinear assemblies of the height of dimAβOs were found in the pellet, suggesting that dimAβOs were co-precipitated by αSyn1–108 aggregates (Fig. 6-D-E). This finding is supported by SDS–PAGE analysis of the partitioning of dimAβ into the supernatant and the pellet fractions. Upon coincubation with αSyn1–108 aggregates, the largest part of dimAβ 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 αSyn1–108 fibril clusters (Fig. 6D-E). In contrast to αSyn1–108 aggregates imaged in absence of dimAβOs, individual fibrils were not detectable in the coincubation samples. This suggests that dimAβOs promote further clustering of αSyn1–108 fibrils into dense aggregates. Taken together, AFM and SDS–PAGE of the conincubation samples provides evidence of a direct interaction of αSyn1–108 fibrils with dimAβOs, as observed before in the homotypic case of Aβ fibrils and dimAβOs.

Discussion

Here, we find that off-pathway αSyn oligomers interfere with secondary nucleation of αSyn fibrils. This interference with secondary nucleation can explain the previously observed inhibitory effect of αSynOs on fibril formation.[46,44] In addition, by combining a well-established αSyn secondary nucleation assay with the well-characterized Aβ off-pathway oligomer model dimAβ, we were able to observe that heterotypic (i.e., Aβ) oligomers inhibit αSyn secondary nucleation to a similar extent as homotypic (i.e., αSyn) oligomers. With regard to the mechanism underlying inhibition of secondary nucleation, pull-down and AFM experiments show that dimAβO 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 Aβ 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 consists 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 Aβ.[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 dimAβ and their respective purification was carried out as described previously.[52,27,42] The gene encoding dimAβ included an N-terminal methionine, a first Aβ40 unit, a $(G_4S)_4$ linker, and a second Aβ40 unit.

Preparation of αSyn fibrils

αSyn wildtype fibrils were prepared in 25 mM sodium acetate buffer, 50 mM NaCl, pH 5, while αSyn1–108 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 dimAβ oligomers

Off-pathway oligomers were prepared following previously published protocols[35,43,44]: DimAβ oligomers were prepared by dissolving HFIP-treated and lyophilised dimAβ 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 dimAβ concentration of either 10 or 20 μM, depending on the experiment. 0.03% NaN_3 was added to prevent protein decomposition. To induce dimAβ 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 αSyn1–108, 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 αSyn 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 αSyn 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 αsyn1–108 fibrils and 5 μM preformed dimAβ 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 dimAβ 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.].

Acknowledgements

This project has received funding from the European Research Council under the European Union's Horizon 2020 research and innovation program, grant agreement No. 726368.

Received 15 August 2024;
Accepted 23 February 2025;
Available online 25 February 2025

Keywords:
amyloid aggregation;
oligomers;
α-synuclein;
Aβ;
cross-seeding;
protofibrils

References

1. Goedert, M., (2001). Alpha-synuclein and neurodegenerative diseases. *Nat. Rev. Neurosci.* **2** (7), 492–501. https://doi.org/10.1038/35081564.
2. Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., Rohan De Silva, H., Kittel, A., Saitoh, T., (1995). The precursor protein of non-Abeta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* **14** (2), 467–475. https://doi.org/10.1016/0896-6273(95)90302-X.
3. International Parkinson's Disease Genomics Consortium (IPDGC), Parkinson's Study Group (PSG) Parkinson's Research: The Organized GENetics Initiative (PROGENI), 23andMe, GenePD, NeuroGenetics Research Consortium (NGRC), Hussman Institute of Human Genomics (HIHG), The Ashkenazi Jewish Dataset Investigator, Cohorts for Health and Aging Research in Genetic Epidemiology (CHARGE), North American Brain Expression Consortium (NABEC), United Kingdom Brain Expression Consortium (UKBEC), Greek Parkinson's

