Structural basis for the dissociation of α-synuclein fibrils triggered by pressure perturbation of the hydrophobic core

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Parkinson’s disease is a neurological disease in which aggregated forms of the α-synuclein (α-syn) protein are found. We used high hydrostatic pressure (HHP) coupled with NMR spectroscopy to study the dissociation of α-syn fibril into monomers and evaluate their structural and dynamic properties. Different dynamic properties in the non-amyloid-β component (NAC), which constitutes the Greek-key hydrophobic core, and in the acidic C-terminal region of the protein were identified by HHP NMR spectroscopy. In addition, solid-state NMR revealed subtle differences in the HHP-disturbed fibril core, providing clues to how these species contribute to seeding α-syn aggregation. These findings show how pressure can populate so far undetected α-syn species, and they lay out a roadmap for fibril dissociation via pathways not previously observed using other approaches. Pressure perturbs the cavity-prone hydrophobic core of the fibrils by pushing water inward, thereby inducing the dissociation into monomers. Our study offers the molecular details of how hydrophobic interaction and the formation of water-excluded cavities jointly contribute to the assembly and stabilization of the fibrils. Understanding the molecular forces behind the formation of pathogenic fibrils uncovered by pressure perturbation will aid in the development of new therapeutics against Parkinson’s disease.

More than a century has passed since F.H. Lewy (1912) described the intracellular inclusion bodies of PD, but only in the 1990s was the aggregated form of the α-synuclein (α-syn) protein shown to be part of the Lewy bodies (LBs) and neurites1. The decreased levels of dopamine in the substantia nigra pars compacta result in the characteristic motor symptoms of this neurodegenerative disorder. α-Syn aggregation is not linked exclusively to the most common form of PD but also to dementia with Lewy bodies, pure autonomic failure, multiple system atrophy (MSA), and autosomal dominant PD, in which missense mutations (A30P, A53T and E46K) are present2,3. In addition, α-syn gene multiplication and single-nucleotide polymorphisms are associated with several neurological syndromes and increased PD risk, respectively4–6.

Despite its link to neurodegeneration1,2, the physiological role of α-syn has not been fully explored. The 140-residue protein is localized either in the soluble form or bound to membranes in presynaptic nerve terminals (PNTs)7, and participates in the regulatory pathways of synaptic vesicle release and trafficking, modification of neurotransmitter release, neuronal survival, and plasticity8–10. Biochemical and biophysical studies have shown its intrinsically disordered behavior11 not only in vitro but also in neuronal cells12, although a tetrameric form has been described in E. coli (recombinant form), neurons, and red blood cells13,14. The protein has three regions: an N-terminal amphipathic segment that binds to lipids and forms α-helices15,16 a non-amyloid-β component

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(NAC), and an acidic C-terminus. Recent investigations have shown that in addition to the N-terminal region, the NAC and C-terminus also participate in the membrane binding process. The membrane-bound α-syn may also trigger protein aggregation and seeding of the cytosolic form, which might explain some of the features observed in PD.

There is a link between α-syn oligomerization and fibrillation and the cytopathological and neuropathological features of PD brains, which in turn are linked to the clinical symptoms of PD. Current efforts to ameliorate the devastating symptoms include stabilization of the monomeric form and blockage of the toxic supramolecular oligomers and fibrils. High hydrostatic pressure (HHP) has become a strong physicochemical strategy for understanding the assembly of supramolecular structures such as amyloids. Pressure mainly exerts its effects by promoting water infiltration into water-excluded cavities in the folded and assembled states. However, there have been few studies exploring the use of HHP to investigate the molecular mechanisms underlying fibril disassembly and the conversion to species that might act as seeds for disease propagation and transmission. There is substantial evidence supporting that PD is a prion-like disorder but the species that contribute to its seeding behavior have yet to be defined. For example, it is unclear why α-syn derived from patients with MSA exhibits a prion-like transmission character, whereas α-syn from Parkinson’s patients does not. The existence of packing defects in the α-syn fibril core presents a valuable model for exploring the enrichment of potential intermediate species for fibrillogenesis and might shed light on new mechanisms interfering with fibril formation. The recently published structure of a toxic α-syn fibril reveals a structural topology consisting of a Greek-key motif with multiple β-strands intercalated with steric zippers, generating a compact, hydrophobic core that may explain why these fibrils are sensitive to pressure. The folding of proteins into globular or fibrillar states is based on the formation of water-excluded cavities that can be perturbed by pressure.

In this work, we identified the mechanism through which pressure triggers α-syn amyloid fibril dissociation from the monomeric form. We provide molecular evidence of how hydrophobic interaction and the formation of water-excluded cavities jointly contribute to the assembly and stabilization of the fibrils. The high-pressure NMR and osmolyte data reveal that pressure pushes water into the hydrophobic core of the fibril, releasing monomers with altered dynamics. We investigated the structural and dynamic properties of these monomers dissociated from HHP-disturbed fibrils and the remaining fibrillar species at the atomic level, and examined how these species might seed amyloid fibril formation.

**Results**

*Initial characterization of α-syn monomer and fibrils for HHP-NMR analysis.* First, the production of α-syn monomers and fibrils for HHP-NMR was characterized by a combination of spectroscopic and biochemical approaches. Although SDS-PAGE of monomers showed a size of ~17 kD, size exclusion chromatography (SEC) revealed a high-purity α-syn in the retention volume close to the value observed for the 44 kD chicken ovalbumin molecular standard (Supplementary Fig. 1a and inset), confirming the same behavior as previously observed by SEC. Next, NMR assignment of the α-syn monomer was completed (Fig. 1a and Supplementary Fig. 2). The amyloid nature of the fibrils was observed through the increased...
quantum yield of Thioflavin T (ThT), x-ray scattering, and bis-ANS solvent-exposed hydrophobic surface area (Supplementary Fig. 1b–d). In addition, the increased \( \beta \)-sheet secondary structure of fibrils is shown by circular dichroism as a negative peak at approximately 220 nm, in contrast to the random conformation of monomers with a negative peak at approximately 200 nm (Supplementary Fig. 1e).

