Supporting Information for:

Formation Kinetics and physicochemical Properties of mesoscopic Alpha-Synuclein Assemblies modulated by Sodium Chloride and a distinct pulsed electric Field

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Experimental materials and methods

Expression and purification of recombinant α-synuclein protein.

The recombinant plasmid (General Biosystems, USA) composed of the full-length α-synuclein gene and pET-41a (+) vector was transformed to *E. coli* BL21(DE3) cell strain (Invitrogen, USA). A single colony of *E. coli* BL21(DE3) was cultured in 100 mL LB medium (150 mg/L ampicillin) at 37 °C up to OD_{600} between 0.7–1.5. Obtained cultures were induced by adding 1 mM IPTG and the following protein expression was performed up to 3 hours. Afterwards the cells were pelletized and resuspended in 0.75 mL of buffer (50 mM Tris pH 8.0, 10 mM EDTA, 150 mM NaCl). Resuspended cells were boiled for 10 min and then pelletized at 21130 x g for 5 min. The supernatant was transferred to a new tube and spined for 2 min after adding

streptomycin sulfate (136 µL of a 10% solution/mL of supernatant) and glacial acetic acid (228 µL/mL of supernatant), followed by one step of precipitation with saturated ammonium sulfate at 4 °C. Precipitated protein was again pelletized and one time washed with 1 mL of ammonium sulfate solution (1:1 (v/v) saturated ammonium sulfate (4 °C) and ddH_2O). The washed pellet was resuspended in 900 µL of 100 mM ammonium acetate and precipitated by adding 900 µL of ethanol at 20 °C. This step was repeated twice and followed by a resuspension in 20 mM Tris pH 7.4, 250 mM NaCl and 1 mM DTT. The resuspended solution of ASN tagged with 6x histidine amino acids was incubated with TEV protease overnight at 4 °C to the His-tag hydrolysis and ASN was purified by Ni-NTA column. Finally, monomeric ASN was obtained by performing the size exclusion chromatography utilizing a HiLoad 16/60 Superdex 75 pg column (Sigma, Neustadt, Germany) equilibrated with 20 mM Tris buffer pH 7.4, 250 mM NaCl and 1 mM DTT. The purity and dispersed homogeneity of obtained protein material was analyzed by a 12% SDS-PAGE and DLS (**Supplementary Figure 1**), which demonstrated the molecular weight to be 18 kD[1] and showed a hydrodynamic radius of 3.65 ± 0.51 nm (PDI: 15%) of monomeric ASN.

Polarized and depolarized dynamic light scattering (DLS/DDLS).

The in-house built DDLS instrument used for the experiments has been described previously.[2] Briefly, a laser beam with a wavelength of 532 nm and output power of 100 mW is polarized by a vertical polarizer before passing a sample solution. The vertical component and horizontal component of the scattered light were collected simultaneously by an objective (Plan APO ELWD 20 × 0.42 WD = 20) directly at 90° and by a polarizing beam splitter (Qioptic Photonics, Göttingen, Germany), respectively, to generate the DLS and DDLS signals. A transparent quartz cuvette (101.015-QS, Hellma Analytics, Munich, Germany) with inner dimensions of 3 mm × 3 mm × 21 mm was used and immersed into an index matching water bath (1 mm thickness). For applying the pulsed electric fields (EFs) during DLS/DDLS measurement, two platinum (Pt) wires with 0.3 mm diameter, 25 mm length and a resistance (R) of approx. 37.5 mΩ at 20 °C (Sigma, Neustadt, Germany) were inserted at two opposite corners of the cuvette. The up-and-down waveform of the applied pulsed EF was shown in previous publications,[3,4] having a pulse width of 0.6 ± 0.15 ms with a maximum output pulse amplitude of 30 V and no more than 1 V of minimum amplitude on 500 Ω load.