Disease Consortium, Alzheimer Genetic Analysis Group, M.A. Nalls, N. Pankratz, C.M. Lill, C.B. Do, D.G. Hernandez, M. Saad, A.L. DeStefano, E. Kara, J. Bras, M. Sharma, C. Schulte, M.F. Keller, S. Arepalli, C. Letson, C. Edsall, H. Stefansson, X. Liu, H. Pliner, J.H. Lee, R. Cheng, M.A. Ikram, J.P.A. Ioannidis, G.M. Hadjigeorgiou, J.C. Bis, M. Martinez, J.S. Perlmutter, A. Goate, K. Marder, B. Fiske, M. Sutherland, G. Xiromerisiou, R.H. Myers, L.N. Clark, K. Stefansson, J.A. Hardy, P. Heutink, H. Chen, N. W. Wood, H. Houlden, H. Payami, A. Brice, W.K. Scott, T. Gasser, L. Bertram, N. Eriksson, T. Foroud, A.B. Singleton, Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease, Nat. Genet. 46 (9) (2014) 989–993. doi:10.1038/ng.3043.

4. Polymeropoulos, M.H., Higgins, J.J., Golbe, L.I., Johnson, W.G., Ide, S.E., Di Iorio, G., Sanges, G., Stenroos, E.S., Pho, L.T., Schaffer, A.A., Lazzarini, A.M., Nussbaum, R.L., Duvoisin, R.C., (1996). Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. *Science* **274** (5290), 1197–1199. https://doi.org/10.1126/science.274.5290.1197.
5. Di Maio, R., Barrett, P.J., Hoffman, E.K., Barrett, C.W., Zharikov, A., Borah, A., Hu, X., McCoy, J., Chu, C.T., Burton, E.A., Hastings, T.G., Greenamyre, J.T., (2016). Alpha-Synuclein binds to TOM20 and inhibits mitochondrial protein import in Parkinson's disease. *Sci. Transl. Med.* **8** (342), (Jun. https://doi.org/10.1126/scitranslmed.aaf3634.
6. Iyer, A., Claessens, M.M., (2019). Disruptive membrane interactions of alpha-synuclein aggregates. *Biochim. et Biophys. Acta (BBA)-Prot. Proteom.* **1867** (5), 468–482. https://doi.org/10.1016/j.bbapap.2018.10.006.
7. Calabresi, P., Mechelli, A., Natale, G., Volpicelli-Daley, L., Di Lazzaro, G., Ghiglieri, V., (2023). Alpha-synuclein in parkinson's disease and other synucleinopathies: from overt neurodegeneration back to early synaptic dysfunction. *Cell Death & Disease* **14** (3), 176. https://doi.org/10.1038/s41419-023-05672-9.
8. Bousset, L., Pieri, L., Ruiz-Arlandis, G., Gath, J., Jensen, P.H., Habenstein, B., Madiona, K., Olieric, V., Böckmann, A., Meier, B.H., et al., (2013). Structural and functional characterization of two alpha-synuclein strains. *Nat. Commun.* **4** (1), 2575. https://doi.org/10.1038/ncomms3575.
9. Li, B., Ge, P., Murray, K.A., Sheth, P., Zhang, M., Nair, G., Sawaya, M.R., Shin, W.S., Boyer, D.R., Ye, S., et al., (2018). Cryo-em of full-length α-synuclein reveals fibril polymorphs with a common structural kernel. *Nat. Commun.* **9** (1), 3609. https://doi.org/10.1038/s41467-018-05971-2.
10. Yang, Y., Shi, Y., Schweighauser, M., Zhang, X., Kotecha, A., Murzin, A.G., Garringer, H.J., Cullinane, P.W., Saito, Y., Foroud, T., et al., (2022). Structures of α-synuclein filaments from human brains with lewy pathology. *Nature* **610** (7933), 791–795. https://doi.org/10.1038/s41586-022-05319-3.
11. Bengoa-Vergniory, N., Roberts, R.F., Wade-Martins, R., Alegre-Abarrategui, J., (2017). Alpha-synuclein oligomers: a new hope. *Acta Neuropathol.* **134** (6), 819–838. https://doi.org/10.1007/s00401-017-1755-1.
12. Ingelsson, M., (Sep. 2016). Alpha-synuclein oligomers—neurotoxic molecules in Parkinson's disease and other Lewy body disorders. *Front. Neurosci.* **10** https://doi.org/10.3389/fnins.2016.00408.
