This item is the archived peer-reviewed author-version of:

Vibrational circular dichroism sheds new light on the competitive effects of crowding and β -synuclein on the fibrillation process of α -synuclein

Reference:

Van de Vondel Evelien, Baatsen Pieter, van Elzen Roos, Lambeir Anne-Marie, Keiderling Timothy A., Herrebout Wouter, Johannessen Christian.- Vibrational circular dichroism sheds new light on the competitive effects of crowding and β -synuclein on the fibrillation process of α -synuclein
Biochemistry / American Chemical Society - ISSN 0006-2960 - 57:41(2018), p. 5989-5995
Full text (Publisher's DOI): <https://doi.org/10.1021/ACS.BIOCHEM.8B00780>
To cite this reference: <https://hdl.handle.net/10067/1554850151162165141>

Article

Vibrational circular dichroism sheds new light on the competitive effects of crowding and α -synuclein on the fibrillation process of α -synuclein

Evelien Van de Vondel, Pieter Baatsen, Roos Van Elzen, Anne-Marie Lambeir, Timothy A Keiderling, Wouter A. Herrebout, and Christian Johannessen

Biochemistry, **Just Accepted Manuscript** • DOI: 10.1021/acs.biochem.8b00780 • Publication Date (Web): 21 Sep 2018

Downloaded from <http://pubs.acs.org> on September 22, 2018

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

ACS Publications

is published by the American Chemical Society, 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Vibrational circular dichroism sheds new light on the competitive effects of crowding and β -synuclein on the fibrillation process of α -synuclein

Evelien Van de Vondel¹, Pieter Baatsen², Roos Van Elzen³, Anne-Marie Lambeir³, Timothy A. Keiderling⁴, Wouter A. Herrebout^{1*}, Christian Johannessen^{1*}

¹Molecular Spectroscopy Group, Department of Chemistry, University of Antwerp, Belgium
²VIB-KU Leuven Center for Brain & Disease Research, Belgium and VIB-KU Leuven Bio Imaging Core
³Laboratory of Medical Biochemistry, Department of Pharmacy, University of Antwerp, Belgium
⁴Department of Chemistry, University of Illinois at Chicago, Chicago, USA

* e-mail: wouter.herrebout@uantwerpen.be; christian.johannessen@uantwerpen.be.
Telephone: +32 032653373; +32 032653505

Abstract: The effects of crowding, using the crowding agent Ficoll 70, and the presence of beta-synuclein on the fibrillation process of alpha-synuclein were studied by spectroscopic techniques, transmission electron microscopy and ThT-assays. This combined approach, where all techniques were applied to the same original sample, generated an unprecedented understanding of the effects of these modifying agents on the morphological properties of the fibrils. Separately, crowding gives rise to shorter mutually aligned fibrils while beta-synuclein leads to branched, short fibrils. The combination of both effects leads to short, branched, mutually aligned fibrils. Moreover, it is shown that the non-destructive technique of vibrational circular dichroism is extremely sensitive towards the length and the higher order morphology of the fibrils.

INTRODUCTION

Aggregates having alpha-synuclein (asyn) as a key component occur in a number of neurodegenerative diseases, including Parkinson's disease.^{1,2} In vitro, monomeric asyn is found to be intrinsically disordered³, but it can undergo structural change in defined conditions to a form with a high beta-sheet structural content, inducing the formation of fibrils.⁴ The formation of these fibrils, and possible inhibitors or promoters of that process, are of high interest, as interference with this process could prevent the formation of aggregates, hence obstructing the onset of disease.⁵

One of the promoters of the fibrillation process is the crowding effect.⁶ The theory of crowding states that the space, occupied by macromolecules, is mutually impenetrable. As the overall concentration of macromolecules in the cell can amount to $\sim 400 \text{ mg mL}^{-1}$, this so-called excluded volume induces nonspecific steric repulsion, hence rendering a part of the intracellular volume unavailable for other macromolecules.⁷ Studies in the past have shown that the presence of crowding agents, macromolecules used to mimic the crowded environment of the cell, accelerates the fibrillation process.^{6,8-10} Moreover, the resulting fibrils are suggested to aggregate in clumps, rather than long, singular fibrils.⁶