For DLS/DDLS measurements a 40 µL ASN solution was pipetted into the cuvette and sealed with a cover slide. Each data point was collected with an acquisition time of 20 s, followed by a delay time of 20 s. The hydrodynamic radii (R_h) of the proteins were calculated based on the Stokes-Einstein equation (equation 1)[5] and Stokes-Einstein-Debye equation (equation 2)[6]:

$$D_t = \frac{K_B T}{6\pi\eta R_h} \quad (1)$$

$$D_r = \frac{K_B T}{8\pi\eta R_h^3} \quad (2)$$

where D_t and D_r represent the translational and rotational diffusion coefficients, respectively, obtained from the corresponding autocorrelation function applying the CONTIN algorithm.[7] K_B is the Boltzmann coefficient; T is the absolute temperature and η is the viscosity value of the solution.

An appropriate viscosity value of each sample solution was considered for the R_h calculations. ASN solutions were prepared in 20 mM Tris buffer pH 7.4 and a gradient of NaCl (0, 50, 150, 250 mM). To monitor the radii and structural evolution of ASN clusters 15% PEG8000 was introduced into 50 µM ASN solutions. All sample conditions were used for experiments without and with exposing sample solutions with EFs for 2 hours.

Imaging via optical brightfield microscope and transmission electron microscopy (TEM).

The phase diagram of ASN was investigated at 20 °C in a protein concentration range of 5-500 µM, applying PEG8000 as crowding agent from 0 up to 20% (w/v) 20 mM Tris buffer pH 7.4, 150 mM NaCl. Images of droplets were recorded applying a cold-light source Leica M205C microscope after 1, 24, 48, and 72 hours.

For TEM imaging the same sample conditions were applied as for the DDLS experiment. After 3 days incubation at 20 °C, a 4 µL of ASN solution was pipetted onto a quantifoil holey carbon coated copper grid (quantifoil 1.2/1.3, 400 mesh, Science Services, Germany), which was glow-discharged for 30 s at 25 mV (GloQube Plus, Quorum) before using. Samples were blotted with a Whatman paper after 30 s incubation, following by two times of grid washing with 10 µL ultrapure water. Afterwards, the grid was placed on the droplet of 10 µL of 2% (w/v) uranyl acetate for negative staining. After 30 s, the grid was removed from the uranyl acetate droplet, blotted and dried on the Whatman paper. TEM images were recorded using a JEM 2100-Plus with 200 kV acceleration voltage.[8]

Congo red staining and Thioflavin T (ThT) fluorescence assays.

0.1% (w/v) Congo red solution and 1 mM Thioflavin T (ThT) solution were prepared in 20 mM Tris buffer pH 7.4 and filtered through a 0.2 μm filter (SARSTEDT, Germany). For Congo red staining, 0.3 μL of 0.1% (w/v) Congo red solutions were added to 3 μL of ASN droplets. After 5-minute incubation at 20 ℃, the droplets were observed applying a Leica M205C microscope.

For ThT assays 1 μL of ThT solution was added to 50 μL ASN sample using a Corning 384 flat bottom black polystyrene plate (Sigma, Neustadt, Germany). The fluorescence intensity was recorded from the top at 20℃ using a TECAN plate reader (Infinite 200 PRO, Tecan Trading AG, Switzerland) at an excitation wavelength of 440 nm and recording an emission wavelength of 480 nm. Assays were performed in triplicates.

Determining the diffusion interaction parameter (K_D) and analyzing the thermostability of α-synuclein via DLS.

A Wyatt Mobius instrument (Wyatt Technology, Santa Barbara, CA, USA) with a sample temperature controller (4-70 ℃) and) and laser wavelength of 532 nm was used to analyze the thermostability and determine K_D of ASN. The translational diffusion coefficient D_t determined by DLS is a function of concentration c [9]:

$$D_t=D_0(1+K_Dc) \quad (3)$$

where D_0 is the diffusion coefficient at infinite dilution, and c is the concentration of the protein in mg/mL, K_D is the first-order diffusion interaction parameter.

For the determination of ASN K_D values in different NaCl solutions, six concentrations of ASN ranging from 1 to 11 mg/mL were applied. Each sample, centrifuged for 1 hour at 21130 x g and 4 ℃ prior to DLS measurements, triplicates with a recording time of 5 s and an interval time of 1 s were measured. The corresponding K_D value of ASN under each NaCl condition was calculated and analyzed applying the software DYNAMICS, applying equation 3.