13. Cline, E.N., Bicca, M.A., Viola, K.L., Klein, W.L., (2018). The Amyloid-β oligomer hypothesis: beginning of the third decade. *J. Alzheimer's Disease: JAD* **64** (s1), S567–S610. https://doi.org/10.3233/JAD-179941.
14. Irwin, D.J., Hurtig, H.I., (2018). The contribution of Tau, Amyloid-Beta and Alpha-Synuclein pathology to dementia in lewy body disorders. *J. Alzheimer's Disease & Parkinson.* **08** (04) https://doi.org/10.4172/2161-0460.1000444.
15. Clinton, L.K., Blurton-Jones, M., Myczek, K., Trojanowski, J.Q., LaFerla, F.M., (2010). Synergistic Interactions between Aβ, Tau, and α-synuclein: acceleration of neuropathology and cognitive decline. *J. Neurosci.* **30** (21), 7281–7289. https://doi.org/10.1523/JNEUROSCI.0490-10.2010.
16. Hamilton, R.L., (2000). Lewy bodies in Alzheimer's disease: a neuropathological review of 145 cases using alpha-synuclein immunohistochemistry. *Brain Pathol. (Zurich, Switzerland)* **10** (3), 378–384. https://doi.org/10.1111/j.1750-3639.2000.tb00269.x.
17. Irwin, D.J., Lee, V.M.-Y., Trojanowski, J.Q., (2013). Parkinson's disease dementia: convergence of α-synuclein, tau and amyloid-β pathologies. *Nat. Rev. Neurosci.* **14** (9), 626–636. https://doi.org/10.1038/nrn3549.
18. Ge, W.-Y., Deng, X., Shi, W.-P., Lin, W.-J., Chen, L.-L., Liang, H., Wang, X.-T., Zhang, T.-D., Zhao, F.-Z., Guo, W.-H., Yin, D.-C., (2023). Amyloid protein cross-seeding provides a new perspective on multiple diseases in vivo. *Biomacromolecules* **24** (1), 1–18. https://doi.org/10.1021/acs.biomac.2c01233.
19. Köppen, J., Schulze, A., Machner, L., Wermann, M., Eichentopf, R., Guthardt, M., Hähnel, A., Klehm, J., Kriegeskorte, M.-C., Hartlage-Rübsamen, M., et al., (2020). Amyloid-beta peptides trigger aggregation of alpha-synuclein in vitro. *Molecules* **25** (3), 580. https://doi.org/10.3390/molecules25030580.
20. Masliah, E., Rockenstein, E., Veinbergs, I., Sagara, Y., Mallory, M., Hashimoto, M., Mucke, L., (2001). beta-amyloid peptides enhance alpha-synuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's disease and Parkinson's disease. *Proc. Natl. Acad. Sci. USA* **98** (21), 12245–12250. https://doi.org/10.1073/pnas.211412398.
21. Noguchi-Shinohara, M., Ono, K., (2023). The mechanisms of the roles of α-synuclein, amyloid-β, and tau protein in the lewy body diseases: pathogenesis, early detection, and therapeutics. *Int. J. Mol. Sci.* **24** (12), 10215. https://doi.org/10.3390/ijms241210215.
22. Mandal, P.K., Pettegrew, J.W., Masliah, E., Hamilton, R.L., Mandal, R., (2006). Interaction between Abeta peptide and alpha synuclein: molecular mechanisms in overlapping pathology of Alzheimer's and Parkinson's in dementia with Lewy body disease. *Neurochem. Res.* **31** (9), 1153–1162. https://doi.org/10.1007/s11064-006-9140-9.
23. P.H. Jensen, P. Hojrup, H. Hager, M.S. Nielsen, L. Jacobsen, O.F. Olesen, J. Gliemann, R. Jakes, Binding of Abeta to alpha- and beta-synucleins: identification of segments in alpha-synuclein/NAC precursor that bind Abeta and NAC, Biochem. J. 323 (Pt 2) (Pt 2) (1997) 539–546. doi:10.1042/bj3230539.
24. Benilova, I., Karran, E., De Strooper, B., (2012). The toxic Aβ oligomer and Alzheimer's disease: an emperor in need