To rule out the contribution of residual monomeric or oligomeric \( \alpha \)-syn species to the observed effect of increased pressure on fibrils, we excluded any remaining species from the fibril samples subjected to HHP-NMR studies (Fig. 1b,c). Fibrils were confirmed by transmission electron microscopy (Supplementary Fig. 1f) and subjected to a washing/centrifugation (w/c) protocol (see Methods) to remove undesired species without significantly affecting the \( \alpha \)-syn fibrils in solution. Only at the eighth wash was there an apparent decrease in fibril concentration (Supplementary Fig. 3a). Following each washing, the oligomeric species washed away from fibril samples were indirectly assessed by dot-blot for an oligomer-sensitive antibody\(^{35,36}\). After eight washings, a very weak signal was observed (Fig. 1b,c). The exclusion of monomers after the w/c steps was confirmed by the \( ^{1}H-^{15}N \) HSQC spectra of fibrils at 1 bar, in which no signals were observed at the same threshold level as the monomer (Supplementary Fig. 3b). Thus, we established an initial condition for sample preparation in which \( \alpha \)-syn fibrils are the predominant species sensing HHP effects when observed by NMR.

**Species released from \( \alpha \)-syn fibrils upon HHP.** We used HHP to obtain insights on the species released from \( \alpha \)-syn fibrils. Washed fibrils subjected to increasing HHP revealed a major population of monomeric species as evaluated by size exclusion, but no oligomeric species were detected (Fig. 2a). Furthermore, the ThT fluorescence of fibrils after 516 and 1,033 bar treatment revealed an abrupt signal decrease and, by circular dichroism, a major shift from the \( \beta \)-sheet signal to the random conformation expected for monomeric \( \alpha \)-syn (Fig. 2b,c).

To better understand the disassembly of fibrils at 1,033 and 2,067 bar, we recorded the ThT kinetics at these pressures after ThT signal stabilization at 1 bar (i.e., sedimented fibrils; Fig. 2d,e and Supplementary Fig. 3c,d).

The data show that fibril disassembly is pressure dependent and a complex process, especially at 1,033 bar, where two decaying profiles are observed throughout the kinetics (Fig. 2d). To determine further details of the effects of pressure on the fibril's secondary structure, we measured the real-time pressure dependence by Fourier transform infrared spectroscopy (FTIR) and circular dichroism (Fig. 2f). HHP-CD measurements at 516 bar over time revealed a continuous decrease of the \( \beta \)-sheet signal at 220 nm, consistent with the process of fibrillization. \( ^{1}H/^{1}H \) fibrils were used to follow amide I changes by HHP-FTIR. We observed a systematic change in time revealed a continuous decrease of the \( \beta \)-sheet signal at 220 nm, consistent with the process of fibrillization. \( ^{1}H/^{1}H \) fibrils were used to follow amide I changes by HHP-FTIR. We observed a systematic change in the amidic band intensity (Fig. 2f). To determine further details of the effects of pressure on the fibril's secondary structure, we measured the real-time pressure dependence by Fourier transform infrared spectroscopy (FTIR) and circular dichroism (Fig. 2f). HHP-CD measurements at 516 bar over time revealed a continuous decrease of the \( \beta \)-sheet signal at 220 nm, consistent with the process of fibrillization. \( ^{1}H/^{1}H \) fibrils were used to follow amide I changes by HHP-FTIR. We observed a systematic change in time revealed a continuous decrease of the \( \beta \)-sheet signal at 220 nm, consistent with the process of fibrillization. \( ^{1}H/^{1}H \) fibrils were used to follow amide I changes by HHP-FTIR. We observed a systematic change in

Further evaluation of small-angle x-ray scattering profiles from fibrils after HHP increments has shown a lower scattering intensity as a function of the scattering vector \( s \) (Fig. 3a) and Kratky plots after 1 bar/2,067 bar with increased values of \( s^2 \) (vs. \( s \) (Kratky-plot)), which is consistent with species adopting increased flexibility due to fibril disassembly (Fig. 3b). The scattering of monomers at different concentrations (Supplementary Fig. 5a–e) revealed similar scattering patterns and Kratky plots as compared to the results obtained from fibrils after HHP treatment at 2,067 bar. To observe the frequency distribution of the species dissociated from fibrils, we performed an ensemble optimization method (EOM) on the scattering data of monomers not subjected to pressure and of species obtained from HHP-disturbed fibrils (Fig. 3c–f and Supplementary Fig. 5d,e). We observed that the species dissociated from fibrils presented a narrower population enriched in conformers of approximately 45–50 Å in radius of gyration, \( R_g \), and 140–150 Å in size distribution (Fig. 3c–f), revealing that the ensemble of species released from fibrils are not the same as the species in the absence of HHP treatment and are not uniformly distributed throughout the conformational space.

\( \alpha \)-Syn fibril dissociation into monomers, as monitored by HHP-NMR. \( \alpha \)-Syn fibrils in solution. Only at the eighth wash was there an apparent decrease in fibril concentration (Supplementary Fig. 3a). Following each washing, the oligomeric species washed away from fibril samples were indirectly assessed by dot-blot for an oligomer-sensitive antibody\(^{35,36}\). After eight washings, a very weak signal was observed (Fig. 1b,c). The exclusion of monomers after the w/c steps was confirmed by the \( ^{1}H-^{15}N \) HSQC spectra of fibrils at 1 bar, in which no signals were observed at the same threshold level as the monomer (Supplementary Fig. 3b). Thus, we established an initial condition for sample preparation in which \( \alpha \)-syn fibrils are the predominant species sensing HHP effects when observed by NMR.

