

Solution conditions define morphological homogeneity of α -Synuclein fibrils

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ABSTRACT

The intrinsically disordered human α -Synuclein (α Syn) protein exhibits considerable heterogeneity in *in vitro* fibrillization reactions. Using atomic force microscopy (AFM) we show that depending on the solvent conditions, A140C mutant and wild-type α Syn can be directed to reproducibly form homogeneous populations of fibrils exhibiting regular periodicity. Results from Thioflavin-T fluorescence assays, determination of residual monomer concentrations and native polyacrylamide gel electrophoresis reveal that solvent conditions including EDTA facilitate incorporation of a larger fraction of monomers into fibrils. The fibrils formed in 10 mM Tris-HCl, 10 mM NaCl and 0.1 mM EDTA at pH 7.4 display a narrow distribution of periodicities with an average value of 102 ± 6 nm for the A140C mutant and 107 ± 9 nm for wt α Syn. The ability to produce a homogeneous fibril population can be instrumental in understanding the detailed structural features of fibrils and the fibril assembly process. Moreover, the availability of morphologically well-defined fibrils will enhance the potential for use of amyloids as biological nanomaterials.

Keywords: α -Synuclein, morphology, fibrillization, periodicity, homogeneous.

Abbreviations: α Syn, α -Synuclein; AFM, Atomic Force Microscopy; A140C α Syn, substitution mutant of α Syn with residue 140 mutated from Alanine to Cysteine; EDTA, Ethylenediaminetetraacetic acid; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; DTT, Dithiothreitol; *E.coli*, *Escherichia coli*; ThT, Thioflavin-T; DTNB, [5, 5'-dithiobis-(2-nitrobenzoic acid)].

1. Introduction

Self-assembled protein structures have been associated with several age related degenerative diseases like Parkinson's disease (PD), Alzheimer's disease (AD) and diabetes [1-3]. However, the propensity of a large number of proteins to form amyloid fibrils *in vitro* irrespective of their primary sequence has resulted in an extension of amyloid science from not only understanding mechanisms involved in fibrillization, diagnostics [4, 5] and potential therapeutics [6-8] but also to studying their mechano-physical properties and designing functionalized fibrils *via* biochemical modifications for custom applications [9, 10]. In the past decade, functionalities of amyloids have been extensively explored for use as nanomaterials. Fibrillar amyloids have been shown to be promising candidates as bio-templates for tissue engineering and bio-mineralization [11] and as metal or polyelectrolyte conjugated nanowires [12-15]. However, at present the applicability of the fibrils as bio-nanomaterials is considerably limited by the inherent heterogeneity in the *in vitro* fibrillization.

Fibrillization is a nucleation dependent polymerization process, in which nucleation is usually induced *de novo* by protein monomers but can also be circumvented by including pre-formed fibrils (seeded aggregation) [16, 17]. The early phase of aggregation is suggested to be a complex molecular mix, where at different time points, alternative competing reaction pathways like fragmentation or surface catalyzed nucleation (secondary nucleation) may be favored [18-22]. The plateau phase of the aggregation reaction is an assortment of monomers, oligomers and fibrils with the morphology of the mature fibrils exhibiting appreciable heterogeneity [21, 23-25]. Morphological heterogeneity or structural polymorphism can be inherent as seen with proteins like Amyloid β [26], α Syn [21, 27, 28], Ig light chains [29], ovalbumin[30], β -lactoglobulin and lysozyme [31], which form morphologically distinct assemblies under the same solvent conditions. Also, specific solvent conditions may favor certain morphology. Numerous reports have shown the critical role of solvent conditions like ionic strength, pH, temperature, inclusion of metal ions, and other small molecules on the aggregation kinetics and morphology of the fibrils [6-8, 32-41]. In general, factors favoring faster fibrillization like higher salt, higher temperature and low pH are shown to result in more heterogeneous aggregations [42].

Features like height, periodicity and length are typically used to describe the morphology of amyloid fibrils. Of these, height and periodicity are shown to be dependent on the number of protofilaments in the fibrils and can be determined accurately by AFM at single fibril level in a direct and model free regime [34, 35, 37, 38, 40, 43]. Length on the other hand is critically affected by agitation during the aggregation reaction and stochastic shear forces inherent with handling, both of which favor fragmentation [33, 39]. Moreover, length analysis of the fibrils deposited on the mica surface is reported to have adsorption (during sample preparation) and detection (during analysis) bias towards shorter or longer fibrils [36, 44].

In the present study we use α Syn as a model protein and report the formation of mature α Syn fibrils with homogeneous morphology. α Syn is a 140 amino acid predominantly neuronal protein implicated in the pathogenesis of Parkinson's disease and other synucleopathies [1, 2]. We systematically imaged a large number of single fibrils at high resolution (3.3 nm/pixel) with AFM and quantified the fibril morphology with respect to their height and periodicity. α Syn, like other amyloidogenic proteins assembles into fibrillar species *in vitro* under a range of solvent conditions and displays highly polymorphic mature fibrils [21, 24, 33, 42, 45]. Furthermore, aggregation kinetics of α Syn (wt and mutants) is known to be highly irreproducible and recently a number of studies have reported on new approaches to improve reproducibility of *in vitro* fibrillization assays [32, 46, 47].

In our study, the mutant carrying a cysteine at position 140 instead of an alanine (A140C) and wild type (wt) protein were used. Given the relative ease of functionalization of cysteine through the -SH group, replacement of alanine by

cysteine facilitates biochemical modifications and makes this mutant an important sequence polymorph for functionalization studies. Also being at the carboxyl terminus, the residue is exposed to the solvent in the mature fibril and as such is less likely to interfere in the process of fibrillization [43, 48, 49]. Incubation of A140C α Syn in solvent conditions with 10 mM NaCl including 2 mM DTT (Dithiothreitol) and 0.1 mM EDTA (Ethylenediaminetetra acetic acid) at pH 7.4 for fibrillization followed by AFM imaging and subsequent image analysis revealed the formation of a homogeneous pool of fibrils with an average periodicity of 102 ± 6 nm and height of 6.4 ± 0.4 nm. EDTA was included in the reaction to enhance the half-life of DTT (used to prevent dimerization of A140C by intermolecular disulfide bond formation). Similar experiments using wt α Syn also showed that inclusion of EDTA influences the fibrillization reaction leading to formation of fibrils exhibiting uniform periodicities with an average value of 107 ± 9 nm and fibril height of 6.9 ± 0.6 nm. Together, these results show that the inclusion of EDTA in the given fibrillization reaction conditions induces the formation of fibrils with uniform heights and periodicities.

2. Materials and Methods

2.1 Expression and purification of α Syn

wt and A140C α Syn were used in the present study. *E. coli* BL21(DE3) cells transformed with the pT7-7 plasmid carrying the wt α Syn gene were cultured in 1 liter of LB medium with 100 μ g/ml Ampicillin. At an OD of 0.6-0.7 the T-7 promoter was induced by 1 mM IPTG for 4 hours. Cells were harvested by centrifugation at 6000 x g for 10 min. The cell pellet was resuspended in 1/10th of the culture volume in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM PMSF, and stirred for 1 hour at 4 °C. Cells were lysed by sonication for 2 min. Cellular debris was removed by centrifugation at 10,000 x g for 20 min. at 4 °C. Nucleic acids were removed from the lysate by adding 1% (w/v) of streptomycin sulfate and stirring for 15 min. at 4 °C, followed by centrifugation at 13,500 x g for 30 min. at 4 °C. α Syn was salted-out from the solution by slow addition of 0.295 g/ml of ammonium sulfate and mild stirring for 1 hour at 4 °C. Precipitated protein was collected by centrifugation at 13,500 x g for 30 min. at 4 °C. The ammonium sulfate pellet was gently resuspended in 1/20th of the culture volume in 10 mM Tris-HCl, pH 7.4 and filtered through a 0.22 μ m filter. The solution was loaded onto a 6 ml ResourceQ column using an Äkta Purifier system (GE Healthcare). α Syn was eluted using a linear gradient of NaCl (0-500 mM) in 10 mM Tris-HCl, pH 7.4 at a flow-rate of 3 ml/min over 20 column volumes and 1 ml fractions were collected. Fractions were checked for α Syn using SDS-PAGE and pooled. The pooled sample was concentrated (Vivaspin-20, 10 kDa; GE Healthcare) to a volume of <2.5 ml. The sample was desalted with a PD-10 column (GE Healthcare) using 10 mM Tris-HCl pH 7.4. The volume was adjusted with Tris-HCl, pH 7.4 to a concentration of 250 μ M, and divided in aliquots of 0.5 ml and stored at -80 °C. The A140C mutant construct was generated by site directed mutagenesis using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene). A140C protein was expressed and purified according to the same protocol as wt α Syn, with additional inclusion of 1 mM DTT in all buffers.

2.2 Fibrillization reaction

250 μ M monomeric stocks of wt and A140C mutant α Syn frozen at -80 °C were thawed and filtered through 0.02 μ m, 10 mm Anotop10 Whatman syringe filters. Aggregation reactions were set up with 100 μ M A140C α Syn, 10 mM Tris-HCl, 1 or 10 mM NaCl, (+/-) 0.1 mM EDTA, 2 mM fresh DTT at pH 7.4 and with 100 μ M wt α Syn, 10 mM Tris-HCl, 1 or 10 mM NaCl, (+/-) 0.1 mM EDTA at pH 7.4. All reactions were prepared in triplicate with volumes of 400 μ l each in 2 ml Lo-Bind round bottom Eppendorf centrifuge tubes and were incubated at 37 °C with 500 rpm orbital shaking in an Eppendorf Thermo-mixer comfort. Fibrillization using Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-Fluka BioChemika) was done in a similar manner as with DTT with minor differences. The protein aliquots from -80 °C were desalted using a Zeba spin desalting column (7k MWCO; Pierce Biotechnology) to remove residual DTT and 100 μ M A140C α Syn solutions were prepared in 10 mM Tris-HCl, 10 mM NaCl, 2 mM TCEP (final concentrations) at pH 7.4.