of clothes. *Nat. Neurosci.* **15** (3), 349–357. https://doi.org/10.1038/nn.3028.
25. Jan, A., Hartley, D.M., Lashuel, H.A., (2010). Preparation and characterization of toxic Abeta aggregates for structural and functional studies in Alzheimer's disease research. *Nat. Protoc.* **5** (6), 1186–1209. https://doi.org/10.1038/nprot.2010.72.
26. Dear, A.J., Meisl, G., Šarić, A., Michaels, T.C.T., Kjaergaard, M., Linse, S., Knowles, T.P.J., (2020). Identification of on- and off-pathway oligomers in amyloid fibril formation. *Chem. Sci.* **11** (24), 6236–6247. https://doi.org/10.1039/c9sc06501f.
27. Hasecke, F., Miti, T., Perez, C., Barton, J., Schölzel, D., Gremer, L., Grüning, C.S.R., Matthews, G., Meisl, G., Knowles, T.P.J., Willbold, D., Neudecker, P., Heise, H., Ullah, G., Hoyer, W., Muschol, M., (2018). Origin of metastable oligomers and their effects on amyloid fibril self-assembly. *Chem. Sci.* **9** (27), 5937–5948. https://doi.org/10.1039/C8SC01479E.
28. Muschol, M., Hoyer, W., (2023). Amyloid oligomers as on-pathway precursors or off-pathway competitors of fibrils. *Front. Mol. Biosci.* **10**, 1120416. https://doi.org/10.3389/fmolb.2023.1120416.
29. Alam, P., Bousset, L., Melki, R., Otzen, D.E., (2019). α-synuclein oligomers and fibrils: a spectrum of species, a spectrum of toxicities. *J. Neurochem.* **150** (5), 522–534. https://doi.org/10.1111/jnc.14808.
30. Lacor, P.N., Buniel, M.C., Furlow, P.W., Clemente, A.S., Velasco, P.T., Wood, M., Viola, K.L., Klein, W.L., (2007). Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J. Neurosci.: Off. J. Soc. Neurosci.* **27** (4), 796–807. https://doi.org/10.1523/JNEUROSCI.3501-06.2007.
31. L. Forny-Germano, N.M. Lyra e Silva, A.F. Batista, J. Brito-Moreira, M. Gralle, S.E. Boehnke, B.C. Coe, A. Lablans, S. A. Marques, A.M.B. Martinez, W.L. Klein, J.-C. Houzel, S. T. Ferreira, D.P. Munoz, F.G. De Felice, Alzheimer's disease-like pathology induced by amyloid-β oligomers in nonhuman primates, J. Neurosci.: Off. J. Soc. Neurosci. 34 (41) (2014) 13629–13643. doi:10.1523/JNEUROSCI.1353-14.2014.
32. Yasumoto, T., Takamura, Y., Tsuji, M., Watanabe-Nakayama, T., Imamura, K., Inoue, H., Nakamura, S., Inoue, T., Kimura, A., Yano, S., Nishijo, H., Kiuchi, Y., Teplow, D.B., Ono, K., (2019). High molecular weight amyloid β1-42 oligomers induce neurotoxicity via plasma membrane damage. *FASEB J.: Off. Publicat. Federation Am. Soc. Exp. Biol.* **33** (8), 9220–9234. https://doi.org/10.1096/fj.201900604R.
33. Tomiyama, T., Matsuyama, S., Iso, H., Umeda, T., Takuma, H., Ohnishi, K., Ishibashi, K., Teraoka, R., Sakama, N., Yamashita, T., Nishitsuji, K., Ito, K., Shimada, H., Lambert, M.P., Klein, W.L., Mori, H., (2010). A mouse model of amyloid beta oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. *J. Neurosci.: Off. J. Soc. Neurosci.* **30** (14), 4845–4856. https://doi.org/10.1523/JNEUROSCI.5825-09.2010.
34. Söderberg, L., Johannesson, M., Nygren, P., Laudon, H., Eriksson, F., Osswald, G., Möller, C., Lannfelt, L., (2023). Lecanemab, aducanumab, and gantenerumab - binding profiles to different forms of amyloid-beta might explain efficacy and side effects in clinical trials for Alzheimer's disease. *Neurotherapeut.: J. Am. Soc. Exp. NeuroTherapeut.* **20** (1), 195–206. https://doi.org/10.1007/s13311-022-01308-6.