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consistent with α-syn monomers and assigned at a specific pressure value, and compared to the initial monomer assignment at the same pressure. Among the 100 correlations assigned for initial monomers at 1 bar, we were able to assign for the dissociated species from fibrils, 40 correlations at 500 bar, 55 at 1,000 bar, 59 at 1,500 bar, 34 at 2,500 bar, and 93 upon pressure release (Fig. 4b). The intensities from each peak in the 1H–15N HSQC spectra relative to the values at 1 bar for monomers that were not incorporated into the fibrils (Fig. 4c, black lines) and to the values of 500 bar for dissociated species released from the α-syn fibrils (Fig. 4c, blue lines) were plotted as a function of pressure increase. This plot revealed a different behavior of the monomeric sample compared...
to monomeric species released from fibrils upon HHP treatment. While there were no changes in the relative intensities (line-broadening effects) for the initial monomers, the monomers released from fibrils exhibited a bell-shaped behavior (26 studied correlations) and were fitted to a second order equation that reflects conformational exchange motions \(^37,38\). The second-order coefficients (\(b_2\)) of 7 correlations in the NAC region and 14 correlations in the acidic C-terminus were monitored as a function of residue number and revealed significant negative values consistent with conformational exchange motions in these regions (Fig. 4d). These results suggest that the monomeric species released from \(\alpha\)-syn fibrils are structure-modified monomers (SMMs) presenting dynamic behavior in the conformational exchange regime. Interestingly, part of the mapped region belongs to the hydrophobic core of the fibril that is stabilized by water-excluded cavities and a salt bridge, with steric zippers forming the dry interface \(^31\). Water-excluded cavities and salt bridges are highly sensitive to pressure \(^20,32\).

For an in-depth exploration of the dynamic behavior of the SMMs released from \(\alpha\)-syn fibrils, we evaluated the pressure dependence of these species and compared them with free monomers by performing \(^1\)H CPMG relaxometry experiments. We found that they do indeed have a different morphology, as detected by negatively stained transmission electron microscopy images.

**HHP effects on fibril structure.** To evaluate if remaining fibrils adopt a different structure after mild pressure challenges we designed electron microscopy and solid-state NMR experiments. We found that they do not adopt a different structure after mild pressure challenges we designed electron microscopy and solid-state NMR experiments. We found that they do indeed have a different morphology, as detected by negatively stained transmission electron microscopy images.
(Fig. 6c). After pressure treatment, fibrils seem to get thicker and form clusters, probably due to pressurization effects on fibril dissociation. Of note, pre-dissociated and pre-denatured states have also been found in small proteins\(^{20,39}\).

To test whether the core of the \(\alpha\)-syn fibrils was affected (before disassembly), a solid-state NMR approach was applied to assess the structural differences between fibrils before and after HHP treatment at 1,033 bar. Because \(\alpha\)-syn includes ten threonine residues, and nine belong to the fibrillar core\(^{31,40,41}\), the effects of HHP on the fibril structure were probed by the difference in threonine chemical shifts. Based on the \(^{13}\)C–\(^{13}\)C correlation spectra, it was possible to verify a slight difference in the threonine chemical shifts before and after HHP treatment, suggesting small changes in the remaining fibrillar population (Fig. 6d). To confirm our results, a second batch of fibrils was prepared, corroborating the result obtained for the first batch (Supplementary Fig. 8). For a more in-depth evaluation of fibril changes under pressure, it would be important to observe the linewidth of threonine peaks. In such a case, a less polymorphic sample should be used to provide narrow lines.

**Seeding mechanism of HHP-disturbed fibrils.** Following the identification and characterization of the main species released from fibrils and the remaining fibrils after 1,033 bar treatment, we examined the effect of these species on seeding the aggregation of \(\alpha\)-syn monomers. By recording the fluorescence intensity of ThT as a function of time, we followed the aggregation of \(\alpha\)-syn when it was incubated with different seeds (Fig. 7 and Supplementary Figs 9a–d and 10a–h). Because the conditions used to produce \(\alpha\)-syn seeds affect the final concentration of fibrils, we first developed a reliable method to determine fibril concentrations after seed production (Supplementary Fig. 9a). As expected, the sonication protocol did not affect the fibril concentration compared to non-treated fibrils. In contrast, seeds produced by HHP at 1,033 and 2,067 bar resulted in an average reduction of ca. 34% and 85% compared to non-treated fibrils, respectively, as determined by \(A_{280}\) nm. Our data show that the remaining species (Rm-F) formed at 1,033 bar (meaning SMMs + Rm-F) were able to seed \(\alpha\)-syn aggregation, but less efficiently than seeds formed after fibril sonication, as revealed by the \(t_1/2\) values (Fig. 7a,b and Supplementary Fig. 9b). The species remaining after 2,067 bar treatment (SMMs + Rm-F) and non-treated fibrils have a modest effect on seeding only compared to the species formed after the 1,033 bar treatment (Supplementary Fig. 9b–d). Of note, we did not observe changes in the slope values of transitions when
comparing increasing amounts of seeds from remaining fibrils after 1,033 bar treatment, in contrast to the values obtained for sonicated fibrils (Supplementary Fig. 10i–l). Altogether, we show that different seeding mechanisms may contribute to the balance between the primary nucleation and elongation phases.