A possible inhibitor of the fibrillation process of asyn is the presence of beta-synuclein. Beta-synuclein (bsyn) has been shown to prolong the lag phase, and affects the speed of the nucleation phase, of fibrillation.¹¹ Because it is lacking the stretch of hydrophobic residues that are found in the middle region of the asyn protein, bsyn was not incorporated into the fibrils.¹¹ It is of vital interest to determine the combined effect of a crowded environment, mimicked by the presence of Ficoll 70 (F70) and bsyn on the fibrillation process of asyn, as both effects will occur simultaneously in cellular environments.^{9,12} In order to examine this combined effect, the fibrillation process in different experimental conditions was studied. A survey of the performed experiments can be found in Table 1.

Table 1. A survey of the performed experiments in this study.

Sample name	Contents solution
asyn	alpha-synuclein (asyn)
F7oasyn150	asyn and 150 mg mL ⁻¹ Ficoll 70 (F7o)
F7oasyn300	asyn and 300 mg mL ⁻¹ Ficoll 70 (F7o)
asynbsyn	asyn and beta-synuclein (bsyn) (1:1 ratio)
F7oasynbsyn150	asyn, bsyn (1:1 ratio) and 150 mg mL ⁻¹ Ficoll 70 (F7o)
F7oasynbsyn300	asyn, bsyn (1:1 ratio) and 300 mg mL ⁻¹ Ficoll 70 (F7o)

The effect of these different conditions on the kinetics of the fibrillation study was monitored using a Thioflavin T-assay (ThT-assay). Because of its enhanced fluorescence upon binding to amyloid fibrils, ThT is often used to monitor the formation of beta-fibrils.¹³ The results, plotting the amount of fluorescence versus time, can be fitted to the Finke-Watzky equation^{14,15}, resulting in quantitative information on the kinetics of the fibrillation process.

In order to study the resulting morphology of the fibrils formed at the different conditions, the secondary structure of the fibrils was estimated from electronic circular dichroism (ECD) and Fourier transform infrared (FTIR) spectroscopy, while the nature of the supramolecular structures was studied using transmission electron microscopy (TEM) and vibrational circular dichroism (VCD). This non-destructive technique shows unprecedented spectroscopic sensitivity towards the presence of fibrils¹⁶, even to the morphology of those fibrils.^{17–19} As is detailed in the supporting information, the use of VCD gives rise to additional structural and morphological information, often not accessible with traditional methods.

The combined results of these experiments gave, in our opinion, exceptional insight in the effect of both the presence of crowding agents and bsyn on the fibrillation process of asyn. It was found that the promotion of the fibrillation process by the crowding effect can be counteracted by the presence of bsyn. The resulting structure of the fibrils, which are short and mutually aligned, is rationalized as a combination of the crowding effect and the effect of the presence of bsyn, and is neither dominated by the effect of the presence F7o, nor by the effect of the presence of bsyn. Importantly, the ensuing morphological differences in the fibril samples can be monitored using VCD spectroscopy.

MATERIALS AND METHODS

Asyn was expressed and purified by using a previously published method.²⁰ The bsyn construct was a gift of Prof. Veerle Baekeland (KU Leuven). The expression and purification of bsyn is described in the supplementary information. The con-

concentration of the protein preparations were determined to be 14 mg mL⁻¹ for asyn and 5.6 mg mL⁻¹ for bsyn. The protein concentration was determined by A280nm ($\epsilon_{\text{bsyn}} 5960 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{asyn}} 5800 \text{ M}^{-1} \text{ cm}^{-1}$) and, independently, by Pierce BCA protein assay (calibrated with BSA). F70 was acquired from Sigma-Aldrich®.