35. Schützmann, M.P., Hasecke, F., Bachmann, S., Zielinski, M., Hänsch, S., Schröder, G.F., Zempel, H., Hoyer, W., (2021). Endo-lysosomal Aβ concentration and pH trigger formation of Aβ oligomers that potently induce Tau missorting. *Nat. Commun.* **12** (1), 4634. https://doi.org/10.1038/s41467-021-24900-4.
36. Hasecke, F., Niyangoda, C., Borjas, G., Pan, J., Matthews, G., Muschol, M., Hoyer, W., (2021). Protofibril-fibril interactions inhibit amyloid fibril assembly by obstructing secondary nucleation. *Angew. Chem.* **133** (6), 3053–3058. https://doi.org/10.1002/ange.202010098.
37. Linse, S., (2017). Monomer-dependent secondary nucleation in amyloid formation. *Biophys. Rev.* **9** (4), 329–338. https://doi.org/10.1007/s12551-017-0289-z.
38. Gaspar, R., Meisl, G., Buell, A.K., Young, L., Kaminski, C. F., Knowles, T.P.J., Sparr, E., Linse, S., (2017). Secondary nucleation of monomers on fibril surface dominates α -synuclein aggregation and provides autocatalytic amyloid amplification. *Q. Rev. Biophys.* **50**, e6. https://doi.org/10.1017/S0033583516000172.
39. Brody, D.L., Jiang, H., Wildburger, N., Esparza, T.J., (2017). Non-canonical soluble amyloid-beta aggregates and plaque buffering: controversies and future directions for target discovery in Alzheimer's disease. *Alzheimer's Res. Therapy* **9** (1), 62. https://doi.org/10.1186/s13195-017-0293-3.
40. Buell, A.K., Galvagnion, C., Gaspar, R., Sparr, E., Vendruscolo, M., Knowles, T.P.J., Linse, S., Dobson, C. M., (2014). Solution conditions determine the relative importance of nucleation and growth processes in α-synuclein aggregation. *Proc. Nat. Acad. Sci.* **111** (21), 7671–7676. https://doi.org/10.1073/pnas.1315346111.
41. Van Der Wateren, I.M., Knowles, T.P.J., Buell, A.K., Dobson, C.M., Galvagnion, C., (2018). C-terminal truncation of α-synuclein promotes amyloid fibril amplification at physiological pH. *Chem. Sci.* **9** (25), 5506–5516. https://doi.org/10.1039/C8SC01109E.
42. Schulz, C.M., Agerschou, E.D., Gardon, L., Alexander, M., Stoldt, M., Heise, H., Tamgüney, G., Hoyer, W., (2024). Disordered regions of inhibitor-bound α-synuclein suppress seed-induced fibril nucleation in cells. *Cell Rep. Phys. Sci.* **5** (9),102180. https://doi.org/10.1016/j.xcrp.2024.102180.
43. Giehm, L., Svergun, D.I., Otzen, D.E., Vestergaard, B., (2011). Low-resolution structure of a vesicle disrupting α-synuclein oligomer that accumulates during fibrillation. *Proc. Nat. Acad. Sci.* **108** (8), 3246–3251. https://doi.org/10.1073/pnas.1013225108.
44. Lorenzen, N., Nielsen, S.B., Buell, A.K., Kaspersen, J.D., Arosio, P., Vad, B.S., Paslawski, W., Christiansen, G., Valnickova-Hansen, Z., Andreasen, M., et al., (2014). The role of stable α-synuclein oligomers in the molecular events underlying amyloid formation. *J. Am. Chem. Soc.* **136** (10), 3859–3868. https://doi.org/10.1021/ja411577t.
45. Rösener, N.S., Gremer, L., Wördehoff, M.M., Kupreichyk, T., Etzkorn, M., Neudecker, P., Hoyer, W., (2020). Clustering of human prion protein and α-synuclein oligomers requires the prion protein n-terminus. *Commun. Biol.* **3** (1), 365.