For the thermostability measurements, the R_h of monomeric ASN at 100 μM in 20 mM Tris buffer pH 7.4 and at different NaCl concentrations (0, 50, 150, and 250 mM) was measured firstly applying a ramp-up temperature (20 ℃ up to 70 ℃) followed by a ramp-down temperature gradient (70 ℃ to 20 ℃). R_h values of ASN assemblies induced by 15% PEG8000 in the presence and absence of EFs in solutions with 20 mM Tris

buffer pH 7.4 and a series of NaCl (0, 50, 150, and 250 mM) were measured with identical temperature gradients, increasing up to 70 °C firstly and then cooling down to 20 °C, in a ramp speed of 1 °C/min. The DLS data were recorded with a temperature step of 1 °C and each data point generated is the average of triplicate acquisitions applying an acquisition time of 5 s in intervals of 1 s.

Wyatt DYNAMICS uses two types of fits for autocorrelation functions, cumulants and regularization fits. The cumulants fit assumes one population of particles with a single average diffusion coefficient and a single standard deviation about the average, while the regularizations fit assumes the presence of any number of populations of particles and each with its own polydispersity. Following the DYNAMICS user guide, the cumulant radii derived from the cumulants fit were used to calculate the K_D values and were used for the temperature-dependence analysis.[10]

Characterizing the autofluorescence of assembled α-synuclein applying confocal laser scanning microscopy (CLSM).

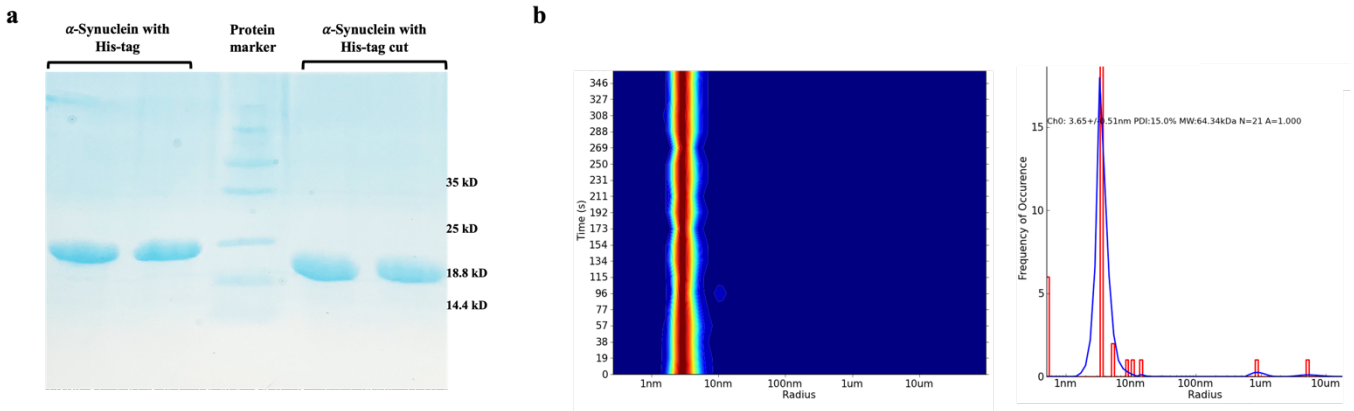
A confocal laser scanning microscope (SP8, Leica) equipped with a 405 nm laser excitation source, spectral detector, and a 63x oil immersion objective with a numerical aperture (N.A.) of 1.4 was used for imaging and fluorescence detection. 150 µL of samples were loaded into the wells of an 8-well chambered coverslip with 300 µL/well capacity and a glass bottom of 170 µm ± 5 µm thickness (μ-Slide 8 Well ^{high} Glass Bottom, Ibidi GmbH, Gräfelfing, Germany). To measure fluorescence spectra an excitation at 405 nm was used and the fluorescence intensities were detected in the wavelength range of 420-780 nm with a detection bandwidth of 20 nm and a step size of 3 nm. The fluorescence spectra of manually chosen background regions were subtracted from the spectra of ASN assemblies. Data analysis was performed applying GraphPad Prism software (Version 9.2.0, San Diego, US) and the normalized spectra were plotted and fitted with a polynomial function of six orders.

