DOI: 10.1002/anie.201200813

Single-Molecule Studies

Molecular Composition of Sub-stoichiometrically Labeled α-Synuclein Oligomers Determined by Single-Molecule Photobleaching**

*Niels Zijlstra, Christian Blum, Ine M. J. Segers-Nolten, Mireille M. A. E. Claessens, and Vinod Subramaniam**

The neuronal protein α-synuclein (aS) is a protein of 140 amino acid which is abundantly expressed in the human nervous system.[1] The aS protein is considered to play a critical role in the onset and progression of Parkinson's disease. Fibrillar aggregates of aS are the main constituents of the Lewy bodies found in the brains of Parkinson patients.[2] Recent reports suggest that aS physiologically occurs as a helically folded tetramer in vivo,[3] but these observations remain a matter of considerable debate.[4] However, growing evidence suggests that oligomeric aggregates are significantly more toxic to cells than fibrillar aggregates,[5] while little is known about the molecular details of these oligomers. Previous reports have proposed sizes up to 70 monomers per oligomer, depending on preparation protocols and specific stage in the aggregation process.[6] Small angle X-ray scattering (SAXS),[7] atomic force microscopy (AFM),[8] and electron microscopy (EM),[9] have been used to study the morphology of aS oligomers, indicating a variety of different shapes. Yet, there is little definitive evidence for the composition of, that is, the number of monomers in, aS oligomers.

For large supramolecular protein assemblies like aS oligomers it is very difficult to accurately determine their molecular weight, and hence the number of monomers per oligomer. One commonly used method for molecular weight determination is size exclusion chromatography (SEC).[9] However, SEC yields unreliable results especially for intrinsically disordered proteins such as aS, which have larger apparent hydrodynamic radii compared to globular proteins. Alternatively, ion mobility mass spectrometry is used on protein complexes.[10] However, mass spectrometry is limited regarding the maximum molecular weight it can determine. This limitation makes the application of the technique to large molecular weight aggregates challenging. Consequently, estimates for the number of monomers forming an aS oligomer range from 8–20 derived from size exclusion chromatography data[11] to 12–60 derived from scanning transmission electron microscopy (STEM) images.[9] To date it still remains unclear if this spread originates from the presence of a variety of different oligomers, or is attributable to the methods used.

Single-molecule photobleaching allows for direct probing of the number of monomers per oligomer without relying on the determination of the molecular mass, a reference, or the need for an extremely high spatial resolution. Conventional single-molecule photobleaching experiments rely on labeling all subunits in a supramolecular assembly.[12] The aggregates are then individually analyzed. Sequential photobleaching of all fluorescent labels incorporated into the oligomer will generate discrete steps in the fluorescence intensity. Counting the bleaching steps yields insight into the number of labels in the respective oligomer, and therefore the number of monomers. However, if the oligomer consists of a large number of monomers, as is expected for aS oligomers, it is impossible to accurately determine the number of photobleaching steps, since the fluorescence intensity decrease converges to an exponentially decaying curve.[13] Additionally, the presence of a large number of fluorescent labels may influence the aggregation process and result in a different oligomeric species. To overcome these problems, we have extended the single-molecule photobleaching method to be used in combination with sub-stoichiometric labeling techniques. In this approach, only a fraction of the monomers contains a fluorescent label.

The exact label probability mass function (PMF) can be calculated for a stochastic incorporation of labeled monomers in an oligomer during the aggregation if the label density, that is, the percentage of fluorescently labeled monomers, at the start of the aggregation is known (see Figure 1). The label PMF gives the probability that an oligomer with a defined number of monomers contains a specific number of labels. In principle, the number of labels can vary between zero and the total number of monomers in an oligomer. The observed number of bleaching steps determined individually for a large number of sub-stoichiometrically labeled single oligomers will always show a distribution.

Hence, in contrast to conventional photobleaching experiments using 100% labeling, the bleaching trace of one oligomer in our experiment, where we use sub-stoichiometric labeling, will not directly yield any information about the number of monomers forming the oligomer. When using sub-stoichiometric labeling techniques, it is essential to count the bleaching steps for a statistically relevant number of oligo-

[*] N. Zijlstra, Dr. C. Blum, Dr. I. M. J. Segers-Nolten, Dr. M. M. A. E. Claessens, Prof. Dr. V. Subramaniam
Nanobiophysics, MESA+ Institute for Nanotechnology and MIRA Institute for Biomedical Technology and Technical Medicine, Faculty of Science and Technology
University of Twente, P.O. Box 217
7500 AE Enschede (The Netherlands)
E-mail: v.subramaniam@utwente.nl

[**] This work was financially supported by the "Nederlandse Organisatie voor Wetenschappelijk Onderzoek" (NWO) through the NWO-CW TOP program number 700.58.302. We further acknowledge support from the Stichting Internationaal Parkinson Fonds. We thank Prof. Alfred Meixner for help with designing and realizing the microscope.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201200813.