2.3 Thioflavin-T assay

Progress of fibrillization was followed by a Thioflavin-T (ThT) fluorescence assay. A stock solution of 1 mM ThT was prepared in 50 mM glycine-NaOH buffer, pH 8.2 and filtered through a 0.22 μm syringe filter. At each time point (every 24 hours), 5 μl aliquots of sample were drawn and diluted in 2 ml of 5 μM ThT working solution diluted in glycine-NaOH buffer, pH 8.2. Fluorescence intensity was measured in triplicate on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA), with excitation at 457 nm and emission detection from 475 to 600 nm using slit widths of 10 nm. ThT curves were prepared by plotting the emission intensity readings at 485 nm *versus* aggregation time; triplicate values were averaged and blank subtracted.

2.4 Residual Monomer Concentration (RMC)

The amount of monomers left in the aggregation reaction after attaining the plateau phase in a ThT assay was determined by centrifugation of 200 μl aliquots of aggregation reactions at 21,000 \times g for 1 hour in an IEC Micromax microcentrifuge (Thermo Fisher Scientific Holding B.V., Breda, The Netherlands). The supernatant recovered from the centrifuged samples was filtered through 0.02 μm , 10 mm Anotop10 Whatman syringe filters and the absorbance was measured at 280 nm and 330 nm in a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Benelux B.V., s'-Hertogenbosch, The Netherlands). The absorption values at 280 nm were corrected for scattering contributions before calculating the residual monomer concentration [50].

2.5 Atomic Force Microscopy – fibril morphology analysis

AFM samples were prepared by adsorbing 20 μl of fibril sample, 5 to 10 times diluted in 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 on freshly cleaved mica (Muscovite mica, V-1 quality, EMS) for 4 minutes, followed by 2 gentle washes with 100 μl of fresh MilliQ water and drying in a gentle stream of nitrogen gas. AFM images were acquired on a Bioscope Catalyst (Bruker, Santa Barbara, CA, USA) in soft tapping mode in air using a silicon probe, NSC36 tip B with force constant of 1.75 N/m (MikroMasch, Tallin, Estonia). All images were captured with a resolution of 512 samples/line with a scan rate of 0.5 Hz and a scan size of 1.7 \times 1.7 μm . For each solvent condition about 100 non-overlapping fibrils more than 1 μm in length were qualitatively analyzed by Scanning Probe Image Processor - 6.02 (Image Meterology A/S, Hørsholm, Denmark) software; for quantitative analysis of height (h) and periodicity (p) a custom written Matlab script using DIPimage toolbox (version 2.3, TU Delft, Delft, The Netherlands) was used. The script is based on quantitative analysis of AFM images as described elsewhere [27].

2.6 DTNB assay

DTNB [5, 5'-dithiobis-(2-nitrobenzoic acid)] assay was carried out to quantify the amount of active DTT (free thiols) in aggregation reactions with and without EDTA. 10 mM DTNB stock solution was prepared in 0.1 M sodium phosphate buffer, pH 8.0 and filtered through a 0.22 μm syringe filter. For each time point reading, a standard curve for DTT over a concentration range of 5 to 50 μM was prepared with a working concentration of 1 mM DTNB. 5 μl aggregation aliquots were drawn at each time point and added to 1 ml of 1 mM DTNB solution in 0.1 mM sodium phosphate buffer pH 8.0 and absorbance was measured at 412 nm on a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Benelux B.V., s'-Hertogenbosch, The Netherlands). The concentration of the free thiols in aggregation reactions was calculated using $A_{412\text{nm}}$ absorbance readings and the slope of the standard curve.

2.7 Native Polyacrylamide Gel Electrophoresis

Clear native polyacrylamide gel electrophoresis (nPAGE) was performed to probe the conformers/oligomeric assemblies formed during the aggregation reaction. 12% resolving nPAGE was cast with 4% stacking gel and electrophoresed in 300

mM of Bis-Tris at pH 7.4 as the anode buffer and 300 mM of Bis-Tris, 500 mM Tricine at pH 7.4 as the cathode buffer. A constant current of 4 mA was applied while running in the stacking gel and of 6 mA in the resolving gel. The sample for electrophoresis was prepared in 2x sample loading buffer (300 mM of Bis-Tris pH 7.4, 3 M Aminocaproic acid, 0.001% Ponceau S). 10 μ l protein samples were drawn fresh from the aggregation reaction and electrophoresed. Electrophoresed gels were stained in Coomassie Brilliant Blue R250 staining solution in acetic acid: methanol: water (1:4:5) for 20 minutes and destained in acetic acid: methanol: water (1:4:5).

3. Results and Discussion

3.1 Thio-T fluorescence assay

A number of *in vitro* studies on amyloidogenic proteins have shown the crucial role of salt concentration on aggregation kinetics. In general, higher salt concentrations lead to faster self-assembly [45, 51-54]. This enhanced aggregation rate is attributed to sufficient charge screening at higher salt concentrations thus reducing the repulsive forces among the monomers and facilitating intra and intermolecular interactions governed by hydrophobicity [34, 55]. Intramolecular hydrophobic interactions induce partially folded conformations in intrinsically disordered proteins like α Syn, one or more of which could be a nucleation promoting conformation [56]. The possibility of attaining conformations with subtle structural differences in a given solvent condition decisively influences alternative fibril assembly pathways during nucleation, and is proposed to be the basis of heterogeneity [26, 57, 58]. A recent study on strain like behavior of α Syn amyloids shows formation of fibril (non-periodic) or ribbon (periodic) type of assemblies, highlighting differences in monomer conformations induced by specific reaction conditions [58, 59].

With an aim to reduce heterogeneity, we set up fibrillization experiments of A140C α Syn in 10 mM Tris-HCl and 2 mM DTT at pH 7.4 and at low salt concentrations of 1 and 10 mM NaCl, instead of more frequently used concentrations of 50 mM or 100 mM NaCl. Recent reports studying the effect of solvent conditions on α Syn fibrillization clearly demonstrate the tendency of fibrils to form higher order assemblies and floccules at higher NaCl concentrations [32, 41]. Such supra-molecular aggregates are not amenable to single fibril level morphological studies by AFM. 0.1 mM EDTA was included in the reactions to enhance the half-life of DTT, which was incorporated to prevent intermolecular disulfide bond formation by the introduced cysteine residue. Inclusion of 0.1 to 1 mM of EDTA has been shown to enhance the stability of thiol additives in protein purification against oxidation by trace metal ions [60]. An experiment to determine the optimal EDTA concentration to maximize half-life of DTT showed 0.1 mM EDTA to be as effective as higher concentrations (data not shown). Thus 0.1 mM EDTA was chosen as the working concentration in EDTA supplemented aggregation reactions.

We observed two clear trends in the ThT curves following fibrillization of A140C mutant in the above-mentioned solvent conditions. Reactions in the presence of 10 mM NaCl showed higher fluorescence intensities compared to those at 1 mM NaCl salt concentration. Also, reactions in the presence of 0.1 mM EDTA reproducibly showed higher fluorescence intensities in the plateau phase of the aggregation reaction as compared to reactions without EDTA (Fig. 1). The measured fluorescence intensities of the reactions with 1 mM NaCl in the presence of EDTA were comparable to those of the reactions with only 10 mM NaCl. In a control experiment, A140C fibrils prepared in 10 mM Tris-HCl, 10 mM NaCl and 2 mM DTT at pH 7.4, were titrated with 0.08 mM to 2.5 mM EDTA at pH 7.4 to examine a possible effect of EDTA on the ThT assay. The results showed no noticeable difference in ThT intensities in the presence/absence of EDTA (data not shown).

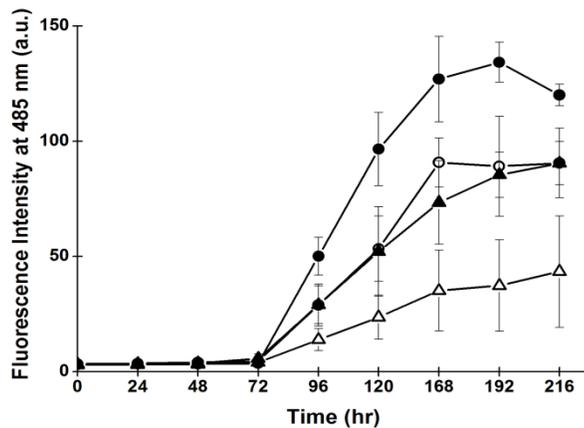


Figure 1. Kinetics of A140C α Syn aggregation measured by ThT fluorescence as a function of NaCl concentration in the absence or presence of 0.1 mM EDTA. Aggregation buffer included 10 mM Tris-HCl, 2 mM DTT at pH 7.4, and 1 mM NaCl (Δ , \blacktriangle) or 10 mM NaCl (\circ , \bullet); without EDTA (open symbols) or with 0.1 mM EDTA (filled symbols). Error bars denote standard deviation among triplicates.