Measuring the secondary structure of monomeric α-synuclein applying circular dichroism.

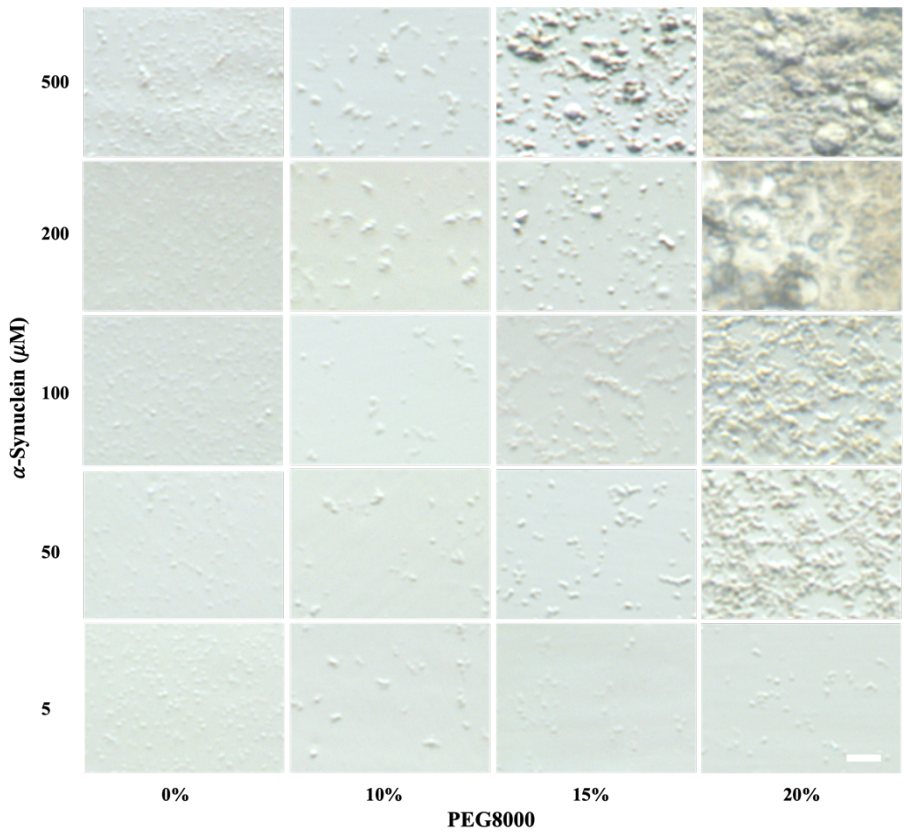
ASN at 1 mg/mL in 20 mM Tris buffer pH 7.4 was prepared with four different concentrations of NaF (0, 50, 150, 250 mM) and centrifuged at 16000 x g, 4 °C for 1 h. For investigating the effect of EFs on the secondary structure of ASN, a 50 µL ASN solution was exposed to EFs for 1 hour, followed by 5-minutes centrifugation

at 21130 x g and 20 °C to remove aggregates. All samples not exposed or exposed to EFs were diluted by the corresponding buffer to a final concentration of 0.2 mg/mL ASN. Finally, all diluted samples were transferred to a quartz cuvette with 1 mm path length (Hellma- Analystics, Germany) and were measured applying a JASCO-815 spectropolarimeter (JASCO, Japan). In each measurement 10 individual spectra were averaged, recorded within a wavelength range of 260-190 nm, with a scanning speed of 100 nm/min and a bandwidth of 1 nm. The spectra were evaluated by the Software Spectra Manager from JASCO.