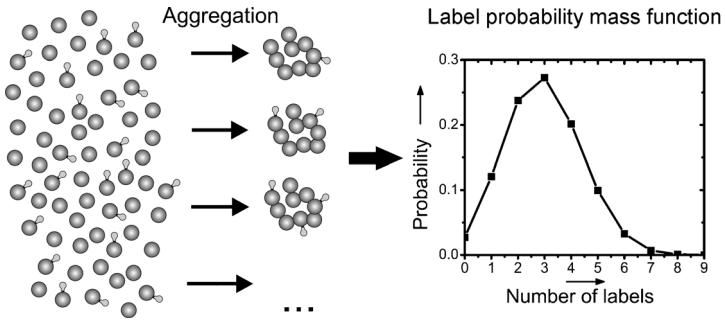

Figure 1. During the aggregation process, the incorporation of labeled monomers in the oligomer is a stochastic process. Therefore, the number of labels can vary between zero and the total number of monomers. Because of the stochastic incorporation we can predict the label probability mass function and link the number of fluorescent labels to the total number of labeled and unlabeled monomers.

mers to determine the distribution of the number of labels in the oligomers. This distribution then is linked to the total number of monomers, labeled and unlabeled, in the oligomer through the label PMF. By using different label densities, it is even possible to investigate the influence of fluorescent labels on the aggregation process.

If the prepared oligomers consist of multiple species with a distribution of the number of monomers rather than a single species with a specific number of monomers, the measured histogram of bleaching steps will be broadened compared to the bleaching histogram for a single well-defined oligomeric species. The histogram of bleaching steps for a single well-defined oligomeric species is fully explained by the label PMF for a fixed number of monomers per oligomer. Any broadening of the histogram of bleaching steps compared to the label PMF must originate from a distribution of the number of monomers per oligomer. This broadening can be directly accessed by comparing the measured histogram of bleaching steps with the label PMF calculated for a fixed number of monomers per oligomer to obtain information about any distribution of the number of monomers per oligomer.

Expression and purification of aS wild-type and mutant aS A140C was performed as previously published;[14] for more details see section S1 in the Supporting Information. Fluorescently labeled aS oligomers were prepared with different label densities, namely 7.5, 15, 20, and 30%, according to the protocol described in section S1 in the Supporting Information. Since wild-type aS does not contain a cysteine residue required for labeling, we used the A140C mutant with Alexa Fluor 488 as fluorescent label. To check for influences of the fluorescent label on the aggregation, the preparation at a label density of 15% was repeated with Atto 488 as label. After the aggregation, the oligomers were purified using a gel filtration column to separate nonaggregated monomers from oligomers. The oligomeric species were spread over four elution fractions. A typical elution profile for a label density of 7.5% is shown in section S2 in the Supporting Information.

A thin polyvinylalcohol (PVA) layer containing the oligomers at low concentrations was spincoated on top of a glass microscope cover slip to spatially separate and immobilize the oligomers (for details see section S3 in the Supporting Information). The bleaching traces were recorded using a custom-built confocal microscope (see section S4 in the Supporting Information for details).

We also analyzed the bleaching behavior of the labeled monomers. These studies showed the expected typical one step bleaching (see section S5 in the Supporting Information), confirming the presence of a single fluorescent label on the monomer and excluding any effects arising from dye/protein or dye/matrix interaction giving rise to multiple step bleaching.

Sequential photobleaching of all the fluorescent labels in an oligomer results in discrete steps in the fluorescence intensity, see Figure 2. The number of fluorescent labels present in each oligomer was