An analogous set of experiments with 100 μ M wt α Syn in 10 mM Tris-HCl, with 1 or 10 mM NaCl in the absence and presence of 0.1 mM EDTA at pH 7.4 was done to ascertain if the trend observed in A140C is also seen with wt α Syn. Fibrillization reactions in the presence of 0.1 mM EDTA reproducibly showed higher ThT fluorescence values as compared to reactions without EDTA (Fig. S1). Varying the concentration of EDTA from 0.05, 0.1 to 0.2 mM for further optimization of wt aggregations did not show any significant difference in the final fluorescence values (Fig. S2). α Syn aggregation is reported to be a first order nucleation dependent polymerization reaction [16]. In fibrillization with monomers, primary nucleation is dominant and is characterized by a sigmoidal growth curve in the ThT assay [61]. Higher ThT intensities in the presence of EDTA are thus indicative of more cross- β structure. Determination of residual monomer concentration at the plateau phase of ThT curves showed that reactions with EDTA had lesser monomers left for A140C and wt aggregations (Fig. S3). Together, data from ThT assays and residual monomer concentration analysis suggest that a larger fraction of monomers is incorporated in ThT positive fibrils in the presence of EDTA (Fig. 1, S1 and S3). Lower final fluorescence intensities and higher residual monomer concentrations found for 1 mM NaCl reactions similarly suggest lower cross- β content and less uptake of monomers in fibrils. However, it is also possible that ThT may exhibit differential binding to fibrils prepared under different solvent conditions. Calculation of per μ M contribution of fluorescence from each fibril sample using residual monomer concentration and final fluorescence values show that fibrils prepared in the presence of EDTA have 10 to 40 % higher fluorescence per μ M of protein incorporated into fibrils (Table S1). Thus in solvent conditions with EDTA not only more monomers are included into the fibrils but also the fibrils formed likely bind ThT in a more efficient manner. The same may hold for fibrils formed in 10 mM *versus* 1 mM aggregation conditions.

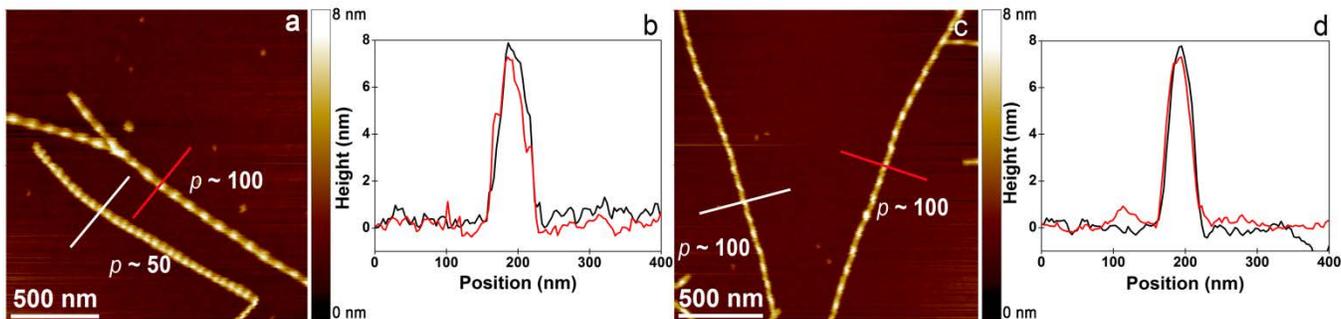


Figure 2. Representative AFM height images of A140C α Syn (a, c) and corresponding height profiles (b, d). Tapping mode AFM height images of A140C α Syn fibrils prepared in 10 mM Tris-HCl, 10 mM NaCl, 2 mM DTT at pH 7.4 and in the absence (a) or presence (c) of 0.1 mM EDTA. White and red lines in (a) and (c) indicate positions of line profiles depicted in (b) and (d). Labels (50, 100) signify approximate value for periodicity (p) of the fibrils in nm. AFM image size: 1.7 x 1.7 μ m.

3.2 AFM imaging and morphology analysis

Fibrils from the end phase of fibrillization reactions (after 192-216 hours), were imaged by AFM using soft tapping mode in air. The fibrils (A140C and wt) were typically several micrometers long, and exhibited distinctive twisted morphology (Fig. S4 and S5) [27]. The key morphological features of twisted amyloid fibrils are their height and periodicity, characteristics that can be experimentally measured with high accuracy using AFM [35, 38]. For each solvent condition, around 100 non-overlapping fibrils of at least 1 μ m length were quantitatively analyzed for height and periodicity.

The periodicities of A140C fibrils grown in 10 mM NaCl as well as 1 mM NaCl showed a broad distribution, with average periodicities of 99 ± 32 nm and 109 ± 20 nm, respectively. Interestingly, the fibril population produced in 10 mM NaCl appeared to have three distinct groups with average periodicities around 50, 100 and 150 nm (Fig. 2a, 2b 3a, and 3c). However, fibrils grown in the same salt conditions, but in the presence of 0.1 mM EDTA, showed a significantly narrower distribution of periodicities with an average value of 102 ± 6 nm for 10 mM NaCl and 105 ± 6 nm for 1 mM NaCl salt concentrations (Fig. 2c, 2d, 3b, 3d and Table 1). Thus, the homogeneity in periodicity of the A140C fibrils appears to be sensitive to the presence of EDTA. In other amyloid forming proteins like β -lactoglobulin [34] and insulin [40] fibril periodicity is reported to vary as a function of fibril height, which is explained by the Hierarchical Assembly Model (HAM), where successive association of protofilaments into protofibrils and fibrils accompanied by mutual twisting gives rise to various periodicities that correlate with measured heights [34, 35, 37, 40, 43]. The measured fibril heights in our study however, were not considerably different for fibrils prepared in 1 or 10 mM NaCl and with or without EDTA. The average heights for fibrils were 6.5 ± 0.7 (1 mM NaCl), 6.3 ± 0.4 (10 mM NaCl), 6.3 ± 0.6 (1 mM NaCl with EDTA) and 6.4 ± 0.4 (10 mM NaCl with EDTA). Also it is noteworthy that fibrils formed in 10 mM NaCl without EDTA and displaying distinct periodicities of 50, 100 and 150 nm did not show differences in height expected for hierarchical assembly (Fig. 2, 3 and Table 1). This observation shows that the morphological parameters of height and periodicity in mature α Syn fibrils formed under the given solvent conditions are not coupled.