The mechanism of fibril disassembly triggered by pressure needs water. To better understand the mechanism under which pressure leads to fibril disassembly we used the recent solid-state NMR structure of the \( \alpha \)-syn fibril and a Monte Carlo algorithm to explore the existence of non-exposed cavities (Fig. 8a–c). Interestingly, the side chains of the intermolecular salt-bridge pair E46-K80 form a non-exposed cavity along the perpendicular axis of the fibril, as clearly visualized in Fig. 8. Furthermore, the cavity-prone hydrophobic core formed by the side chains of Q79, V82, and A89 accommodates two additional cavities immediately inward from the Greek-key topology that might be facilitated by the presence of several small residues in the turn segments (Fig. 8a–c). The locking mechanism of the \( \alpha \)-syn fibril core consisting of the charges of the E46-K80 pair and the corresponding non-exposed cavity formed by these residues would likely make it a vulnerable site into which water molecules get pushed and ultimately hydrate the cavity-prone hydrophobic core (Fig. 8). To assess the relevance of water on fibril disassembly we have used glycerol. Because this osmolyte preferentially excludes water molecules from around the fibril assembly and also from the immediate vicinity of the dissociated species, we expected the pressure-induced release of monomers to be progressively less effective as the concentration of glycerol increased. The measurement of light scattering as a function of pressure increments revealed that fibrils are less affected as increasing glycerol concentrations is used, confirming the important role of water molecules on fibril disassembly (Fig. 8d). The three aligned non-exposed cavities may explain the peeling of monomeric species from the fibril at mild pressure due to water penetration (Figs 6c,d and 8a–d).

Discussion

We demonstrate that the major ensemble of species released from HHP-disturbed fibrils are monomers that present a different dynamic behavior at the NAC and acidic C-terminal regions. Moreover, the remaining fibrils are slightly different at the level of the core region. Interestingly, while we do not detect appreciable amounts of oligomers upon dissociation of \( \alpha \)-syn, we do detect slightly different fibrils as pre-dissociated species. The presence of key elements including a salt-bridge and water-excluded cavities along the perpendicular axis of the dried hydrophobic core explains its susceptibility to pressure (Fig. 8a–c).

It has been shown that the aggressive variants A30P and A53T are more susceptible to pressure than the wild-type as monitored by light scattering measurements23. This finding reveals the existence of different packing defects and hydrophobic pockets in the fibril core24, rendering \( \alpha \)-syn fibrils a valuable model for investigating HHP-induced intermediates and unveiling fibrillation pathways. Furthermore, it has been shown that HHP strongly reduces the toxicity levels of A30P protofibrils in mesencephalic and cortical neurons by breaking them into smaller aggregates24. Finally, a recent study has shown that in neuronal cells challenged by HHP, \( \alpha \)-syn loses contact with its PLC\( \beta \)-1 partner, thereby triggering \( \alpha \)-syn protein aggregation inside the cell22. These HHP studies
on α-syn provide a wide range of HHP applications for improving our understanding of aggregation pathways, not only mechanistically but also functionally. Nevertheless, the molecular mechanism by which HHP triggers the dissociation of α-syn fibrils has never been addressed at the atomic level, limiting current interpretations to the field of bioimaging and biochemical approaches. Here, we explored the mechanism by which pressure dissociates α-syn fibrils, and add new information regarding the presence of so far invisible and dynamically modified monomeric species released from fibrils (Figs 2 and 4) and the remaining slightly modified fibrils (Fig. 6). Indeed, the hydration of exposed nonpolar residues, the presence of non-exposed cavities, and the electrostriction of exposed charges are key elements in explaining protein unfolding triggered by pressure20,32–34. The recent ssNMR structure of the Greek-key α-syn fibril provided the framework for understanding fibril stability31 and insights into how pressure would affect its core (Fig. 8e).

Although appreciable studies have shown the disaggregation of different fibrils triggered by osmotic and chemical agents43,44 the challenges of identifying and characterizing these transient and heterogeneous intermediate species remain notoriously difficult. Using single-molecule strategies and near-physiological conditions, α-syn fibrils were shown to disaggregate into monomers as well as oligomeric species, the latter dissociating into monomers after longer incubation times45. Here, the combination of pressure-based approaches not only provided insights into the mechanism of α-syn fibril disassembly but also yielded structural information on the dynamic properties of these intermediate monomeric species and the remaining fibrils formed after compression.

Several studies support that PD exhibits prion-like behavior25–30 but there is still no clear evidence regarding what species contribute to the mechanism of seeding and cell-to-cell spreading. Both monomeric and aggregated

Figure 6. HHP effects on fibril structure. (a) Solid-state NMR structure of full-length α-syn monomer (PDB: 2n0a) highlighting the N- and C-terminus (blue) and the Greek-key arrangement of the core (green). (b) Solid-state NMR structure of the α-syn fibril core (residues 46–96, PDB: 2n0a) showing the key elements for fibril stability: the salt-bridge between E46-K80 (gray), steric zippers involving V49, V77 and V82 (cyan), the glutamine ladder Q79 (purple) and the hydrophobic packing involving I88, A91 and F94 (orange). Threonine positions (yellow) are highlighted to show the effects of HHP on the α-syn fibril core. (c) Negatively stained transmission electron microscopy of fibrils before and after 1 h treatment at 1,033 bar. (d) 13C–13C correlation spectrum of the α-syn fibril core acquired in a static magnetic field of 600 MHz before (blue) and after (red) 1 h at 1,033 bar. Dashed lines show the changes.
forms of α-syn have been previously detected in the cerebrospinal fluid and plasma in humans and found to be secreted by rat primary cortical neurons in our study, we show that the fibril-to-monomer transition occurs upon HHP challenge without appreciable amounts of oligomers, and that the dynamic properties of these SMMs differ from those of the initial α-syn monomers.

Considering that cells take up all three species of α-syn (monomers, oligomers, and fibrils) from the extracellular space in our study, we show that the fibril-to-monomer transition occurs upon HHP challenge without appreciable amounts of oligomers, and that the dynamic properties of these SMMs differ from those of the initial α-syn monomers.