Samples were made with compositions detailed in Table 2. Each sample contained 0.06 mM ThT, and 6 times 100 μL were loaded into a 96-well plate. The fibrillation assay was run on Tecan infinite M200 with following settings: 37 °C, 600 kinetic cycles, 20 minutes shaking before assay, 15 minutes shaking in between measurements, measuring intervals of 20 minutes for a total of 150 hours, absorbance wavelength 350 nm, excitation wavelength 446 nm and emission wavelength 482 nm. After fibrillation, the curves were analyzed, after which the Y-axis was modified to represent the percentage of RFU's relative to the total fluorescence amplitude at the end of the fibrillation reaction. All samples were harvested at the end of the fibrillation process of 150 hours, after all samples had reached an equilibrium state. However, the 'plateau' at the end of the fibrillation process showing this equilibrium phase was left out of figure 2 for clarity.

After the fibril formation, the samples were frozen at -20°C. To measure the ECD-spectra, the original samples were diluted to a concentration of 0.2 mg mL⁻¹ alpha-synuclein or 0.05 mg mL⁻¹ beta-synuclein. 3 accumulations from 250 to 180 nm were taken with 0.5 s and 1 nm on a ChiraScan® spectrometer in a cell with a path length of 0.05 cm. Corresponding backgrounds were measured and subtracted from the results. Deconvolution of the spectra was not attempted for this study, as spectra recorded down to 180 nm are needed for an accurate description of the amount of beta-sheet present in the sample. However, below 190 nm, ECD measurements were unreliable due to interference of the solvent and of Ficoll 70. Moreover, most deconvolution software is based on globular protein basis set, and the reliability towards fibril structures has not been proven.

Table 2. A survey of the performed experiments in this study.

Sample name	Concentration F70 [mg mL ⁻¹]	Concentration bsyn [mg mL ⁻¹]	Concentration asyn [mg mL ⁻¹]
F70	300	0	0
Bsyn	0	2	0
F70bsyn300	300	2	0
F70bsyn150	150	2	0
asyn	0	0	2
F70asyn300	300	0	2

F7oasyn150	150	0	2
Asynbsyn	0	2	2
F7oasynbsyn300	300	2	2
F7oasynbsyn150	150	2	2
F7o150	150	0	0

As any method, known to us, used to obtain the increased protein concentration necessary for the IR and VCD measurements, might have an effect on the fibril integrity, it was opted to use lyophilization and a solvent exchange to D₂O on the harvested samples. In this way, both samples with enhanced VCD spectra and ‘normal intensity’ VCD spectra would be treated similarly, and in our opinion a qualitative comparison between the measurements would make sense. Therefore, to acquire the IR and VCD-spectra, the samples, collected after the fibrillation process, were lyophilized to extract all the H₂O, then dissolved in an abundance of D₂O (pD 7.44) and placed at 4 °C overnight to exchange all the exposed H-atoms for D-atoms. Next, the samples were lyophilized one additional time, and re-dissolved in D₂O to achieve a concentration of approximately 20 mg mL⁻¹ of alpha-synuclein, and measured in a 50 μm path length cell for 8 times 60 minutes on a Bruker PMA 37 VCD attachment coupled to a Bruker IFS 66v/s infrared spectrometer.²¹

An ECD spectrum was also measured from each of the VCD samples, 100 times diluted with D₂O, and found to be identical to the ECD spectra in H₂O, indicating that the samples were not destroyed during the lyophilization process. (Figures S6 and 7). The effect of the lyophilization process on the VCD spectrum of asyn fibrils was also deemed minimal (Figure S8).

The resulting spectra are an average of these 8 accumulations. Corresponding backgrounds were measured similarly, and subtracted from the raw spectra. The IR- and VCD-spectra were normalized to A=1 for the amide I region. The intensity of the signal of the VCD-spectra was determined by calculating the peak-to-peak value and dividing this by the corresponding intensity of the IR-signal. The resulting g-factors were divided by the g-factor of the corresponding sample without crowding agent. The resulting factor was determined to be a measure of the level of enhancement in the spectrum. The samples from the ECD-measurements, with a concentration of 0.2 mg mL⁻¹, were also used for the TEM-measurements. To this end a droplet of 2.5 μL of sample was deposited on a 300 mesh copper EM-support grid and blotted after 1,5 mins. Then, the samples were stained for 1 minute with 1% uranyl acetate and subsequently dried. The images were taken at a magnification of 6000x and 10000x on a JEOL JEM 1400 (Japan, Tokyo), equipped with an EMSIS Quemesa 11Mpxl camera operated at 80kV.