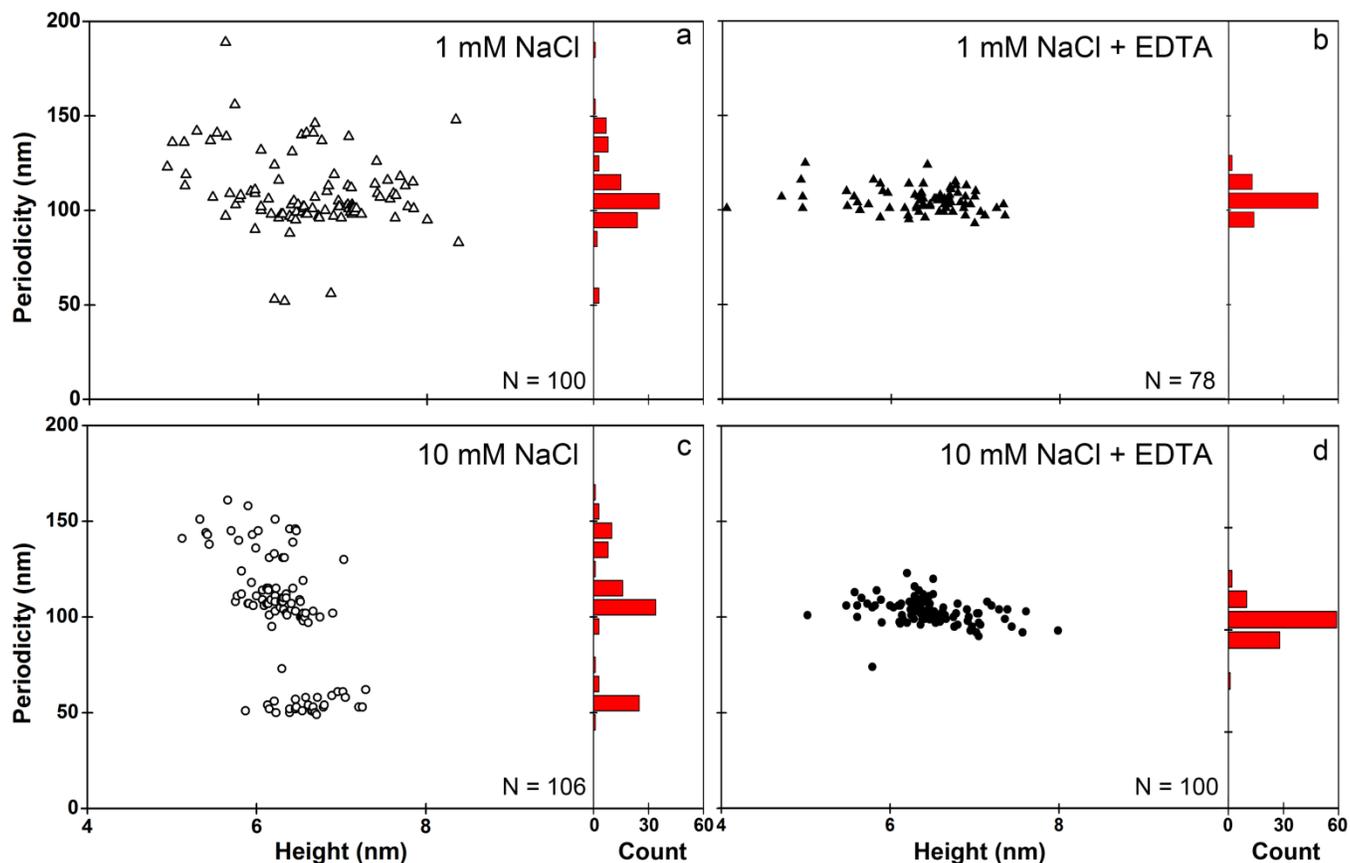


Figure 3. Scatter plots representing quantitative morphological features of A140C α Syn fibrils prepared in 10 mM Tris-HCl, 2 mM DTT at pH 7.4 with 1 mM NaCl (Δ , \blacktriangle) or 10 mM NaCl (\circ , \bullet) in the absence (a, c – open symbols) or presence (b, d – filled symbols) of 0.1 mM EDTA. Next to each scatter plot a histogram in red shows the distribution of periodicities for the fibrils. Bin size: 10 nm. (a,c) Fibrils grown in the absence of EDTA display a broad distribution of periodicities; notably aggregations in 10 mM NaCl result in three fibril groups with average periodicities around 50, 100 and 150 nm. (b,d) Fibrils produced in the presence of EDTA show one dominant population corresponding to an average periodicity of about 100 nm. Fibril periodicities show no apparent correlation with heights in the absence (a,c) or presence (b,d) of EDTA.

A parallel set of experiments probing the effect of inclusion of EDTA on height and periodicity of wt α Syn fibrils was done to assess if the homogeneity in periodicity with EDTA is limited to the A140C mutant or is seen with wt α Syn aggregation as well. Fibrillization was performed using 100 μ M wt α Syn in 1 or 10 mM NaCl, with and without 0.1 mM EDTA at pH 7.4. The fibrils formed at 1 mM NaCl showed homogeneous periodicity of 110 ± 10 nm (no EDTA) and 108 ± 7 nm (0.1 mM EDTA). Akin to A140C, at 10 mM NaCl concentration wt α Syn fibril periodicity was more defined in the presence of EDTA with an average value of 107 ± 9 nm as compared to 119 ± 24 nm for fibrils prepared without EDTA (Fig. 4, 5 and Table 1). The height distribution of wt α Syn fibrils formed in the presence of EDTA however showed a slightly narrower distribution when compared to fibrils aggregated without EDTA. It is notable that only reducing the salt concentration from 10 to 1 mM NaCl, in the absence of EDTA, makes the aggregations more homogeneous regarding morphology of the fibrils formed. The kinetics of fibrillization are however slower, and a smaller protein fraction is included in fibrils indicating that very low salt conditions are less efficient conditions for fibrillization.

0.1 mM EDTA	A140C		wt		Morphological observables
	1 mM NaCl	10 mM NaCl	1 mM NaCl	10 mM NaCl	
-	6.5 ± 0.7 (100)	6.3 ± 0.4 (106)	6.7 ± 0.4 (104)	6.1 ± 0.7 (100)	Height (<i>h</i>) (nm)
+	6.3 ± 0.6 (78)	6.4 ± 0.4 (100)	6.7 ± 0.5 (100)	6.9 ± 0.6 (105)	
-	109.1 ± 20 (100)	99.6 ± 32 (106)	110 ± 10 (104)	119.1 ± 24 (100)	Periodicity (<i>p</i>) (nm)
+	105.1 ± 6 (78)	102.7 ± 6 (100)	108 ± 7 (100)	107.6 ± 9 (105)	

Table 1. Summary of average height and periodicity measurement of A140C and wt α Syn fibrils (\pm stdv). Number of fibrils analyzed for each solvent condition is shown in parentheses.

3.3 DTNB assay

All fibrillization reactions with the A140C mutant included DTT as a reducing agent in order to inhibit intermolecular disulfide bond formation through the cysteine residue at position 140. Formation of cysteine dimers is a favored reaction, such that the protein aliquots thawed from $-80\text{ }^{\circ}\text{C}$ often showed dimer protein bands on native gels (Fig. S6). A typical aggregation reaction starting with monomers, as probed by ThT fluorescence takes on average 7 days to reach the plateau, while DTT has a much shorter half-life. The half-life of DTT has been previously shown to be affected by the presence of free divalent metal ions which enhance its oxidation [62]. Thus, in order to prevent oxidation of DTT by trace metal ions, 0.1 mM EDTA was included in the aggregation reactions. The effect of EDTA on the half-life of DTT was probed by quantitating free thiols in the aggregation reaction by a DTNB assay. In the DTNB assay, DTNB [5, 5'-dithiobis-(2-nitrobenzoic acid)] reacts with free thiols in a 1:1 stoichiometric ratio in aqueous solvents, forming 2-nitro-5-thiobenzoate (NTB²⁻) which is intense yellow in color and can be quantified by absorbance at 412 nm [63]. In reactions without EDTA the half-life of DTT was about 1 day whereas in the presence of 0.1 mM EDTA the half-life increased significantly to more than 9 days (Fig. S7). A control experiment to check if presence of EDTA alone could bring about the colorimetric change in the DTNB assay showed negative results (data not shown). In the presence of EDTA, the notable increase in the half-life of DTT as well as the qualitative influence on fibrillization of α Syn is suggestive of two distinct possibilities. EDTA primarily acts as a chelating agent and sequesters the trace divalent metal ions; as a result it prevents oxidation of DTT in A140C fibrillization.

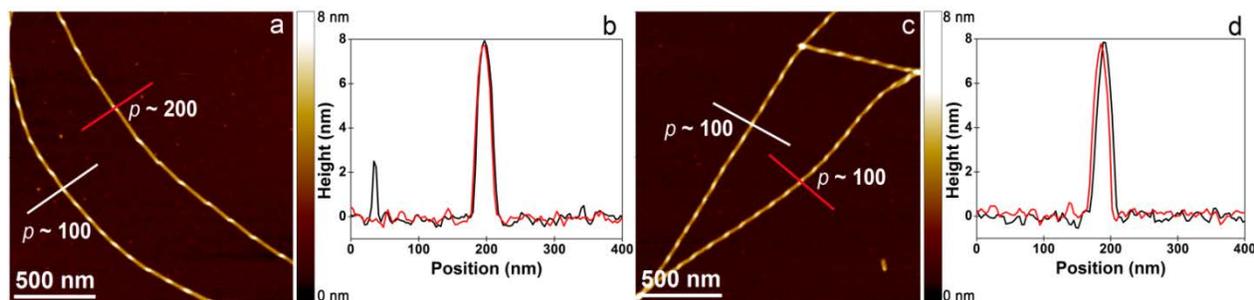


Figure 4. Representative AFM height images of wt α Syn (a, c) and corresponding height profiles (b, d). Tapping mode AFM height images of wt α Syn fibrils prepared in 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 and in the absence (a) or presence (c) of 0.1 mM EDTA. White and red lines in (a) and (c) indicate positions of the line profiles depicted in (b) and (d). Labels (100, 150) signify approximate value for periodicity (*p*) of the fibrils in nm. AFM image size: 1.7 x 1.7 μm .