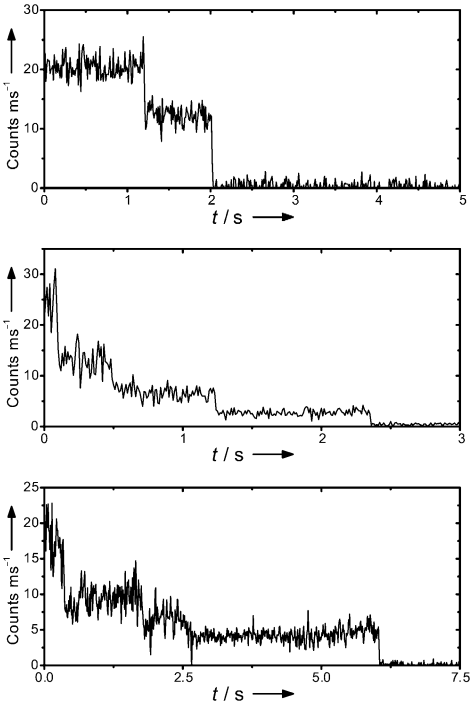

Figure 2. Three typical time traces with increasing number of bleaching steps. The graphs show two-step bleaching (top), four-step bleaching (middle), and five-step bleaching (bottom). The intensity is background subtracted.

determined by counting the number of bleaching steps for each time trace. The analysis of a complex time trace is shown in section S6 in the Supporting Information. For each label density and analyzed elution fraction, we recorded bleaching traces from a minimum of 100 distinct oligomers.

To verify that the spread of the oligomers over four elution fractions was solely because of a broadening inherent to the column used and not because of the differences in oligomer size, we determined the number of monomers per oligomer for the outer elution fractions and the center fraction for a label density of 15%, see section S7 in the Supporting Information. We found no differences in oligomer

composition between the three fractions and therefore chose to use the center fraction, containing the highest oligomer concentration, for all other label densities. By counting the number of photobleaching steps for each distinct oligomer, we obtained a histogram of bleaching steps for each label density. Figure 3 shows the photobleaching histograms for label densities of 7.5, 15, 20, and 30%. As expected, there is a clear shift of the histograms to a larger number of steps as a result of the increasing label density.

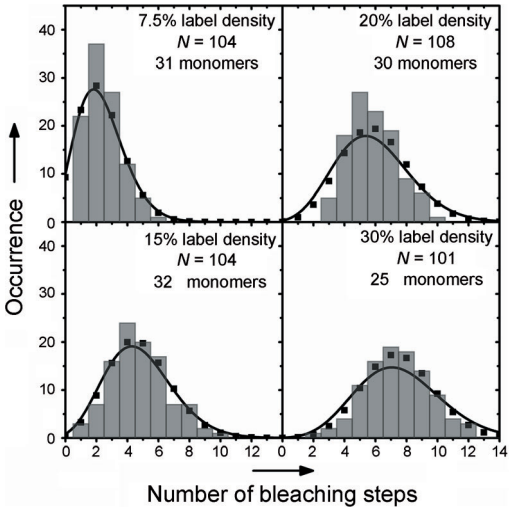

Figure 3. Histograms of the number of bleaching steps observed for the oligomers with label densities of 7.5, 15, 20, and 30%. The histograms are fitted by Poisson distributions (solid lines). The mean values of the Poisson distributions give the average number of labels incorporated into the oligomer, which was then used to calculate the label PMF (squares).

The stochastic incorporation of labeled or unlabeled monomers is described by Poisson statistics. Therefore, the bleaching histogram for each label density was fitted with a Poisson distribution from which the mean number of labels per oligomer was determined (Figure 3, solid lines). Since the width of the distribution is a result of the stochastic incorporation of labeled monomers and hence does not describe the experimental error, we determined the error from the uncertainty in the mean of the distribution. Using the exact label density, the mean number of monomers per oligomer is determined at 31 ± 2, 32 ± 1, 30 ± 2, and 25 ± 1 for the 7.5, 15, 20, and 30% label densities, respectively. The PMF is calculated from the mean number of monomers per oligomer and the label density (Figure 3, squares). The number of monomers that form an oligomer for the label densities of 7.5, 15, and 20% is remarkably uniform.

The small variation in the number of monomers that form an oligomer indicates that the number of labeled monomers does not influence the aggregation process. Furthermore, this small variation shows that the incorporation of labeled monomers during the aggregation process is indeed a truly stochastic process. If the process were not truly stochastic, one would expect a systematic increase or decrease in the number of monomers per oligomer with the label density, reflecting a preference for incorporating a labeled or unlabeled monomer. The number of monomers in combination with the label density gives the label PMF. Comparing the widths of the histograms and the label PMFs yields insight into the distribution of the number of monomers comprising one oligomer. The total widths of the measured histograms are fully explained by the widths of the label PMF, implying that the oligomers are present as a single species.