RESULTS AND DISCUSSION

Crowding effects on asyn fibril formation.

The FTIR spectra, VCD spectra and TEM images for asyn with different concentrations of F70, are presented in figure 1. While asyn is ubiquitously acetylated at the N-terminus *in vivo*,^{22,23} it has been independently determined and reported that the modified protein has very close aggregation propensities in comparison to the non-acetylated protein.²⁴ It was also determined that the resulting fibrils have similar morphology.²⁵

Figure 1. FTIR spectra, vibrational circular dichroism spectra and TEM images of alpha-synuclein fibrillated solely in solution (left), fibrillated in the presence of 150 mg mL⁻¹ Ficoll 70 (middle) and in the presence of 300 mg mL⁻¹ Ficoll 70 (right). The scale bar in the TEM images represents 250 nm. The arrows on the TEM images indicate some clear examples

of the alignment of the fibrils in side-by-side orientation. The IR and VCD spectra have been normalized to a maximum absorbance of 1 in the amide I region.

The sample of asyn alone forms long, unbranched fibrils (see figure 1G). The IR-spectrum (see figure 1A) suggests the presence of both disordered structure and beta-sheet structure in the protein, as determined by the presence of features at 1645 cm^{-1} and 1625 cm^{-1} , respectively, in the second derivative (see figure S4A). The presence of beta-sheet structural content is supported by the VCD-spectrum, as broad negative feature at 1632 cm^{-1} is very similar to the features linked to concanavalin A, a protein with high beta-sheet structural content.²⁶ The ECD-spectrum also indicates the presence of beta-sheet structural content (see figure S1A). The signal intensity of the VCD spectrum (see figure 1D) of this sample is within the expected range for VCD-spectra, indicating that the fibrils do not possess any kind of alignment or order in between themselves, as one would expect an enhancement of the VCD-signal if that were the case.²⁷ It also needs to be noted that the nature of the sample induces artefacts in the baseline in the VCD spectrum, as the signal-to-noise ratio of the measurements of other samples are proven to be comparable to the one in other instruments (see SI). The lack of alignment between the fibrils is confirmed by the TEM-images, as they show long, randomly distributed fibrils. The kinetics of this fibrillation process is taken as a reference point to determine the observed effects qualitatively. The quantitative data, on which this qualitative description is based, can be found in the supporting information, while the results of the ThT-assay can be found in figure 2.

Upon the addition of 150 mg mL^{-1} F70 during the fibrillation process, the lag-time reduces significantly, while the elongation phase is slightly steeper (see figure 2). There also seems to be a difference in the structure of the resulting fibrils, as suggested by the different ECD spectrum of these fibrils. (See figure S1B) The solutions before fibrillation however are identical in all cases, suggesting that the change in structure only occurs upon fibrillation. The VCD-signal (see figure 1E) has a shape similar to the shape of fibrils of previously published VCD for a C-terminal truncated version of asyn,²⁸ and is enhanced by a factor 14. It is important to note that the lyophilization procedure, used in the preparation of the samples, might have decreased the final intensity of the enhanced VCD signal. As all samples were treated similarly, the relative final intensities of the VCD spectra will be used for a qualitative comparison.

This induced change in VCD sign pattern has previously been assigned to an inversion of fibril supramolecular chirality.^{18,29} The TEM-images lack any evidence of a twist in the fibrils, which has previously been linked to this kind of enhancement.^{18,19,27} On the other hand, it can be observed that the fibrils align themselves in a side-by-side orientation. This alignment between fibrils thus suggests a dipolar coupling on the supramolecular level, between the fibrils, and is

most likely the cause of the enhancement in this case. Furthermore, the high level of supramolecular orientation can also be identified as the cause of the previously reported clusters of aggregates observed for fibrils of asyn grown in a crowded environment.⁶

The crowding effect on the fibrillation process also depends on the concentration of the crowding agent, as the addition of 300 mg mL⁻¹ F7o during the fibrillation process reduces the lag-time even further (see figure 2). The elongation phase seems to be less steep than with the F7oasyn150 sample, although still slightly steeper than witnessed with the asyn sample. This difference in kinetics also results in a different fibril morphology, as the TEM-image (see figure 1I) illustrates that the addition of 300 mg mL⁻¹ F7o during the fibrillation process shortens the fibrils. As crowding increases the number of nucleation sites, relatively more fibrils are initiated, which could account for the overall smaller lengths.