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properties to non-treated fibrils, they exhibit different morphologies and slightly different core structures, providing an opportunity for new platform-based studies for combating PD.

The use of seeds accelerates fibrillar aggregation reactions through elongation and surface-catalyzed secondary nucleation and decreases the lag phase, as observed in our seeding experiments (Fig. 7). In contrast to what has been shown for the aggregation of Aβ42, in which the association of primary and secondary events occurs by the growth of oligomeric nuclei and fibrils, for α-syn, the amyloid fibril growth occurs through monomer but not oligomer addition59. The dissociation mechanism triggered by pressure points to the reverse of this assembly reaction. At this point in the study, the lacking information is whether these SMMs populated by HHP are trapped species in the amyloidogenic pathway of α-syn or may participate in the de novo formation of higher-order oligomers and fibrils. By FTIR, we observed the recovery of the α-sheet signal of these monomers released from HHP-disturbed fibrils at physiological temperature (Fig. 2g).

Using preformed α-syn fibrils produced by sonication as seeds, we show that in contrast to the observations of these sonicated fibrils, in which secondary nucleation dominates the elongation rate (as revealed by the increasing slopes at the transition points, Fig. 7a and Supplementary Fig. 10i–l), the seeds of remaining fibrils populated by HHP and non-treated fibrils do not exert any influence on the elongation phase of aggregation (equal slopes, Fig. 7b and Supplementary Fig. 10i–l). Therefore, these remaining fibrils may participate in seeding through primary nucleation processes such as providing hydrophobic-prone surfaces for fibril growth, previously reported as a requirement for α-syn fibrillation51.

In conclusion, we provide biochemical and structural data on the mechanism of how pressure affects the cavity-prone hydrophobic core of α-syn fibrils and leads to fibril dissociation of dynamic monomeric species (Fig. 8e), which were heretofore not detected by conventional techniques. HHP should be considered as a potential tool for developing a new generation of target species and for drug screening studies based on the dynamic properties of intermediate structures uncovered. Intermediate species of amyloidogenic pathways have already been observed in the case of transthyretin (TTR), which is involved in senile systemic amyloidosis and familial amyloidotic polyneuropathy52. Future therapeutics focused on the blockage of de novo aggregation and seeding and the development of new biomarkers for early diagnosis may represent an effective strategy to combat PD.

**Methods**

**Preparation of monomeric α-syn and fibrils.** Recombinant monomers were produced based on the previous protocol53. For protein purification, cells were harvested from 0.8 L culture and resuspended in 30 mL of 20 mM Tris-Cl (pH 8.0) containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, homogenized for 1 to 3 sec in an automated homogenizer (Novatecnica) and lysed by ultrasonication in a Vibra cell machine for 20-sec intervals over 30 min at 300 W (Sonics&Materials, Inc.). The crude extract was then subjected to osmotic shock by decreasing the pH to 3.5 using 1 M HCl and centrifuged at 16,000 g for 10 min.

**Preparation of α-syn fibrils**. Fibrillation reactions were performed using at least two different protein batches to a final volume of 1 mL in 20 mM Tris-Cl (pH 7.4) 100 mM NaCl and centrifuged at 20,000 g, 10 min, 4 °C. The supernatant was neutralized (pH 7.5) using 1M NaOH followed by α-syn precipitation with 50% v/v (NH4)2SO4 at 10 °C. After centrifugation (16,000 g for 20 min, 4 °C), the pellet was resuspended in ca. 30–40 mL of 20 mM Tris-Cl (pH 8.0) containing 1 mM EDTA and dialyzed overnight (ca. 12–16 h) against 2 L of the same buffer followed by two changes with MilliQ water (2 L for each change, same duration). Different aliquots from 8–10 mg (ca. 3–5 mL) of the dialyzed protein were flash frozen in liquid nitrogen and lyophilized for 36–48 h in a Flexi Dry unit (FTS Systems, #FD-1-84A) coupled with a vacuum pump. Lyophilized protein batches were stored at −20 °C and used after an interval no longer than one month. For 15N- or 13C-labelled samples, the protein was produced as described previously54 and purified as above.

For fibril preparation, one lyophilized aliquot (ca. 8–10 mg) was resuspended in 1 mL of 10 mM Tris-Cl (pH 7.4) 100 mM NaCl and centrifuged at 20,000 g, 10 min, 4 °C. The supernatant was then injected four times (250 μL per injection at a flow rate of 0.7 mL min⁻¹) into a Superdex 75 10/300 column previously equilibrated in the same buffer (Supplementary Fig. 1a) using a Ultra Fast Liquid Chromatograph (Shimadzu). At this step, SEC was chosen to obtain α-syn at higher purity (>99%) (Supplementary Fig. 1a, inset). The concentration of monomeric α-syn was estimated by absorbance at 280 nm (A280nm) using a molar extinction coefficient of 5,960 M⁻¹ cm⁻¹. Fibrillation reactions were performed using at least two different protein batches to a final volume of 1 mL in 2 mL low-protein binding tubes (Eppendorf) using 140 μM of α-syn obtained immediately after SEC. Samples were incubated at 37 °C and with continuous shaking at 600 rpm using a Thermomixer comfort (Eppendorf) for 7–10 days.

**Washing protocol of α-syn fibrils.** Fibrils were submitted to a washing/centrifugation protocol to exclude remaining species (α-syn monomers and oligomers) from the fibril solutions. Fibrils were washed (only by swirling the solution) eight times with 1 mL of 10 mM Tris-Cl, 100 mM NaCl pH 7.4 using 15 mL conical tubes and a swing bucket rotor at low velocity, 3,000 g, 10 min, 4 °C. This velocity was chosen because no significant changes were observed in the dissociation rate of α-syn fibrils measured at each washing step based on A280nm of an aliquot of resuspended fibrils dissolved in 5 M guanidinium chloride (Supplementary Fig. 3a). The α-syn fibril concentration used in the experiments was estimated by measuring A280nm from the monomeric fraction released from the fibril after treatment with 5 M guanidinium chloride (i.e., the monomeric α-syn concentration present in the fibril), and used to estimate the concentrations of seeding conditions (Supplementary Fig. 9a).