The deviation that we observe at a label density of 30% clearly shows the limitations of the photobleaching technique. We see different effects that can contribute to an imprecise determination of the distribution of the number of labels in the oligomers. Brighter oligomers are imaged as brighter spots in the scanned area and make the less bright oligomers less visible. As a consequence, the brighter oligomers are inadvertently selected more often, which would result in seemingly larger oligomers since bright oligomers contain more fluorescent labels. However, for an oligomer containing a larger number of fluorescent labels it is more likely that the number of bleaching steps is underestimated since the probability that two fluorophores bleach in a short time period increases. We believe that this effect results in an underestimation of the number of labels that in our case outweighs the apparent increase of the number of labels because of the choice of brighter oligomers. Since this limitation already arises with the sub-stoichiometric labeling approach used here, it is obvious that conventional photobleaching with 100% labeling is not suitable for large aggregates (see section S8 in the Supporting Information for a typical time trace for 100% labeled oligomers). Finally, the variation found for a labeling density of 30% might also be an indication that the relatively high density of the fluorescent label has an influence on the aggregation behavior.

To test whether the results are reproducible, we prepared two different batches of oligomers labeled at label density of 7.5%. We found for both batches the same number of monomers per oligomer. Since the fluorescent label itself can also influence the aggregation process, we prepared two separate batches of oligomers labeled at a label density of 15% with a different fluorescent label, namely Alexa Fluor 488 and Atto 488, and compared both histograms. We found no difference, and can therefore conclude that the labels used at position 140 of the amino acid sequence of aS do not influence the aggregation.

We expect that coupling or energy transfer between fluorophores will have only minor, if any, effect on our study. For small numbers of fluorophores per oligomer, as we used here, the average distance between fluorophores will be larger than the coupling range considering the estimated oligomer diameter of about 10–30 nm.[15] By embedding the oligomers in a PVA matrix, the fluorophores will not be able to rotate freely which will influence both the excitation and detection efficiencies. However, it has been shown that the largest difference between the most efficient and least efficient orientation is a factor of about 3 for a high numerical aperture (NA) objective as used in our experiments.[16] This difference will only influence the step size, and not the number of bleaching steps observed.

In conclusion, we have developed a new method using single-molecule photobleaching in combination with sub-stoichiometric labeling techniques that allows us to analyze large macromolecular protein assemblies. We can determine the composition, probe the distribution of the number of monomers per oligomer, and investigate the influence of the fluorescent label on the aggregation process.

We showed that for α-synuclein aggregation the fluorescent labels used do not have an influence. Moreover, we find no distribution of the number of monomers per oligomer and find a single, well-defined α-synuclein oligomeric species consisting of 31 monomers per oligomer.

However, previous studies reported in the literature showed differences in α-synuclein oligomers, but the origin of these differences is unclear. The variations in oligomer characteristics may reflect different stages in the aggregation process, and possibly a combination of conformational and structural differences rather than only specific numbers of monomers per oligomer.

Received: January 30, 2012
Revised: June 25, 2012
Published online: July 13, 2012