It appears however, that although the fibrils are significantly shorter, the mutual alignment of the fibrils is retained upon increasing the concentration of crowding agent, resulting in an enhancement of a factor 10 in the VCD-spectrum (see figure 1F), compared to the spectrum of asyn fibrillated solely in solution (see figure 1D). While the VCD-spectrum is similar in shape in comparison with the previously discussed VCD-spectrum, we believe that the smaller enhancement of the VCD-signal is due to the shorter fibrils, as mutual alignment of smaller aggregates would occur less frequently, which in turn shows that VCD spectroscopy is sensitive both to the size and alignment of the fibrils. The ECD-signal (see figure S1C) and the IR-signal of the F7oasyn300 fibrils look similar to the spectra of the F7oasyn150 fibrils. This suggests that the presence of F7o induces a small difference in the secondary structure of the fibrils, independent of the concentration of the crowding agent, while the resulting overall morphology of the collection of fibrils depends directly on the concentration crowding agent used during the fibrillation process.

bsyn effects on asyn fibril formation.

The effect of the presence of a 1:1 ratio asyn:bsyn during the fibrillation process significantly increases the lag phase as compared to pure asyn (see figure 2), while the slope of the elongation phase is less pronounced. This results in fibrils that contain, based on analysis of the second derivative and the corresponding IR-spectrum, a higher contribution of disordered secondary structure in comparison with the fibrils of the asyn sample (see Figure S5A). This is supported by analysis of the ECD-and VCD-spectra of this sample, as these spectra clearly are a superposition of a disordered pattern combined with a beta-sheet contribution (see figures 3D and S2A). We believe

Figure 2. Normalized relative fluorescence units of the Thioflavin T-assay of alpha-synuclein (black), alpha-synuclein in the presence of 150 mg mL⁻¹ (dark blue) and 300 mg mL⁻¹ (light blue) Ficoll 70, alpha-synuclein in a 1:1 presence of beta-synuclein (dark green) and alpha-synuclein in a 1:1 presence of beta-synuclein and in the presence of 150 mg mL⁻¹ (brown) and 300 mg mL⁻¹ (light green) Ficoll 70. The fluorescence of ThT was monitored as a function of time under continuous shaking at 37°C. The solid lines represent the average of a minimum of 4 experiments, while the dotted lines represent the fit of the Finke-Watzky function. The corresponding parameters can be found in the SI. The Y-axis represents the percentage of RFU's relative to the total fluorescence amplitude at the end of the fibrillation reaction.

Figure 3. FT-IR spectra, vibrational circular dichroism spectra and TEM images of alpha-synuclein:beta-synuclein (1:1) fibrillated solely in solution (left), fibrillated in the presence of 150 mg mL⁻¹ Ficoll 70 (middle) and in the presence of 300 mg mL⁻¹ Ficoll 70 (right). The scale bar in the TEM images represents 250 nm. The arrows on the TEM images indicate some clear examples of the alignment of the fibrils in side-by-side orientation. The IR and VCD spectra have been normalized to a maximum absorbance of 1 in the amide I region.

that this observation is either due to differences in the secondary structure of asyn when interacting with bsyn, or due to the fact that bsyn itself does not fibrillate in the timescale of this experiment and thus remains present as an unstructured protein in solution. This observation is supported by the TEM-image (see figure 3G), where only small aggregates of protein are visible. However, despite their longer lag time, the resulting fibrils are also shorter and more branched than the

fibrils of the asyn sample. The lack of visible overall alignment of the sample is also confirmed by the lack of enhancement in the VCD-spectrum, as is demonstrated in figure 3D.