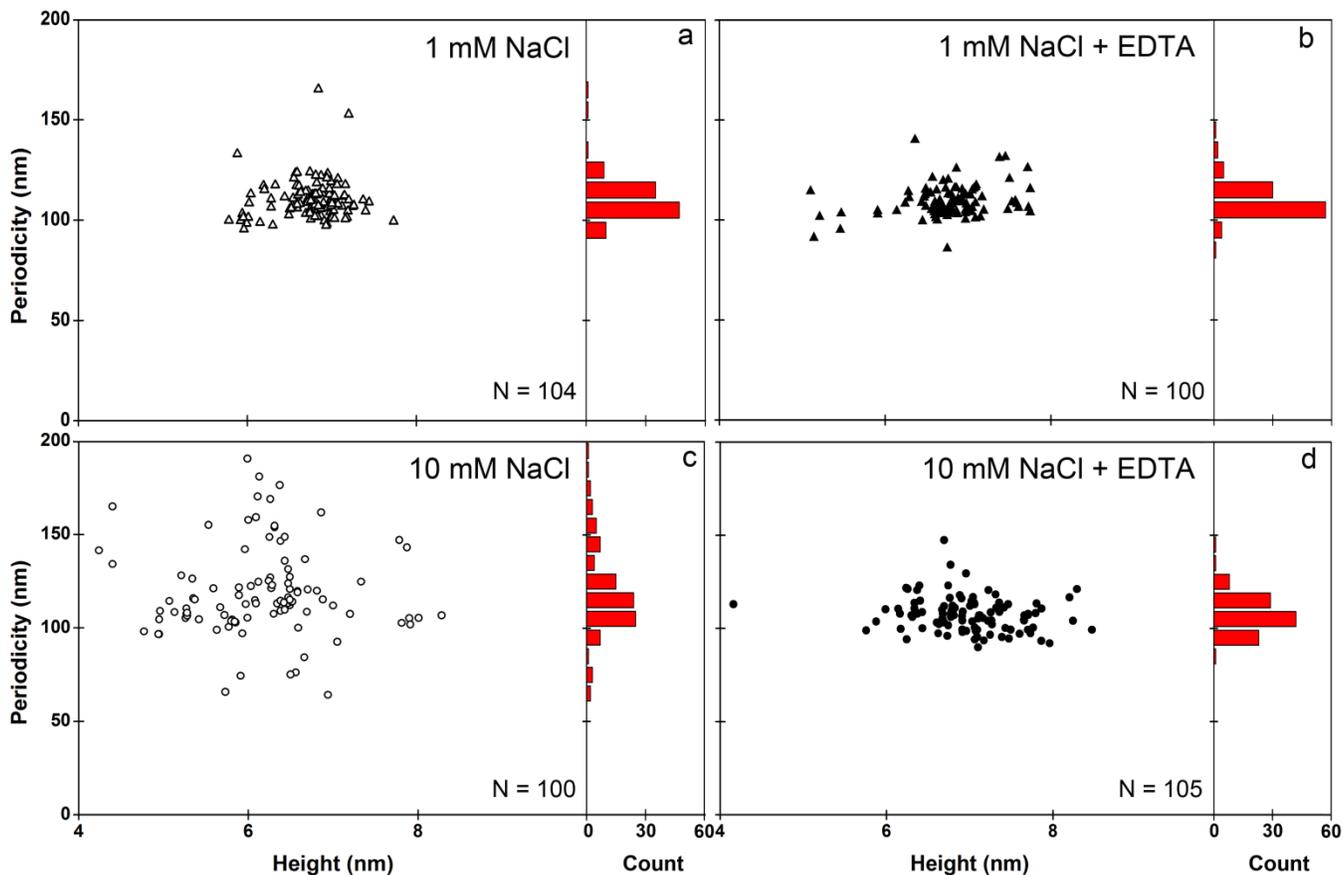


Figure 5. Scatter plots representing quantitative morphological features of wt α Syn fibrils prepared in 10 mM Tris-HCl at pH 7.4 with 1 mM NaCl (Δ , \blacktriangle) or 10 mM NaCl (\circ , \bullet) in the absence (a, c – open symbols) or presence (b, d – filled symbols) of 0.1 mM EDTA. Next to each scatter plot a histogram in red shows the distribution of periodicities for the fibrils. Bin size: 10 nm. (a,c) Fibrils grown in the absence of EDTA display a broad distribution of fibril periodicities notably for reactions aggregated at 10 mM NaCl. (b,d) Fibrils produced in the presence of EDTA show a narrow distribution of fibril periodicities. Fibril periodicities show no apparent correlation with heights in the absence (a,c) or presence (b,d) of EDTA.

Additionally this chelating property possibly curbs interaction of metal ions with the negatively charged C-terminus of α Syn monomers, thereby altering its conformation to one favorable for aggregation [52, 64]. Previous studies have shown that sequestration of metal ions by specific chelators leads to formation of more ordered fibrils, which have a sharper X-ray diffraction pattern owing to better alignment of the sample [65]. Evidence for enhancement of half-life of DTT and modulation of metal ion interaction with α Syn monomer being independent of each other is apparent in fibrillization of wt α Syn in the presence of EDTA but without DTT, where similar ThT kinetics (Fig. S1) and homogeneity in morphology was observed. Also experiments including DTT in aggregation conditions to probe the effect of reducing conditions on wt aggregation did not show any decisive trend in the ThT assay (Fig. S8).

3.4 Native PAGE analysis

Native polyacrylamide gel electrophoresis (nPAGE) is sensitive to the conformations of protein molecules and can be insightful about aggregation events. A140C aggregation at different NaCl concentrations, with and without EDTA was followed on 12% nPAGE for qualitative analysis of conformers and oligomeric assemblies. Initially, all the fractions exhibited a similar profile, but by 72 hrs, concomitant with near depletion of DTT in reactions without EDTA, additional

faster migrating bands (below the monomer band) were apparent together with a dimer band. However, in reactions with EDTA no additional bands could be observed, and over time, monomer bands became fainter indicating exhaustion of the free monomers in the solution (Fig. 6 and S6). The formation of additional faster migrating α Syn conformers and dimers appears to be prevented by DTT (half-life of which is critically influenced by EDTA) as they tend to appear only once DTT is exhausted in the solution. We also performed an aggregation with TCEP as the reducing agent using 100 μ M A140C α Syn in 10 mM Tris-HCl, 10 mM NaCl, 2 mM TCEP at pH 7.4. TCEP is a stronger and a more stable reducing agent than DTT and does not require EDTA. Fibrillization in the presence of TCEP showed faster aggregation kinetics in the ThT assay than reactions with DTT (+/- 0.1 mM EDTA) (Fig. S9). Also on nPAGE no additional bands were observed after 72 hours of aggregation, thus confirming that stable reducing conditions inhibit their formation (Fig. S10).

In wt fibrillization reactions (no DTT) followed on nPAGE similar to A140C, a discernible difference in the monomer band intensity between reactions with and without EDTA could be seen at 72 hours and later. Also in the presence of EDTA no additional bands could be observed (Fig. S11). Together these results, in agreement with ThT and RMC analyses, demonstrate that a higher fraction of monomers is incorporated into wt and A10C fibrils in reactions with EDTA.

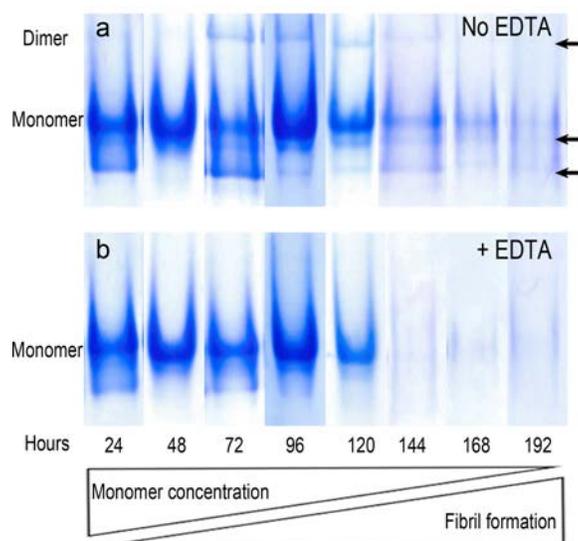


Figure 6. 12% clear native PAGE analysis of A140C α Syn fibrillization reactions. Aggregation reactions were set up in 10 mM Tris-HCl, 10 mM NaCl, 2 mM DTT at pH 7.4 and in the absence (a) or presence (b) of 0.1 mM EDTA. Lanes in panel (a) and (b) show qualitative differences in the active aggregation reactions as a function of time. Note the appearance of additional bands in the fractions without EDTA at 72 hrs and later (indicated by black arrows). Monomer protein concentration decreases with time dependent accumulation of fibrillar species, which do not electrophorese in the polyacrylamide gel. Lanes in panel (a) and (b) are parts of separate gels electrophoresed for each time point. For complete gels refer to Fig. S6.

4. Conclusions

In this work we show that fibrillization of α Syn can be carefully modulated by solution conditions to produce morphologically homogeneous populations of fibrils. The fibrils were prepared in low salt conditions in an effort to reduce stochasticity inherent with amyloid systems. Buffer conditions of 10 mM Tris-HCl, 10 mM NaCl, 2 mM DTT and 0.1 mM EDTA at pH 7.4 yielded A140C α Syn fibrils displaying a narrow distribution of periodicities with an average value of 102 ± 6 nm; in similar solvent conditions but without DTT, wt α Syn formed fibrils with an average periodicity of $107 \pm$

9 nm. Inclusion of EDTA is instrumental in forming fibrils with well-defined periodicity, by two likely effects involving its chelating function. Firstly, in reactions with thiol containing A140C α Syn, it reduces oxidation of DTT by metal ions, significantly enhancing DTT's half-life and thus preventing protein dimerization. Secondly, it prevents the interaction of metal ions with the negatively charged C-terminus of α Syn monomers potentially introducing a conformational bias which may positively influence the nucleation. Morphological analyses by AFM evidently demonstrate the formation of homogeneous fibrils with EDTA. Additionally, ThT assays, RMC and nPAGE results support the proposition of more efficient incorporation of α Syn monomers into fibrils in the presence of 0.1 mM EDTA. The present results are an important step forward for using amyloids as bio-nanomaterials as it provides a practical handle to prepare homogeneous, and reproducible amyloid fibrils.

Acknowledgements

We thank Kirsten van Leijenhorst-Groener, Yvonne Kraan and Nathalie Schilderink for protein expression and purification, and Dr. Martin Bennink, Kees van der Werf and Robert Molenaar for advice on AFM. This work is supported by NanoNextNL, a micro and nanotechnology consortium of the Government of The Netherlands and 130 partners.

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Solution conditions define morphological homogeneity of α -Synuclein fibrils

SUPPLEMENTARY INFORMATION

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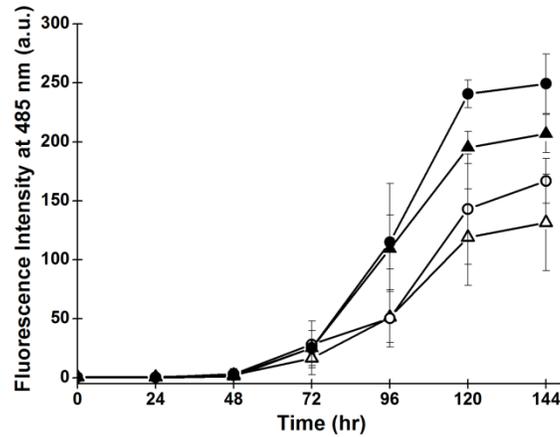


Figure S1 Kinetics of wt α Syn aggregation measured by ThT fluorescence as a function of NaCl concentration in the absence or presence of 0.1 mM EDTA. Aggregation buffer included 10 mM Tris-HCl at pH 7.4, and 1 mM NaCl (Δ , \blacktriangle) or 10 mM NaCl (\circ , \bullet); without EDTA (open symbols) or with 0.1 mM EDTA (filled symbols). Error bars denote standard deviation among triplicates.