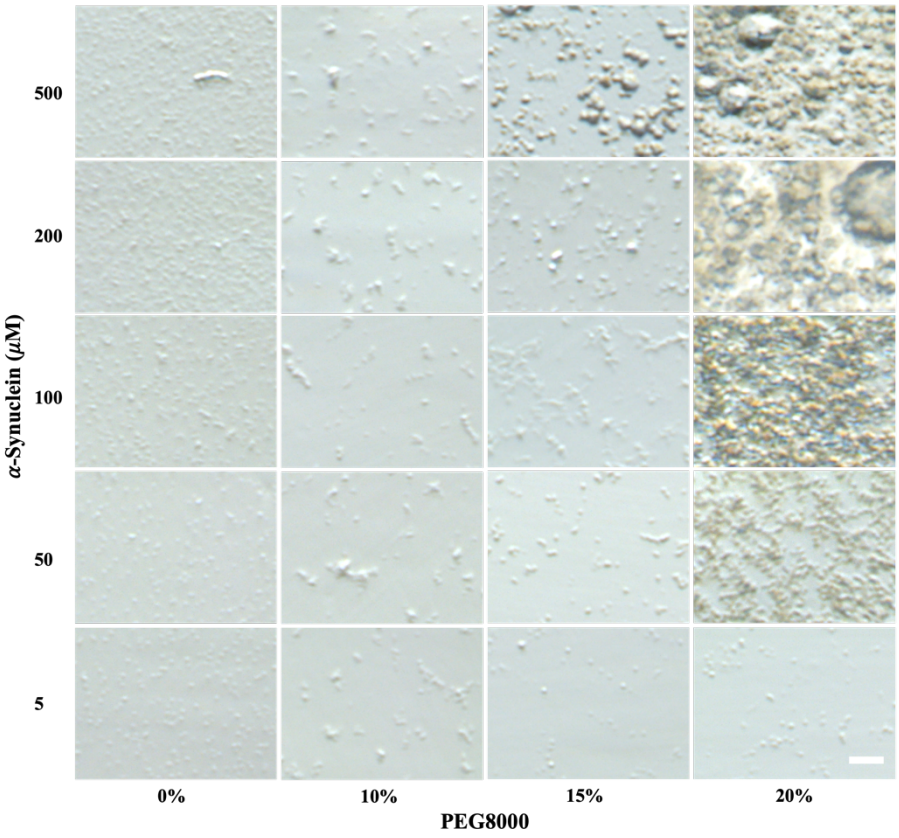
Supplementary figures



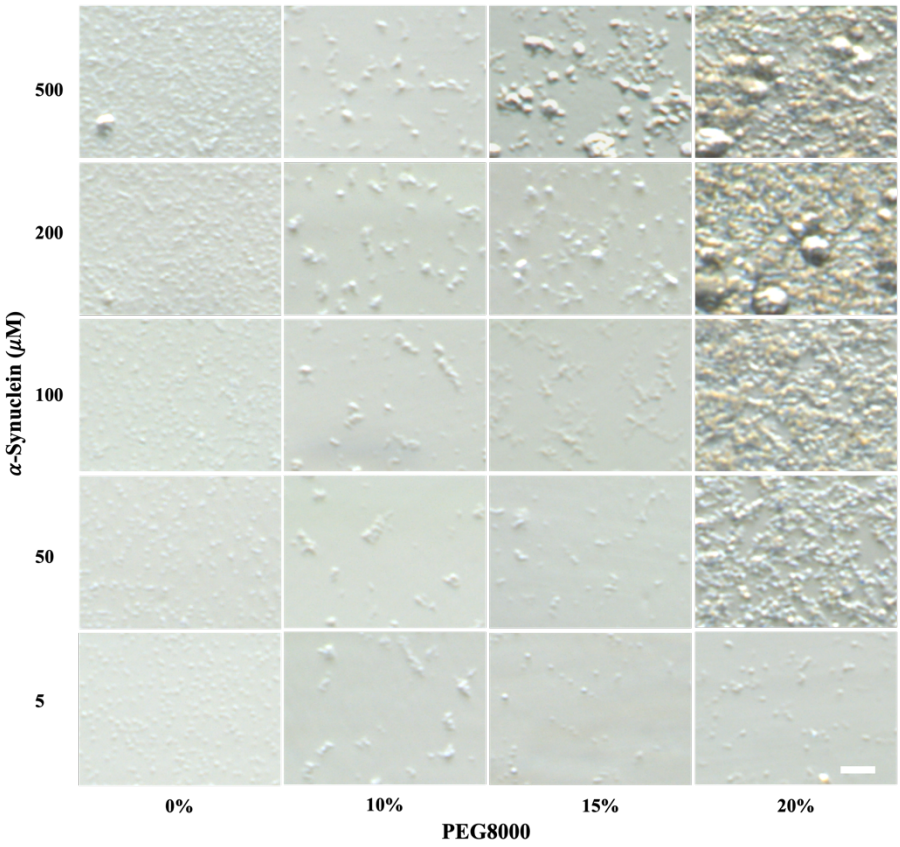
Supplementary Figure 1. Quality control of purified α-synuclein applying (a) 12% SDS-PAGE and (b) DLS.