The addition of 150 mg mL⁻¹ F70 during the fibrillation process once again reduces the lag time of the fibrillation process (see figure 2). This addition does not seem to have a big impact on the secondary structure of the proteins, as the IR- and ECD-spectra are comparable (see figures 3B and S2B). However, the higher order structure of the fibrils is different, as the shape of the VCD-spectrum is similar to the shape of VCD-spectrum of the fibrils of the F70asyn150 sample (see figure 1E). This VCD-signal is also enhanced, but only by a factor 4.5, significantly smaller than the enhancement factors observed before. This is in accordance with the TEM-image (see figure 3H), where the resulting fibrils are significantly shorter than the ones shown in figure 1H and I, but still do align.

Finally, the addition of 300 mg mL⁻¹ F70 during the fibrillation process of the 1:1 mixture of asyn and bsyn decreases the lag time even further (see figure 2), retaining the dependency on the concentration of crowding agent. Although the elongation phase is steeper in comparison with the elongation phase of the asynbsyn sample, it is still less steep than the elongation phase of the asyn sample, suggesting that neither effect completely dominates the kinetics of the fibrillation process, but that the resulting fibrillation process is influenced by a combination of both effects. The effect of the concentration of crowding agents on the structure of the fibrils is also retained, as the addition of 300 mg mL⁻¹ F70 during the fibrillation process results in even shorter fibrils, which even so still do align. This is visible in the TEM-image, but also deductible from the enhancement of the VCD-signal (see figure 3I and F). The enhancement factor only amounts to a factor 2.5, while retaining the shape of the VCD-spectrum similar to the shapes of the VCD spectra of all the fibrils grown in the presence of F70. The IR- and ECD-spectra still suggest a similar secondary structure for these fibrils as the fibrils originating from the F70asynbsyn150 sample and the asynbsyn sample.

CONCLUSION

These results all indicate that neither the effect of bsyn nor the effect of F70 on the fibrillation process of asyn dominate the final fibrillation process. The ThT-results suggest that the presence of bsyn delays the fibrillation process, while the crowding effect promotes it. When both are present, the effects compete with each other, resulting in a fibrillation process that is an intermediate of both separate effects. The higher order structure of the fibrils however seems to result from a combination of both effects, giving rise to short, mutually aligned fibrils. This highlights the importance of taking a combination of several effects into account when studying the fibrillation process of asyn.

Most importantly, these results have also shown that VCD, a non-destructive technique, is a versatile tool and can be used to determine the higher order structural properties of the fibrils, as it is sensitive to and the mutual alignment of the fibrils and their length. The presence of an enhanced VCD signal will also enable the measurement of more complicated, physiologically relevant samples. As the resulting signal is significantly larger than that of interfering proteins, for example as is demonstrated with the asyn:bsyn samples, the interference of the disordered signal of bsyn is rendered trivial in the enhanced spectra. The authors therefore believe that a full understanding of this phenomenon will open the door to the measurement of real pathological samples.

Finally, these results indicate that bsyn is, also in cellular environments, a moderator of the fibrillation process of asyn, and thus an interesting target for research concerning neurodegenerative diseases.

ASSOCIATED CONTENT

Supporting Information. Full details on the expression and purification of bsyn, the kinetic analysis of the ThT plots, the ECD spectra as well as the second derivative of the IR spectra and a brief description on the use of IR and VCD in protein characterization studies can be found in the supporting information.

AUTHOR INFORMATION

Author Contributions

The manuscript was written through contributions of all authors. / E.V.d.V. and R.V.E. performed the expression of the samples and the fibrillation assay, and E.V.d.V. also performed the spectroscopic experiments. P.B. performed the TEM analysis, while A.M.L., C.J., W.H. and T.K. conceived the experiments and contributed to the discussion of the results. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

The authors acknowledge the Agency for Innovation by Science and Technology in Flanders (IWT) and the Fund for Scientific Research (FWO) for a predoctoral scholarship and a travel grant to E.V.d.V. Financial support by the Flemish Community, through the BOF concerned research actions (GOA) is acknowledged. The authors also thank Dr. Yani Sim (Medical Biochemistry group, Department of Pharmacy, University of Antwerp, Belgium) for technical support.