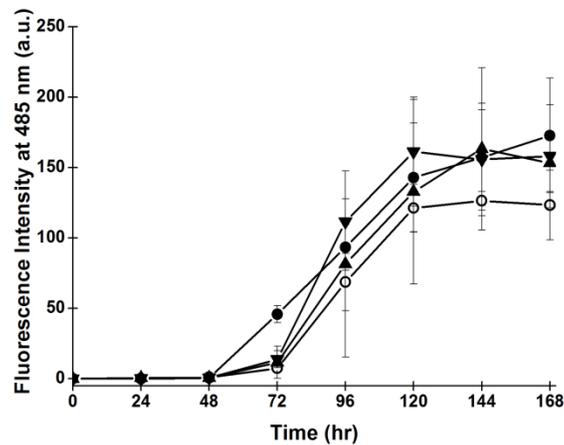


Figure S2 Kinetics of wt α Syn aggregation measured by ThT fluorescence as a function of EDTA concentration. Aggregation buffer included 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 with no EDTA (\circ), 0.05 mM EDTA (\blacktriangle), 0.1 mM EDTA (\bullet) and 0.2 mM EDTA (\blacktriangledown). Error bars denote standard deviation among triplicates.

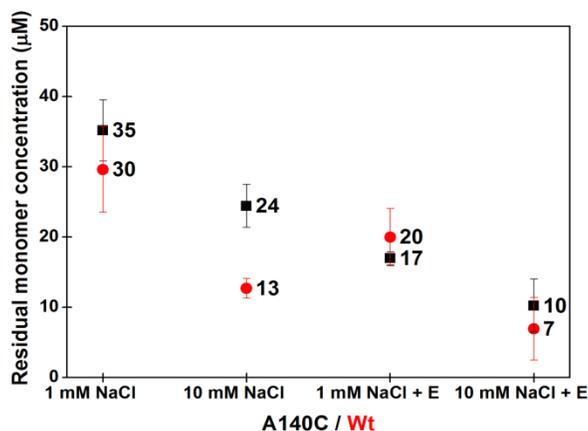


Figure S3 Residual monomer concentration (RMC) determined from aggregation reactions of A140C and wt α Syn at the plateau phase of ThT curves. Error bars denote standard deviations among triplicates.

In general the RMC values for A140C and wt in similar solvent conditions are comparable except for reactions of A140C with 10 mM NaCl and no EDTA. This difference is most likely due to formation of dimers of A140C, which is a favored reaction in the absence of DTT. In reactions without EDTA, DTT gets depleted by 3 days (see DTNB results; Fig S7) and increases the probability of A140C monomers to associate into dimers through cysteines (see nPAGE results; Fig S6). Formation of dimers reduces the net concentration of monomers available for fibrillization hence, the lower final ThT fluorescence (see ThT assay; Fig 1). For estimation of RMC the reactions were passed through 0.02 μ m filters which are not effective in filtering out dimers. Thus the RMC values for A140C reactions in 10 mM NaCl without EDTA are expected to reflect concentration of monomeric as well dimeric species.

		Final fluorescence value	RMC (μM)	aSyn conc forming fibrils (100 μM - RMC)	Fluorescence per μM fibrillar αSyn
A140C	1mM NaCl	43	35	65	0,66
	10 mM NaCl	90	24	76	1,18
	1 mM NaCl +E	90	17	83	1,08
	10 mM NaCl +E	120	10	90	1,33
wt	1 mM NaCl	131	30	70	1,87
	10 mM NaCl	166	13	87	1,91
	1 mM NaCl + E	207	20	80	2,59
	10 mM NaCl + E	250	7	93	2,69
Relative fluorescence per μM of fibrils in +/- EDTA reactions					
	Ratio		%	% higher fluorescence in + EDTA reactions	
A140C	1 mM NaCl +/- EDTA	0,66/1,08	61,0	39,0	
	10 mM NaCl +/- EDTA	1,18/1,33	88,8	11,2	
wt	1 mM NaCl +/- EDTA	1,87/2,59	72,3	27,7	
	10 mM NaCl +/- EDTA	1,91/2,69	71,0	29,0	

Table S1 Estimation of specific ThT fluorescence per μM of αSyn incorporated into fibrils in each solvent condition. Reactions with EDTA have higher ThT fluorescence per μM of αSyn thus suggesting that fibrils formed under different solvent conditions as well as fibrils of different morphology may bind ThT in a different manner.

Per μM fluorescence of αSyn was estimated by calculating the amount of αSyn contributing to the ThT fluorescence at the plateau phase. The amount of αSyn incorporated in fibrils was calculated by subtracting the RMC values for each solvent condition (100 μM - RMC). A ratio of the per μM fluorescence from reactions without and with EDTA was taken to ascertain differential ThT fluorescence in fibril samples prepared in different buffer conditions.

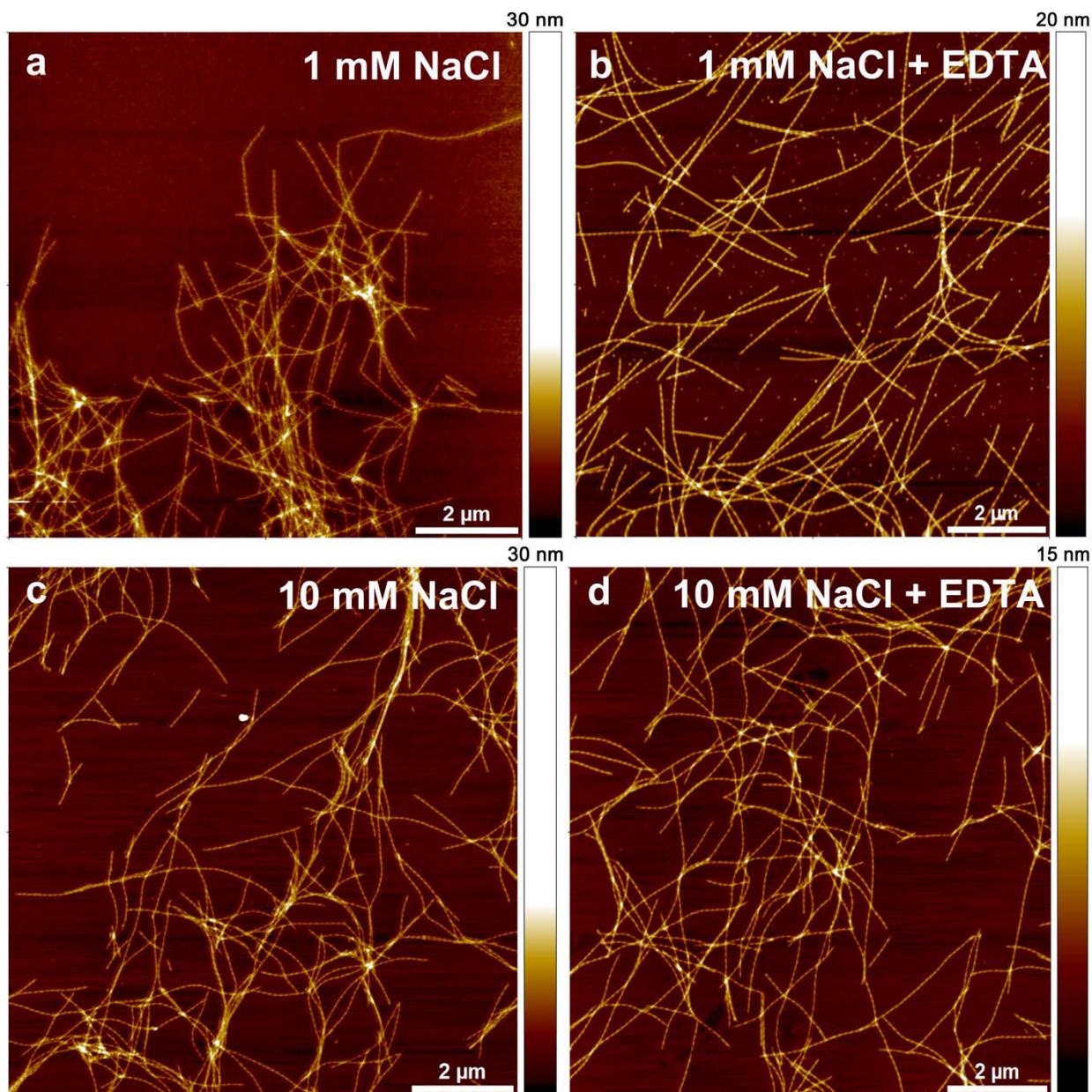


Figure S4 Overview AFM height images of A140C α Syn fibrils from the plateau phase of ThT assays. Aggregation conditions included 10 mM Tris-HCl, 2 mM DTT at pH 7.4 with (a) 1 mM NaCl, (b) 1 mM NaCl and 0.1 mM EDTA, (c) 10 mM NaCl and (d) 10 mM NaCl with 0.1 mM EDTA. Image size: 10x10 μ m. Note: Different dilutions were used to prepare AFM sample from aggregation reactions with different solvent conditions. Thus, the amount of fibrils formed under the given conditions can not be estimated from these representative images.