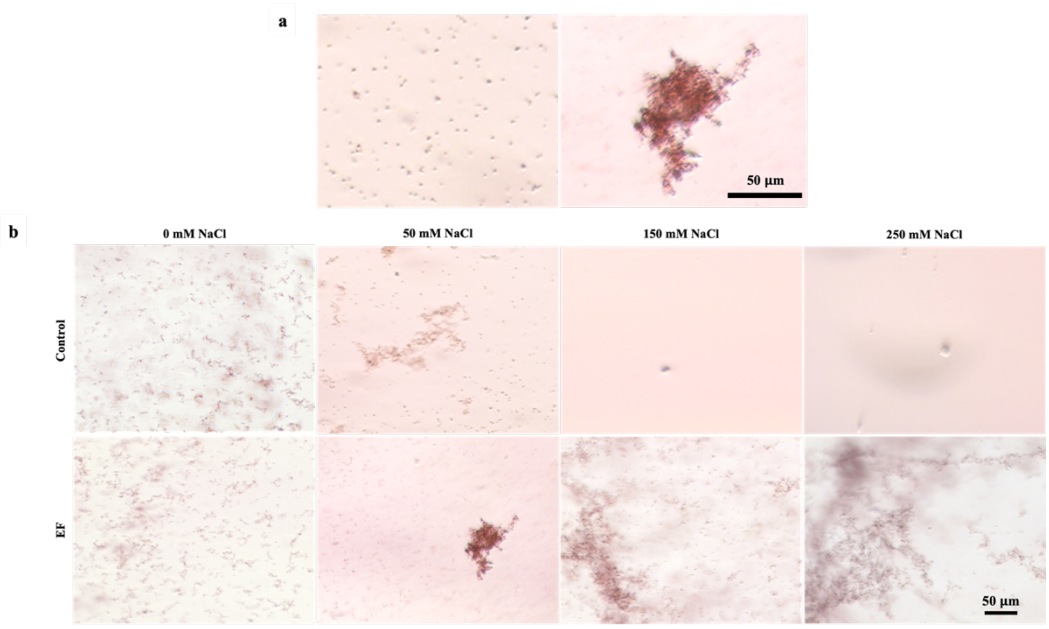
Supplementary Figure 2. Phase diagram of α-synuclein obtained after 24-hour incubation at 20 ℃ in solutions of 20 mM Tris pH 7.4, 150 mM NaCl, and PEG8000. The scale bar corresponds to 10 µm.