ABBREVIATIONS

Asyn, alpha-synuclein; bsyn, beta-synuclein; ThT, Thioflavin T; F70, Ficoll 70; FTIR, Fourier transform infrared; ECD, electronic circular dichroism; VCD, vibrational circular dichroism; TEM, transmission electron microscopy

REFERENCES

(1) Lundvig, D., Lindersson, E., and Jensen, P. H. (2005) Pathogenic effects of alpha-synuclein aggregation. *Mol. Brain Res.* 134, 3–17.

(2) Conway, K. A., Harper, J. D., and Lansbury, P. T. (1998) Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat. Med.* 4, 1318–1320.

(3) Alderson, T. R., and Markley, J. L. (2013) Biophysical characterization of alpha-synuclein and its controversial structure. *Intrinsically Disord. Proteins* 1, 18–39.

(4) Breydo, L., Wu, J. W., and Uversky, V. N. (2012) Alpha-synuclein misfolding and Parkinson's disease. *Biochim. Biophys. Acta* 1822, 261–285.

(5) Uversky, V. N. (2007) Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation. *J. Neurochem.* 103, 17–37.

(6) Munishkina, L. A., Cooper, E. M., Uversky, V. N., and Fink, A. L. (2004) The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *J. Mol. Recognit.* 17, 456–464.

(7) Ellis, R. J. (2001) Macromolecular crowding: Obvious but underappreciated. *Trends Biochem. Sci.* 26, 597–604.

(8) Bai, J., Liu, M., Pielak, G. J., and Li, C. (2017) Macromolecular and small molecular crowding have similar effects on alpha-synuclein structure. *ChemPhysChem* 18, 55–58.

(9) Uversky, V. N., M. Cooper, E., Bower, K. S., Li, J., and Fink, A. L. (2001) Accelerated alpha-synuclein fibrillation in crowded milieu. *FEBS Lett.* 515, 99–103.

(10) Fink, A. L. (2006) The aggregation and fibrillation of alpha-synuclein. *Acc. Chem. Res.* 39, 628–634.

(11) Uversky, V. N., Li, J., Souillac, P., Millett, I. S., Doniach, S., Jakes, R., Goedert, M., and Fink, A. L. (2002) Biophysical properties of the synucleins and their propensities to fibrillate: Inhibition of alpha-synuclein assembly by beta- and gamma-synucleins. *J. Biol. Chem.* 277, 11970–11978.

(12) Biere, A. L., Wood, S. J., Wypych, J., Steavenson, S., Jiang, Y., Anafi, D., Jacobsen, F. W., Jarosinski, M. A., Wu, G. M., Louis, J. C., Martin, F., Narhi, L. O., and Citron, M. (2000) Parkinson's disease-associated alpha-synuclein is more fibrillogenic than beta- and gamma-synuclein and cannot cross-seed its homologs. *J. Biol. Chem.* 275, 34574–34579.

(13) Khurana, R., Coleman, C., Ionescu-Zanetti, C., Carter, S. A., Krishna, V., Grover, R. K., Roy, R., and Singh, S. (2005) Mechanism of thioflavin T binding to amyloid fibrils. *J. Struct. Biol.* 151, 229–238.

(14) Nath, S., Meuvlis, J., Hendrix, J., Carl, S. A., and Engelborghs, Y. (2010) Early aggregation steps in alpha-synuclein as measured by FCS and FRET: Evidence for a contagious conformational change. *Biophys. J.* 98, 1302–1311.

(15) Morris, A. M., Watzky, M. A., Agar, J. N., and Finke, R. G. (2008) Fitting neurological protein aggregation kinetic data via a 2-step, minimal/"Ockham's razor" model: The Finke-Watzky mechanism of nucleation followed by autocatalytic surface growth. *Biochemistry* 47, 2413–2427.

(16) Ma, S., Cao, X., Mak, M., Sadik, A., Walkner, C., Freedman, T. B., Lednev, I. K., Dukor, R. K., and Nafie, L. a. (2007) Vibrational circular dichroism shows unusual sensitivity to protein fibril formation and development in solution. *J. Am. Chem. Soc.* 129, 12364–12365.