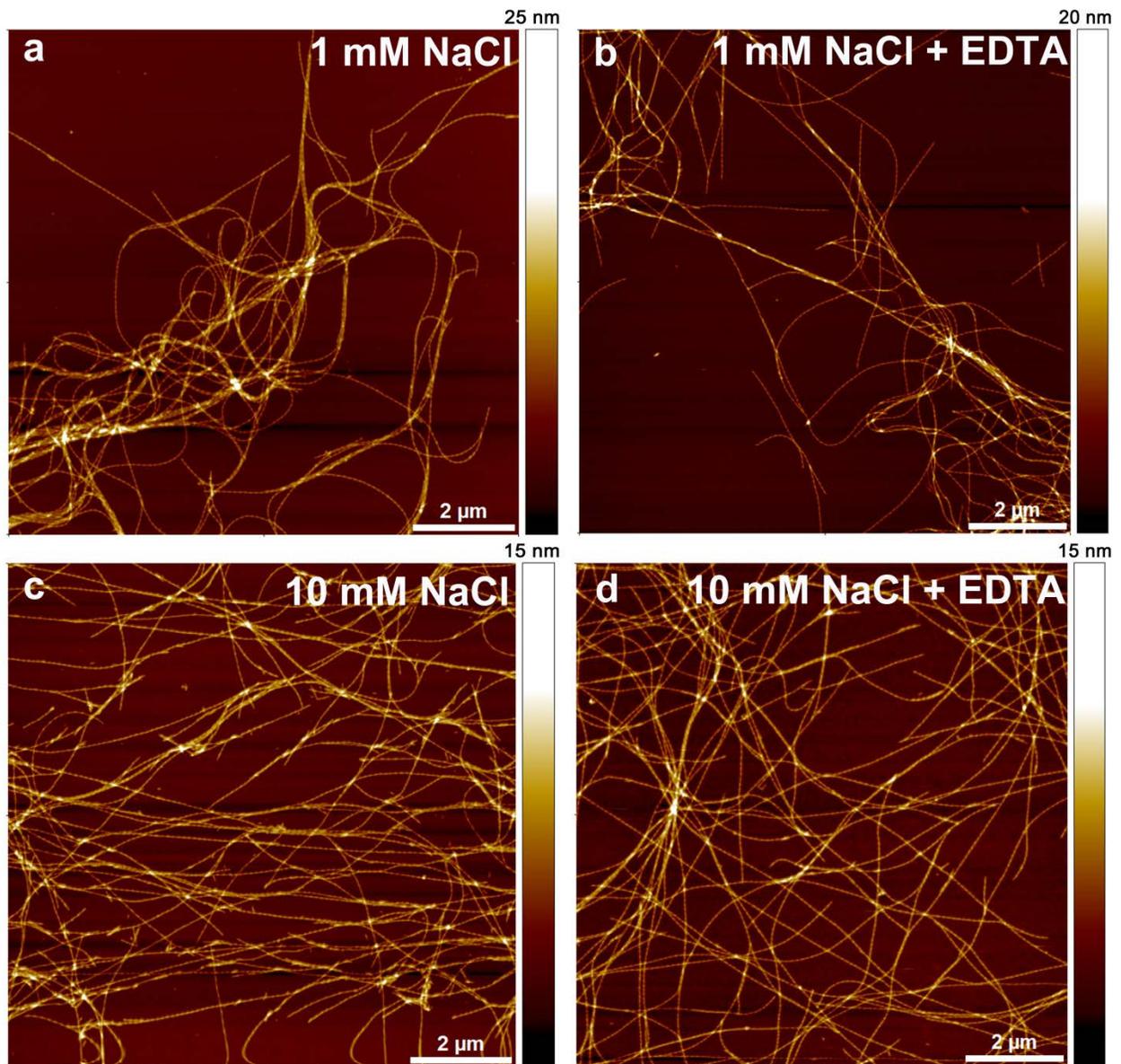


Figure S5 Overview AFM height images of wt α Syn fibrils from the plateau phase of ThT assays. Aggregation conditions included 10 mM Tris-HCl at pH 7.4 with (a) 1 mM NaCl, (b) 1 mM NaCl and 0.1 mM EDTA, (c) 10 mM NaCl and (d) 10 mM NaCl with 0.1 mM EDTA. Image size: 10x10 μ m. Note: Different dilutions were used to prepare AFM sample from aggregation reactions with different solvent conditions. Thus, the amount of fibrils formed under the given conditions can not be estimated from these representative images.

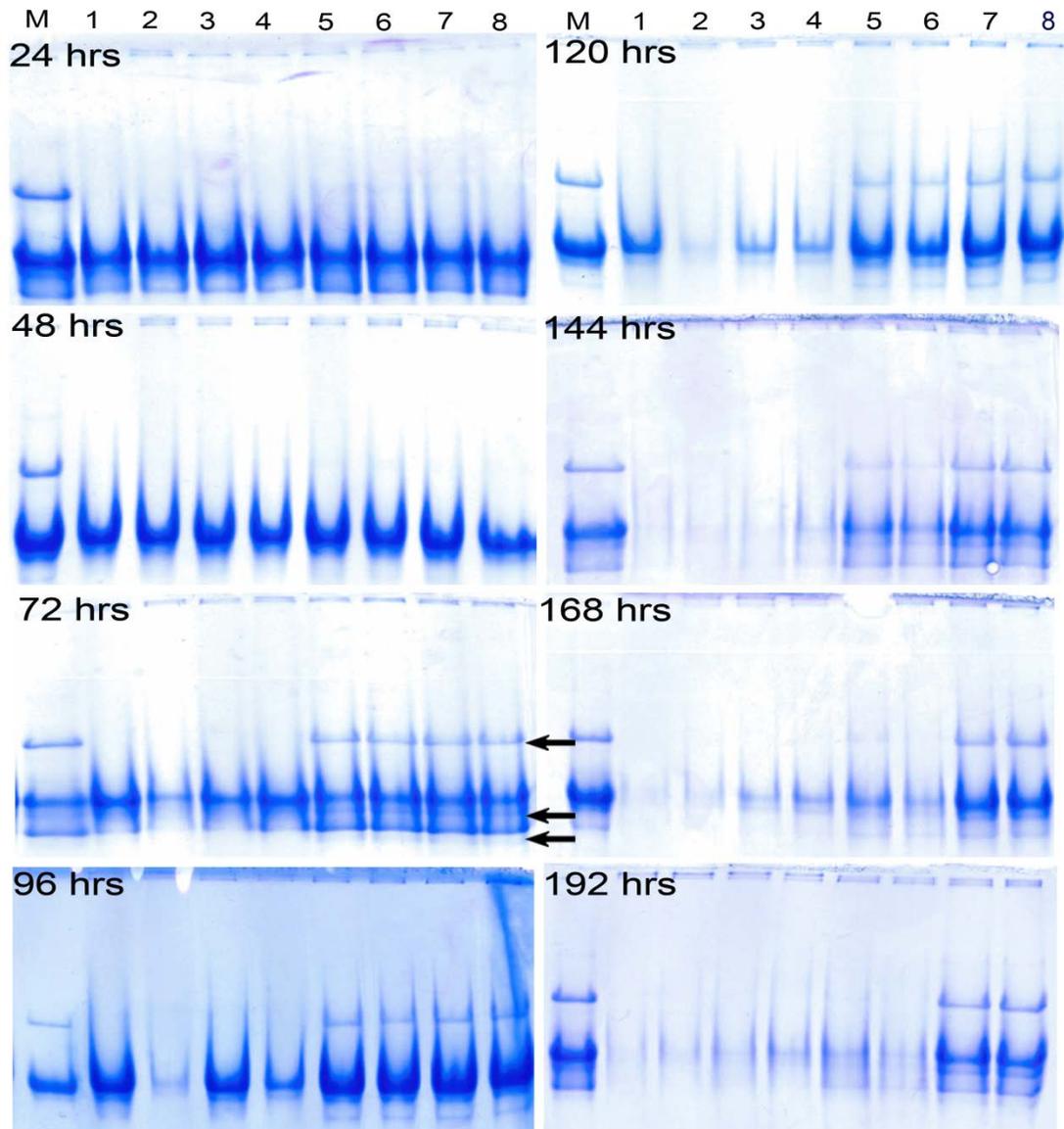


Figure S6 12% clear native PAGE analysis of A140C α Syn fibrillization reactions in 10 mM Tris-HCl, 1 or 10 mM NaCl, 2mM DTT at pH 7.4 and in the absence or presence of 0.1 mM EDTA. Likelihood of dimerization of A140C monomers through cysteines is evident from the dimer band shown by protein aliquot thawed from -80°C (lane M) in which active DTT is very low (see DTNB assay results). The dimer band disappears upon reduction of the disulfide bonds by addition of fresh DTT (see other 24 hrs lanes). The reactions in the absence of EDTA show a dimer band and additional bands by 72 hrs (indicated by black arrows). Lanes: M: A140C α Syn

monomer thawed from -80 °C; 1,2: duplicates of 10 mM NaCl with 0.1 mM EDTA; 3,4: duplicates of 1 mM NaCl with 0.1 mM EDTA; 5,6: duplicates of 10 mM NaCl without EDTA; 7,8: duplicates of 1 mM NaCl without EDTA.