**Size exclusion chromatography in ultra-fast liquid chromatography (UFLC).** Size exclusion chromatography (SEC) for the α-syn species formed after HHP treatment were performed using two different columns: a GPC-250 (Agilent) and a Superdex 200 10/300 (GE Life science). Equal amounts of washed fibrils (87 μM) were subjected to 1, 516, 1,033, and 2,067 bar for 1 h, followed by centrifugation at 20,000 g, 10 min, 4 °C and injection of the supernatants. All UFLC runs were performed in 10 mM Tris-Cl (pH 7.4), 100 mM NaCl at a flow rate of 0.7 mL min⁻¹, and the absorbance was monitored at 214 nm. The elution volumes from each species were
always compared with a molecular weight protein standard (Biorad, #151-1901). Both columns presented similar elution profiles with no detectable amounts of oligomeric species in the studied concentration range.

**Routine transmission electron microscopy (TEM).** TEM images were obtained after 7 days. Samples 3 µL in volume were applied for 3 min to previously discharged carbon film on 200 mesh cooper grids (EMS, #CF200-Cu), gently dried with filter paper and stained for 1 min with 5% uranyl acetate. Negatively stained samples were visualized on a Zeiss 902 microscope operated at 80 kV with different magnifications (30,000 ×, 50,000 ×, 80,000 ×).

**Dot-Blots (DBs).** DBs probed with anti-α-syn (Invitrogen, #AHB0261, batch 1031405c) and A-11 (Millipore EMD, #AB9234, batch 2387440) antibodies were used during the w/c steps of α-syn fibrils to evaluate the rate of oligomeric species washed away from fibril samples. The A-11 antibody has been reported to recognize oligomeric species of different amyloidogenic proteins, including α-syn.

**Far-UV CD and HHP-CD.** Far-UV CD measurements were performed on a Jasco J-715 UV/Vis spectropolarimeter at 25 °C. The set-up for spectrum acquisition was scanning from 190–260 nm, resulting in an average of three spectra with a scanning speed of 50 nm min−1 and 0.2 nm data steps. Data are shown as raw ellipticity in millidegrees (mdeg) and were acquired using a 0.01-cm path length circular cuvette. The α-Syn fibrils were incubated under different HHP conditions (1 h at 516 or 1,033 bar or 10 h at 516 bar) and analyzed after returning to atmospheric pressure. Fibrils at ca. 45–60 μM and monomers at 55 μM were incubated with 10 μM ThT prepared in water or 25 μM bis-ANS for 10 min at 25 °C prior to taking spectra.

**Fluorescence spectroscopy (FS) and HHP-FS.** The fluorescent probes used to measure the α-syn monomers and fibrils were Thioflavin T (ThT) and 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS). Measurements were performed at 25 °C on an ISSK2 spectrofluorometer (ISS, Inc.) equipped with a high-pressure cell (ISS, Inc.). Bis-ANS and ThT binding to α-syn fibrils were monitored by exciting samples at 360 and 450 nm, respectively, and recording the emission spectra at 400–600 nm (for bis-ANS) and 460–540 nm (for ThT) at 1 bar. For ThT binding, samples were incubated for 1 h at 516 and 1,033 bar before spectrum acquisition at atmospheric pressure. Fibrils at ca. 45–60 μM and monomers at 55 μM were incubated with 10 μM ThT in water or 25 μM bis-ANS for 10 min at 25 °C prior to taking spectra.

**HHP-FTIR.** The infrared spectra of α-syn fibrils were recorded and analyzed between 1,660 and 1,550 cm−1, corresponding to the amide I band region of 1,700–1,600 cm−1. Due to the 13C and 15N isotope labeling, the spectra are shifted 45–50 cm−1 to lower wavenumbers. The amide I band is essentially associated with C=O and C–N stretching vibrations, plus C–C–N deformation vibrations of amino-acids. The pressure-dependent FTIR data were recorded using a Nicolet Magna 550 spectrometer, equipped with a liquid-nitrogen-cooled MCT detector. Each FTIR spectrum was obtained by recording 256 scans at a spectral resolution of 2 cm−1. Due to the 13C and 15N isotope labeling, the infrared light was focused onto the pinhole of a gas-membrane-driven diamond anvil cell with a brass spacer (Diacell® VivoDAC, Almaseasy Lab). Fine BaSO4 powder was used as an internal pressure calibrant. The sample concentration was 30 mg mL−1 in 6 μL of pure D2O (no washing steps for this experiment), and using this volume in the DAC cell yielded a sufficiently high signal-to-noise ratio. An external water thermostat served as a temperature control to maintain a temperature of 25 °C. After each pressure change, the sample was equilibrated for 5 min before collection of the IR spectrum. Spectral evaluation was performed using the Thermo GRAMS software as described elsewhere.

Experimental data were fitted using the Voigt function in OriginPro 9.0 G software. Only the bands present in both 2nd derivative and Fourier self-deconvolution (FSD) were used. Peaks were allowed to move ± 2 cm−1 and peak widths were limited to 20 cm−1. Seven bands were analyzed: 1571, 1580, 1588, 1602, 1612, 1624 and, 1638 cm−1. The 1612 band is attributed to α-helix; 1580, 1588 and, 1638 to β-sheet; 1602 to random coils; and 1624 to turns and bends. The 1571 band is attributed to amino-acid side chain vibrations and therefore was omitted from the final plot. However, it was used during the peak-fitting analysis as a baseline correction band.