(17) Shanmugasundaram, M., Kurouski, D., Wan, W., Stubbs, G., Dukor, R. K., Nafie, L. A., and Lednev, I. K. (2015) Rapid filament

supramolecular chirality reversal of HET-s (218-289) prion fibrils driven by pH elevation. *J. Phys. Chem. B* 119, 8521–8525.

(18) Kourouski, D., Lombardi, R. A., Dukor, R. K., Lednev, I. K., and Nafie, L. A. (2010) Direct observation and pH control of reversed supramolecular chirality in insulin fibrils by vibrational circular dichroism. *Chem. Commun.* 46, 7154.

(19) Measey, T. J., and Schweitzer-Stenner, R. (2011) Vibrational circular dichroism as a probe of fibrillogenesis: The origin of the anomalous intensity enhancement of amyloid-like fibrils. *J. Am. Chem. Soc.* 133, 1066–1076.

(20) van Raaij, M. E., Segers-Nolten, I. M. J., and Subramaniam, V. (2006) Quantitative morphological analysis reveals ultrastructural diversity of amyloid fibrils from alpha-synuclein mutants. *Biophys. J.* 91, L96–L98.

(21) Urbanová, M., Setnička, V., and Volka, K. (2000) Measurements of concentration dependence and enantiomeric purity of terpene solutions as a test of a new commercial VCD spectrometer. *Chirality* 12, 199–203.

(22) Bartels, T., Choi, J. G., and Selkoe, D. J. (2011) α -Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. *Nature* 477, 107–111.

(23) Anderson, J. P., Walker, D. E., Goldstein, J. M., De Laat, R., Banducci, K., Caccavello, R. J., Barbour, R., Huang, J., Kling, K., Lee, M., Diep, L., Keim, P. S., Shen, X., Chataway, T., Schlossmacher, M. G., Seubert, P., Schenk, D., Sinha, S., Gai, W. P., and Chilcote, T. J. (2006) Phosphorylation of Ser-129 is the dominant pathological modification of α -synuclein in familial and sporadic lewy body disease. *J. Biol. Chem.* 281, 29739–29752.

(24) Fauvet, B., Fares, M. B., Samuel, F., Dikiy, I., Tandon, A., Eliezer, D., and Lashuel, H. A. (2012) Characterization of semisynthetic and naturally N α -acetylated α -synuclein in vitro and in intact cells: Implications for aggregation and cellular properties of α -synuclein. *J. Biol. Chem.* 287, 28243–28262.

(25) Kang, L., Moriarty, G. M., Woods, L. A., Ashcroft, A. E., Radford, S. E., and Baum, J. (2012) N-terminal acetylation of alpha-synuclein induces increased transient helical propensity and decreased aggregation rates in the intrinsically disordered monomer. *Protein Sci.* 21, 911–917.

(26) Keiderling, T. A. (2002) Protein and peptide secondary structure and conformational determination with vibrational circular dichroism. *Curr. Opin. Chem. Biol.* 6, 682–688.

(27) Pazderková, M., Pazderka, T., Shanmugasundaram, M., Dukor, R. K., Lednev, I. K., and Nafie, L. A. (2017) Origin of enhanced VCD in amyloid fibril spectra: effect of deuteration and pH. *Chirality* 29, 469–475.

(28) Iyer, A., Roeters, S. J., Kogan, V., Woutersen, S., Claessens, M. M. A. E., and Subramaniam, V. (2017) C-terminal truncated alpha-synuclein fibrils contain strongly twisted beta-sheets. *J. Am. Chem. Soc.* 139, 15392–15400.

(29) Kourouski, D., Lu, X., Popova, L., Wan, W., Shanmugasundaram, M., Stubbs, G., Dukor, R. K., Lednev, I. K., and Nafie, L. A. (2014) Is supramolecular filament chirality the underlying cause of major morphology differences in amyloid fibrils? *J. Am. Chem. Soc.* 136, 2302–2312.

For Table of Contents Use only

External factors affecting fibrillation in alpha-synuclein