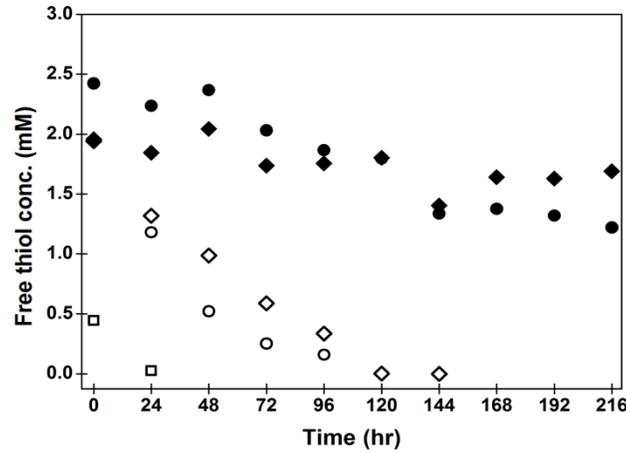


Figure S7 DTNB assay probing free thiols as an indicator of the DTT half-life in the absence or presence of 0.1 mM EDTA as a function of time. Aggregation buffer for the assay included 10 mM Tris-HCl, 10 mM NaCl, 2 mM DTT at pH 7.4. Aliquot of A140C α Syn monomers thawed from $-80\text{ }^{\circ}\text{C}$ (□), A140C α Syn aggregation reaction with fresh 2 mM DTT (○), A140C α Syn aggregation with fresh 2 mM DTT and 0.1 mM EDTA (●), aggregation buffer with fresh 2 mM DTT (◇), aggregation buffer with fresh 2 mM DTT and 0.1 mM EDTA (◆).

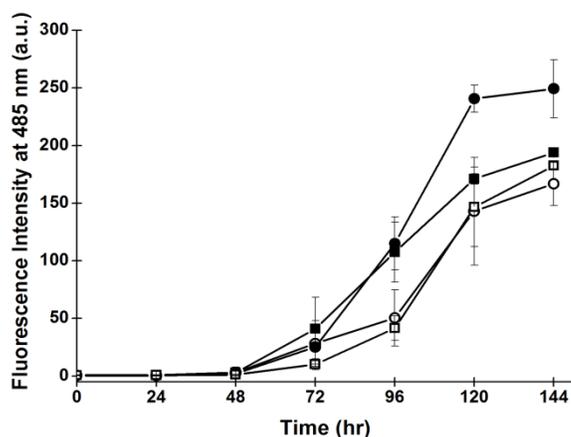


Figure S8 Effect of reducing conditions on wt α Syn aggregation as measured by ThT fluorescence. Aggregation buffer included 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 with no EDTA and DTT (○), 2 mM DTT (□), 0.1 mM EDTA (●) and 2 mM DTT and 0.1 mM EDTA (■). Error bars denote standard deviation among triplicates.

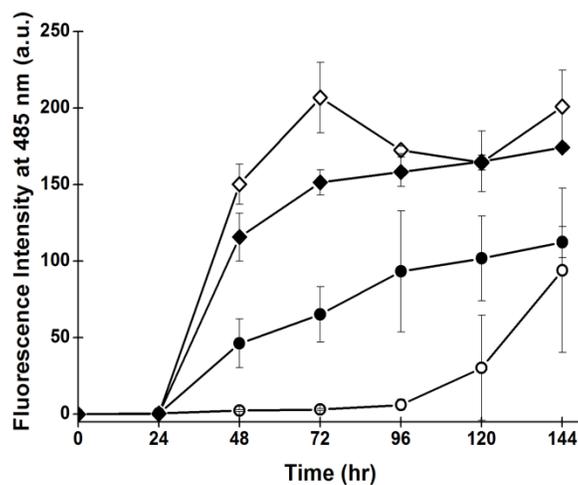


Figure S9 Effect of TCEP as reducing agent on A140C α Syn aggregation as measured by ThT fluorescence. Aggregation buffer included 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 with 2 mM DTT (○), 2 mM DTT and 0.1 mM EDTA (●), 2 mM TCEP (◇) and 2 mM TCEP and 0.1 mM EDTA (◆) at pH 7.4. Error bars denote standard deviation among triplicates.

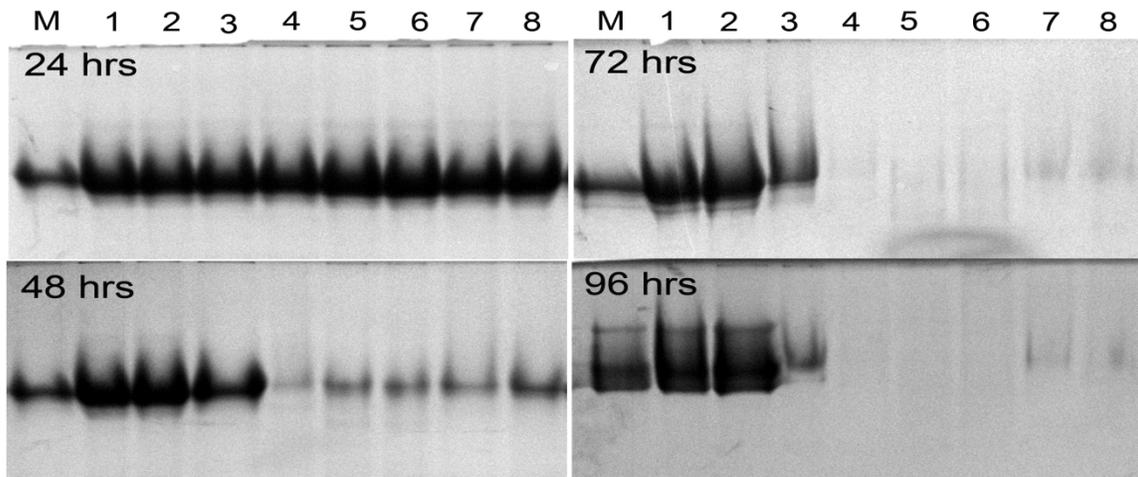


Figure S10 12 % clear native PAGE analysis of A140C α Syn aggregation with TCEP as reducing agent. Aggregation buffer included 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 with +/- TCEP or DTT and +/- EDTA. Lanes: M: Monomers, 1, 2: duplicates with 2 mM DTT; 3, 4: duplicates with 2 mM DTT and 0.1 mM EDTA; 5, 6: duplicates with 2 mM TCEP; 7, 8: duplicates with 2 mM TCEP and 0.1 mM EDTA at pH 7.4.

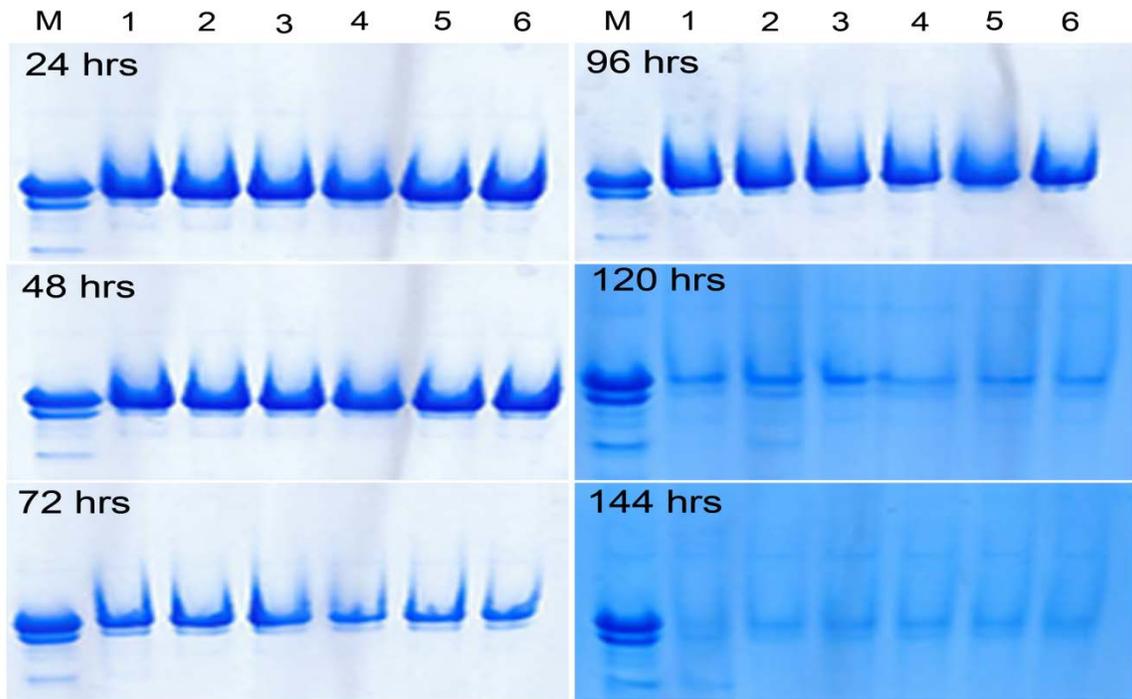


Figure S11 12% clear native PAGE analysis of wt α Syn fibrillization reactions. Aggregation reactions were set up in 10 mM Tris-HCl, 10 mM NaCl at pH 7.4 and in the absence or presence of 0.1 mM EDTA. Monomer protein concentration decreases with time dependent accumulation of fibrillar species, which do not electrophorese in the polyacrylamide gel. Faint additional bands could be observed around 120 and 144 hrs along with discernible difference in the monomer band intensity. Lanes: M = wt monomers; 1, 2, 3: triplicates of wt aggregation without 0.1 mM EDTA; 4, 5, 6: triplicates of wt aggregation with 0.1 mM EDTA.