46. Yang, X., Wang, B., Hoop, C.L., Williams, J.K., Baum, J., (2021). NMR unveils an N-terminal interaction interface on acetylated-α-synuclein monomers for recruitment to fibrils. *Proc. Nat. Acad. Sci.* **118** (18) https://doi.org/10.1073/pnas.2017452118. e2017452118.
47. Hoyer, W., Cherny, D., Subramaniam, V., Jovin, T.M., (2004). Impact of the acidic c-terminal region comprising amino acids 109–140 on α-synuclein aggregation in vitro. *Biochemistry* **43** (51), 16233–16242. https://doi.org/10.1021/bi048453u.
48. Thacker, D., Sanagavarapu, K., Frohm, B., Meisl, G., Knowles, T.P., Linse, S., (2020). The role of fibril structure and surface hydrophobicity in secondary nucleation of amyloid fibrils. *Proc. Nat. Acad. Sci.* **117** (41), 25272–25283. https://doi.org/10.1073/pnas.2002956117.
49. Agerschou, E.D., Schützmann, M.P., Reppert, N., Wördehoff, M.M., Shaykhalishahi, H., Buell, A.K., Hoyer, W., (2021). β-turn exchanges in the α-synuclein segment 44-tkeg-47 reveal high sequence fidelity requirements of amyloid fibril elongation. *Biophys. Chem.* **269**, 106519. https://doi.org/10.1016/j.bpc.2020.106519.
50. Wells, C., Brennan, S., Keon, M., Ooi, L., (2021). The role of amyloid oligomers in neurodegenerative pathologies. *Int. J. Biol. Macromol.* **181**, 582–604. https://doi.org/10.1016/j.ijbiomac.2021.03.113.
51. Andreasen, M., Lorenzen, N., Otzen, D., (2015). Interactions between misfolded protein oligomers and membranes: a central topic in neurodegenerative diseases?. *Biochim. et Biophys. Acta (BBA)-Biomembr.* **1848** (9), 1897–1907. https://doi.org/10.1016/j.bbamem.2015.01.018.
52. Agerschou, E.D., Flagmeier, P., Saridaki, T., Galvagnion, C., Komnig, D., Heid, L., Prasad, V., Shaykhalishahi, H., Willbold, D., Dobson, C.M., et al., (2019). An engineered monomer binding-protein for α-synuclein efficiently inhibits the proliferation of amyloid fibrils. *Elife* **8**, e46112. https://doi.org/10.7554/eLife.46112.