**Small-angle scattering data collection and analysis.** Small angle X-ray scattering (SAXS) studies were performed using the scattering beamline of the National Synchrotron Light Laboratory (LNLS, Campinas,
Brazil). We used a 300 K Pilatus detector, 84 mm × 107 mm (Dectris), a mica sample cell holder for liquids at 25 °C, and a wavelength of 1.55 Å. The direct beam position in the detector was calibrated using silver behenate\(^2\). The sample-detector distance was set to 1,537.945 mm, enabling detection over the s range of 0.01–0.2 Å\(^{-1}\). X-ray photons were scattered from α-syn monomers at different concentrations (140, 500, and 915 μM) or from fibrils after 1 h treatment at 1, 516, 1,033, or 2,067 bars. At each pressure increment, the sample was left for 20 min for accommodation before spectrum acquisition. The NMR assignment of α-syn monomers and fibrils was acquired using a zirconia NMR tube with an internal diameter of 3 mm and an outer diameter of 5 mm (Daedalus Innovations). An Xtreme-60 syringe pump system (Daedalus Innovations) was used to generate pressure increments from 1 to 250, 500, 750, 1,000, 1,250, 1,500, 1,750, 2,250, and 2,500 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift increments (500, 1,000, and 2,067) and 1 bar.

Ensemble optimization method (EOM). For polydisperse or flexible systems, each conformer makes a scattering contribution to the final scattering profile. The average scattering intensity \( I(s) \) is represented by equation (1):

\[
I(s) = \sum_{k=1}^{K} v_k I_k(s)
\]

(1)

where \( v_k \) and \( I_k(s) \) are the volume and the scattering intensity, respectively, from the \( k \)-th conformers. For very flexible or intrinsically disordered proteins such as α-syn, the deconvolution of scattering data cannot be clearly rationalized due to the large number of solutions, and an ensemble approach is thus the method of choice to interpret scattering data\(^6\). Based on the input of the amino-acid composition, EOM (i) generates a large pool of disordered conformers (pool = 10,000) that approximate the conformational space, (ii) computes the theoretical scattering profiles for each conformer using the CRYOSOL suite\(^2\) and (iii) using a Genetic algorithm (GA) implemented in the GAJOE suite, selects the subset of conformers that best fits the collected experimental data and minimizes the discrepancies (\( \chi^2 \)) as follows by equation (2):

\[
\chi^2 = \frac{1}{K-1} \sum_{j=1}^{K} \left( \mu I(s_j) - I \exp(\sigma(s_j)) \right)^2
\]

(2)

where \( \exp(s) \) is the experimental scattering, \( K \) is the number of experimental points, \( \sigma(s) \) are the standard deviations, and \( \mu \) is a scaling factor\(^6\). Scattered samples of α-syn monomers and α-syn species released from fibrils after HHP treatment (2,067 bar) were subjected to EOM analysis. Three GA trials were taken, and very low s points (judged by Guinier plots) representing less than 5–8% of the whole scattering curve were depleted. The \( R_g \) and \( D_{max} \) distributions for each condition are expressed as avg. ± s.d. from the three GA trials.

NMR assignment of α-syn monomers and HHP-NMR spectroscopy. For assignment, heteronuclear \(^1\)H-\(^1\)N HSQC NMR spectra were acquired at 288 K using a Bruker Avance III 800-MHz spectrometer. We used α-syn monomers at 600 μM in 10 mM Tris-Cl (pH 7.4), 100 mM NaCl containing 10% D2O. \(^1\)H chemical shifts were referenced to the signal of DSS (diluted to 1 mM in the sample), and \(^1\)H chemical shifts were indirectly referenced to DSS using a scaling factor of 0.104 obtained from the \(^1\)H, \(^1\)H gyromagnetic ratio (\( \gamma_H/\gamma_N \)). The numbers of increment points used were 1,024 for the \(^1\)H dimension and 702 for the \(^1\)N dimension, with 2 scans at each increment. We used the \(^1\)H-\(^1\)N correlations from the biological magnetic resonance data bank - BMRRB (BMRRB accession number 16543) to aid in the monomer assignment process. We were able to analyze changes in 100 of 140 \(^1\)H-\(^1\)N correlations by NMR.

For the HHP-NMR experiments, we used the same set-up as described above, and the spectra of α-syn monomers and fibrils were acquired using a zirconia NMR tube with an internal diameter of 3 mm and an outer diameter of 5 mm (Daedalus Innovations). An Xtreme-60 syringe pump system (Daedalus Innovations) was used to generate pressure increments from 1 to 250, 500, 750, 1,000, 1,250, 1,500, 1,750, 2,250, and 2,500 bar. At each pressure increment, the sample was left for 20 min for accommodation before spectrum acquisition. The NMR assignment of the α-syn monomer HSQC spectra at different pressures was performed by tracking the systematic shifts using the assigned HSQC at 1 bar. This step was important to exclude the compressibility contribution of pressure on monomers and to aid in finding the \(^1\)H-\(^1\)N correlations of monomeric fragments released from fibrils.

For line-broadening analysis, the relative intensity of each correlation obtained for the α-syn monomers and monomeric species released from fibrils was plotted as a function of pressure increase. The monomeric species released from the fibrils presented a bell-shaped behavior and were fitted to a second-order polynomial equation (\( y = bx^2 + ax + c \), where \( y \) is relative intensity and \( x \) is pressure) using the Origin 8.0 software (Northampton, MA, USA).