Keywords: α-synuclein · photobleaching · proteins · single-molecule studies

[1] D. F. Clayton, J. M. George, *Trends Neurosci.* **1998**, *21*, 249 – 254.
[2] M. G. Spillantini, M. L. Schmidt, V. M.-Y. Lee, J. Q. Trojanowski, R. Jakes, M. Goedert, *Nature* **1997**, *388*, 839 – 840.
[3] a) T. Bartels, J. G. Choi, D. J. Selkoe, *Nature* **2011**, *477*, 107 – 123; b) W. Wang, I. Perovic, J. Chittuluru, A. Kaganovich, L. T. T. Nguyen, J. Liao, J. R. Auclair, D. Johnson, A. Landeru, A. K. Simorellis, S. Ju, M. R. Cookson, F. J. Asturias, J. N. Agar, B. N. Webb, C. Kang, D. Ringe, G. A. Petsko, T. C. Pochapsky, Q. Q. Hoang, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17797 – 17802.
[4] B. Fauvet, M. K. Mbefo, M.-B. Fares, C. Desobry, S. Michael, M. T. Ardah, E. Tsika, P. Coune, M. Prudent, N. Lion, D. Eliezer, D. J. Moore, B. Schneider, P. Aebischer, O. M. El-Agnaf, E. Masliah, H. A. Lashuel, *J. Biol. Chem.* **2012**.
[5] a) K. M. Danzer, D. Haasen, A. R. Karow, S. Moussaud, M. Habeck, A. Giese, H. Kretzschmar, B. Hengerer, M. Kostka, *J. Neurosci.* **2007**, *27*, 9220 – 9232; b) C. Haass, D. J. Selkoe, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 101 – 112; c) M. S. Goldberg, P. T. Lansbury, *Nat. Cell Biol.* **2000**, *2*, E115 – E119.
[6] a) T. Högen, J. Levin, F. Schmidt, M. Caruana, N. Vassallo, H. Kretzschmar, K. Bötzel, F. Kamp, A. Giese, *Biophys. J.* **2012**, *102*, 1646 – 1655; b) S. Hüls, T. Högen, N. Vassallo, K. M. Danzer, B. Hengerer, A. Giese, J. Herms, *J. Neurochem.* **2011**, *117*, 868 – 878; c) M. J. Roberti, J. Foelling, M. S. Celej, M. Bossi, T. M. Jovin, E. A. Jares-Erijman, *Biophys. J.* **2012**, *102*, 1598 – 1607; d) M. Kostka, T. Högen, K. M. Danzer, J. Levin, M. Habeck, A. Wirth, R. Wagner, C. G. Glabe, S. Finger, U. Heinzelmann, P. Garidel, W. Duan, C. A. Ross, H. Kretzschmar, A. Giese, *J. Biol. Chem.* **2008**, *283*, 10992 – 11003; e) H. A. Lashuel, D. Hartley, B. M. Petre, T. Walz, P. T. Lansbury, *Nature* **2002**, *418*, 291 – 291; f) K. E. Paleologou, A. Oueslati, G. Shakked, C. C. Rospigliosi, H.-Y. Kim, G. R. Lamberto, C. O. Fernandez, A. Schmid, F. Chegini, W. P. Gai, D. Chiappe, M. Moniatte, B. L. Schneider, P. Aebischer, D. Eliezer, M. Zweckstetter, E. Masliah, H. A. Lashuel, *J. Neurosci.* **2010**, *30*, 3184 – 3198; g) B. Winner, R. Jappelli, S. K. Maji, P. A. Desplats, L. Boyer, S. Aigner, C. Hetzer, T. Loher, M. Vilar, S. Campionic, C. Tzitzilonis, A. Soragni, S. Jessberger, H. Mira, A. Consiglio, E. Pham, E. Masliah, F. H. Gage, R. Riek, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4194 – 4199.
[7] L. Giehm, D. I. Svergun, D. E. Otzen, B. Vestergaard, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3246 – 3251.
[8] A. Quist, I. Doudevski, H. Lin, R. Azimova, D. Ng, B. Frangione, B. Kagan, J. Ghiso, R. Lal, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10427 – 10432.
[9] H. A. Lashuel, B. M. Petre, J. Wall, M. Simon, R. J. Nowak, T. Walz, P. T. Lansbury, *J. Mol. Biol.* **2002**, *322*, 1089 – 1102.
[10] a) M. Ionut Iurașcu, C. Cozma, N. Tomczyk, J. Rontree, M. Desor, M. Drescher, M. Przybylski, *Anal. Bioanal. Chem.* **2009**, *395*, 2509 – 2519; b) B. T. Ruotolo, J. L. P. Benesch, A. M. Sandercock, S.-J. Hyung, C. V. Robinson, *Nat. Protocols* **2008**, *3*, 1139 – 1152.
[11] A. Rekas, R. B. Knott, A. Sokolova, K. J. Barnham, K. a. Perez, C. L. Masters, S. C. Drew, R. Cappai, C. C. Curtain, C. L. L. Pham, *Eur. Biophys. J.* **2010**, *39*, 1407 – 1419.
[12] a) K. D. Dukes, C. F. Rodenberg, R. K. Lammi, *Anal. Biochem.* **2008**, *382*, 29 – 34; b) H. Ding, P. T. Wong, E. L. Lee, A. Gafni, D. G. Steel, *Biophys. J.* **2009**, *97*, 912 – 921; c) D. Shu, H. Zhang, J. Jin, P. Guo, *EMBO J.* **2007**, *26*, 527 – 537.
[13] C. Eggeling, J. Widengren, R. Rigler, C. A. M. Seidel, *Anal. Chem.* **1998**, *70*, 2651 – 2659.
[14] M. E. van Raaij, I. M. J. Segers-Nolten, V. Subramaniam, *Biophys. J.* **2006**, *91*, L96 – L98.
[15] D.-P. Hong, A. L. Fink, V. N. Uversky, *J. Mol. Biol.* **2008**, *383*, 214 – 223.
[16] J. Hohlbein, C. G. Huebner, *J. Chem. Phys.* **2008**, *129*, 094703.