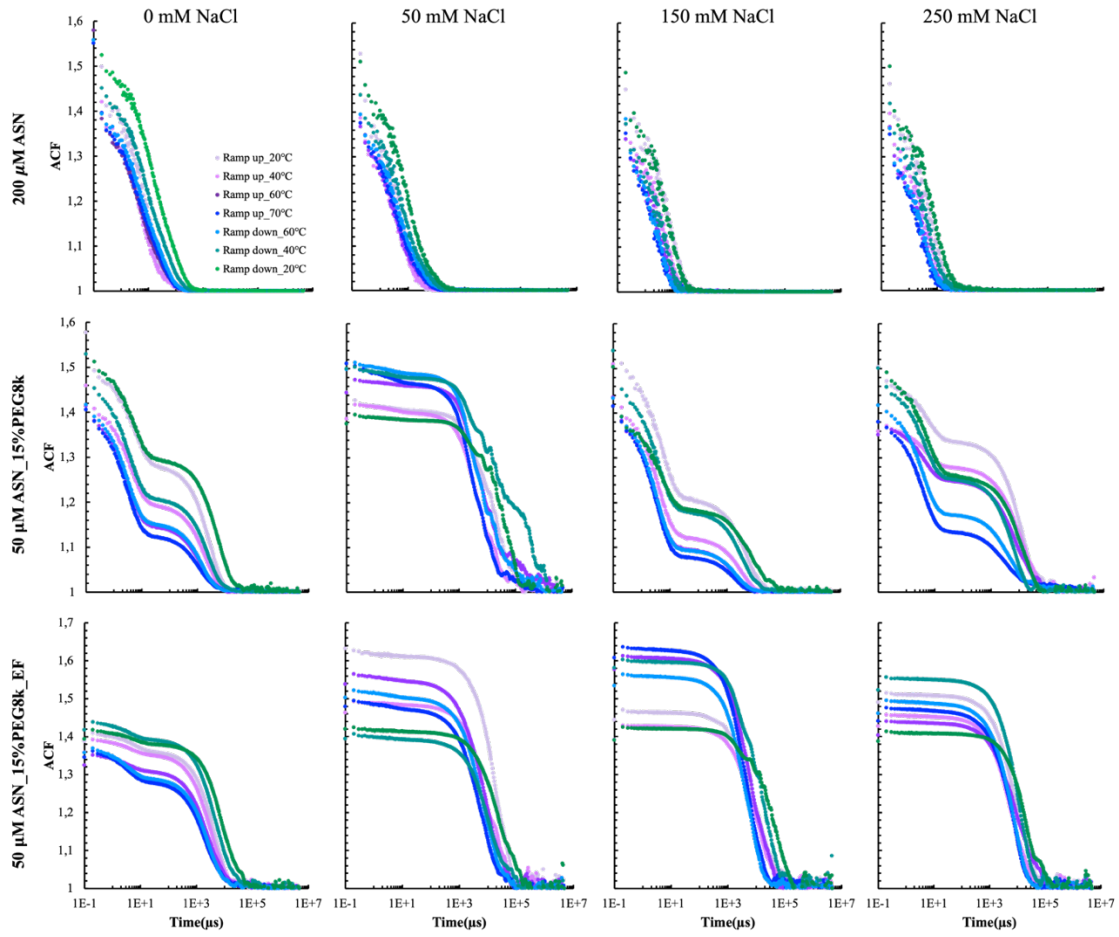
Supplementary Figure 3. Phase diagram of α-synuclein observed after 48-hour incubation at 20 ℃ in solutions of 20 mM Tris pH 7.4, 150 mM NaCl, and PEG8000. The scale bar corresponds to 10 µm.