Chemical shift perturbation (CSP) analysis of the α-syn monomers was performed at 500, 1,000, and 2,500 bar against \(^1\)H-\(^1\)N HSQC NMR spectra at 1 bar using equation (3):

\[
CSP = \left[ (\Delta \delta_H)^2 + 0.1(\Delta \delta_N)^2 \right]^{1/2}
\]

(3)

where \( \Delta \delta_H \) and \( \Delta \delta_N \) represent the chemical shift variations of \(^1\)H and \(^1\)N, respectively, between different pressure increments (500, 1,000, and 2,500) and 1 bar. All spectra were processed using Topspin 3.11, and chemical shift values were measured using the CCPN Analysis 2.4.1 suite\(^3\).
CPMG-RD measurements. Carr-Purcell-Meiboom-Gill relaxation dispersion (CPMG-RD) measurements at mild pressures (500 and 750 bar) were acquired on a Bruker Avance III 800-MHz spectrometer at 288 K. Relaxation rates \( R_{2\text{eff}} \) were calculated from the intensities of resonance in the \( ^{1}H\text{-}^{15}N \) correlation spectra acquired with two CPMG frequencies \( (\nu_{\text{CPMG}} 50 \text{ and } 1000 \text{ Hz}) \) using equation (4):

\[
R_{2\text{eff}} = -\frac{1}{T} \ln \left( \frac{I_0}{I_{\text{CPMG}}} \right)
\]

where \( T \) is time and \( I_0 \) and \( I_{\text{CPMG}} \) are the intensities of resonance obtained from experiments without and with CPMG pulse block. \( R_{2\text{eff}} \) at \( \nu_{\text{CPMG}} \) of 50 and 1000 Hz was plotted as a function of \( \alpha\)-syn residue to probe residues experiencing conformational exchange motions. Samples were kept at the specified pressure for 20 min for accommodation before CPMG-RD measurements.

Solid-state NMR spectroscopy. The experiments were performed in a standard bore with a static magnetic field of 700 MHz (Bruker, Avance III) using a 3.2 mm triple resonance \( (^{1}H\text{-}^{13}C\text{-}^{15}N) \) and flip angle probe head at the National Center of Nuclear Magnetic Resonance, RJ, Brazil, and in a wide bore with a static magnetic field of 600 MHz (Bruker, Avance III) using a 4.0 mm triple resonance probe head at the Leibniz Institute für Molekulare Pharmacologie, Berlin, Germany. The magic angle was calibrated with KBr, the field homogeneity was established using adamantane, and the line width approximately 3 Hz was obtained. The sample temperature was calculated as indicated previously. In all experiments using the 3.2 mm probe head, the temperature was approximately 10°C, and the spinning speed was 14 kHz. With 4.0 mm rotors, the thermostat was set to 2°C. For \( ^{1}C\text{-}^{13}C \) correlation spectra, a DARR mixing period of 20 ms was applied using an RF spinal4 of 85 kHz. The recovery delay was 3 sec because the global T1 was 890 ms. The data processing was performed in a Topspin suite. The applied algorithm used a probe sphere of 1.3 Å, 50 Monte Carlo steps per Å 3 of the molecule, and 20 h, while the ThT signal stabilized. To generate different fibrillation reactions (ca. 75–80%), seeds in the concentration ranges of 1 to around 40 M were prepared to a final volume of 1 mL. The seeds were generated after incubation for 1 h at 1,033 bar or 2,067 bar (SMIs + remaining fibrils [Smt-F]). Preformed seeds were generated by subjecting fibrils to a sonication pulse of 10 sec using 50% power amplification (Sonics & Materials, Inc.). \( \alpha\)-Syn seeds in the concentration ranges of 1 to around 40 \( \mu \text{g} \), in a final reaction volume of 100 M/L/well were incubated with 140 \( \mu \text{M} \), \( \alpha\)-syn monomers.

Seeding experiments. Fibrillation reactions were performed using 140 \( \mu \text{M} \), \( \alpha\)-syn monomers diluted in 10 mM Tris-Cl, 100 mM NaCl pH 7.4 and monitored by the fluorescence of 10 \( \mu \text{M} \), ThT. We recorded emission points at ThT \( \lambda_{\text{max}} \) (i.e., 477 nm) upon excitation at 450 nm. We used a black-bottom 96-well plate (Thermo Scientific) with a final reaction volume of 100 \( \mu \text{L} \) in each well. Plates were sealed with clear polystyrene sealing tape (Fisher Scientific), loaded into a SpectraMax Paradigm Multi-Mode Microplate reader ( Molecular Devices) and incubated at 37 °C. Samples were subjected to orbital agitation. ThT emission was measured at 4-min intervals for 20 h, while the ThT signal stabilized. To generate different \( \alpha\)-syn seeds, equal amounts of washed fibrils from the same fibrillation reaction (ca. 75–80%), seeds in the concentration ranges of 1 to around 40 \( \mu \text{g} \), in a final reaction volume of 100 M/L/well were incubated with 140 \( \mu \text{M} \), \( \alpha\)-syn monomers.

Computational analysis. Internal cavities and surface clefts were mapped in the ssNMR structure of the \( \alpha\)-syn fibril [Protein Data Bank (PDB) 2D0a] based on the Monte Carlo method included in the McVold suite. The applied algorithm used a probe sphere of 1.3 Å, 50 Monte Carlo steps per Å 3 of the molecule, and 2,500 dots per atom on the dotted surface. The minimum volume for cavities to be considered was 7 Å. The identified cavities were visualized using PyMOL.

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Author Contributions
G.A.P.d.O., M.S.F. and J.L.S. designed research; G.A.P.d.O., M.d.A.M., C.C.S., Y.C., C.S. and M.S.F. performed research; J.L.S. contributed new reagents/analytic tools; G.A.P.d.O., M.S.F., C.S., A.H.M., D.F., Y.C., R.W., H.O. and J.L.S. analyzed the data; and G.A.P.d.O., M.S.F. and J.L.S. wrote the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

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