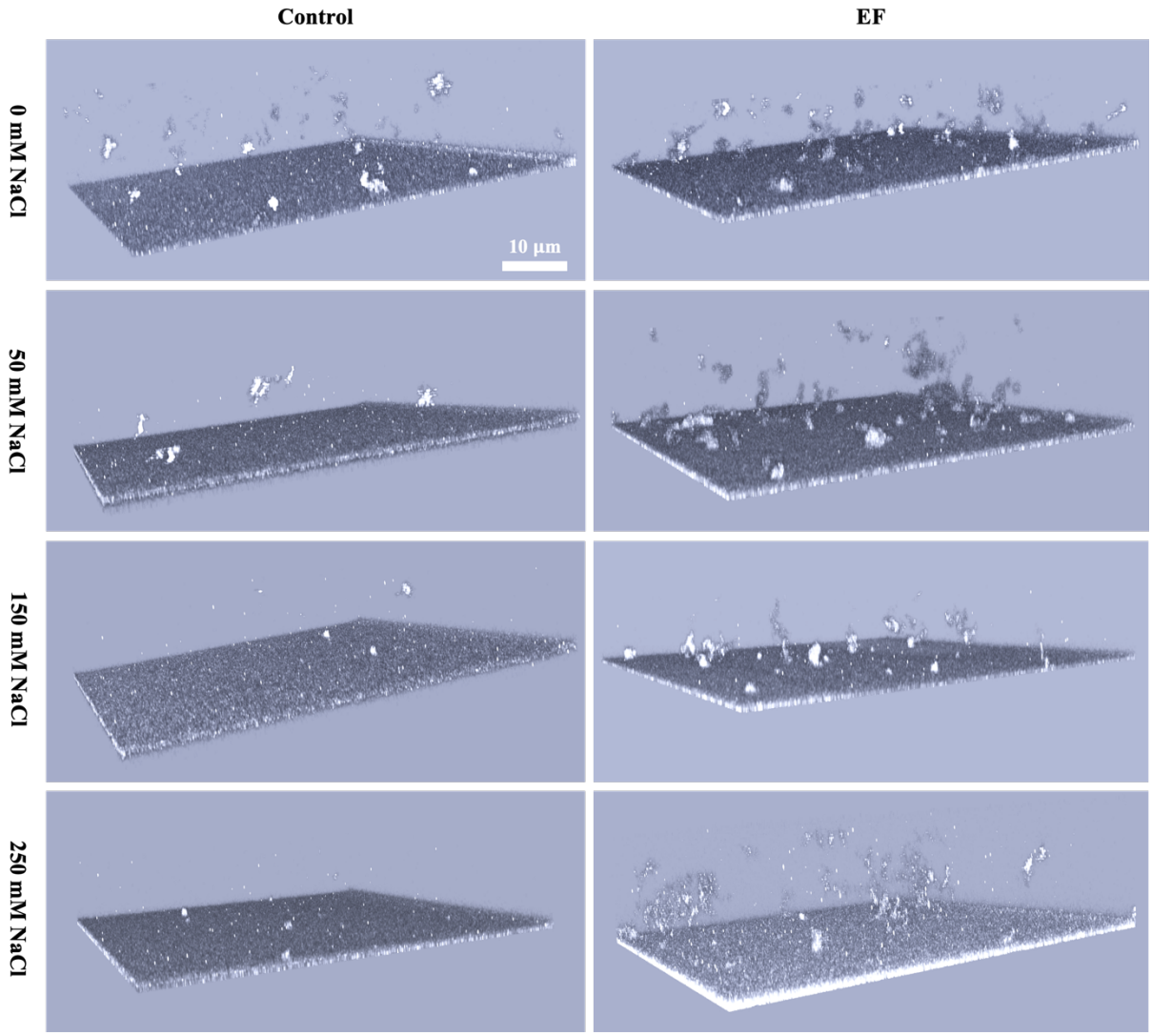
Supplementary Figure 4. Phase diagram of α-synuclein collected after a 72-hour incubation at 20 °C in solutions of 20 mM Tris pH 7.4, 150 mM NaCl, and PEG8000. The scale bar corresponds to 10 µm.



Supplementary Figure 5. (a) Examples of monodispersed and stacked globular assemblies of ASN stained by 0.01% (w/v) Congo red. (b) The Congo red staining results for ASN assemblies formed after 3 days in solutions of 50 µM ASN, 15% PEG8000, and a NaCl gradient without (control) or with the application of a pulsed electric field (EF) at 20 °C.



Supplementary Figure 6. Regularization autocorrelation function (ACF) curves of DLS measurements for α-synuclein with the increase (20 ℃ → 70 ℃) and decrease (70 ℃ → 20 ℃) of the temperature in different solutions.



Supplementary Figure 7. Representative three-dimensional images of α-synuclein solutions scanned in-situ by confocal laser scanning microscopy (CLSM), after 2-hour incubation at 20 ℃ without (control) or with the treatment of a pulsed EF.

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