Supplementary Information

Pharmacological inhibition of α-synuclein aggregation within liquid condensates

Samuel T. Dada, Zenon Toprakcioglu, Maarten C. Hardenberg, Mariana P. Cali, Lena K. Mrugalla, Owen M. Morris, Alexander H.W. Roentgen, Tuomas P.J. Knowles and Michele Vendruscolo*

Centre for Misfolding Diseases, Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, UK

Chemicals

Claramine (Sigma-Aldrich, MO, USA) was synthesised as a trifluoroacetate salt at a purity >98% as measured by high-performance liquid chromatography (HPLC), stored as a lyophilized powder, and solubilised in DMSO (100%) to a final concentration of 10 mM. Molecules were stored at −80 °C and thawed once before each experiment.

Expression and purification of α-synuclein

Wild-type and cysteine variant A90C α-synuclein were expressed as previously described, using *E. coli* BL21 (DE3)-gold competent cells (Agilent Technologies) expressing the pT7-7 plasmid encoding α-synuclein. The expression of α-synuclein was induced by the addition of 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG). Cells were subsequently centrifuged, lysed by sonication, denatured by boiling before the precipitation of α-synuclein using ammonium sulphate. Following this, protein pellets were dialysed and purified through ion exchange and size exclusion chromatography into 50 mM trisaminomethane-hydrochloride (Tris-HCl) pH 7.4 buffer. All buffers used in the dialysis and purification of the α-synuclein A90C cysteine variant contained 1 mM dithiothreitol (DTT) to prevent the formation of disulfide bonds. The final protein concentration was measured using ultraviolet-visible (UV-vis) spectroscopy on a Cary 100 system (Agilent Technologies). All proteins were aliquoted, flash-frozen in liquid nitrogen, stored at -80 °C and thawed once before each experiment.

α-Synuclein labelling

The α-synuclein A90C cysteine variant was labelled with 1.5-fold molar excess of C5 maleimide-linked Alexa Fluor 647 (Invitrogen Life Technologies) overnight at 4 °C under constant gentle stirring. The unbound dye was removed using Amicon Ultra-15 Centrifugal Filter Units and buffer exchanged into 50 mM Tris-HCl at pH 7.4 by size exclusion chromatography. The final protein concentration was measured using ultraviolet-visible (UV-vis) spectroscopy on a Cary 100 system (Agilent Technologies). All proteins were aliquoted, flash-frozen in liquid nitrogen, stored at -80 °C and thawed once before each experiment.

α-Synuclein phase separation assay

To induce liquid droplet formation, non-labelled wild-type α-synuclein was mixed with the A90C variant labelled with Alexa Fluor 647 at a 100:1 molar ratio in either 50 mM Tris-HCl pH 7.4 and 10% polyethylene glycol 10,000 (PEG) (Thermo Fisher Scientific) by volume at room temperature (20-22 °C). Additionally, claramine, dimethyl sulfoxide (1% DMSO) and preformed fibrils were added to the protein phase separation assay for various experiments. 10 µL of the final mixture was pipetted on a 35 mm glass bottom dish (P35G-1.5-20-C, MatTek Life Sciences) and immediately imaged on a Leica Stellaris Will inverted confocal microscope using a 40×/1.3 HC PL Apo CS2 oil objective (Leica Microsystems). The excitation wavelength was set to 633 nm for all experiments. All images were processed and analysed in ImageJ (NIH).

α-Synuclein aggregation assay within condensates

Thioflavin T (ThT) (20 µM (Sigma)), claramine, 1% DMSO and preformed fibrils, depending on the experiments, were mixed with monomeric wild-type α-synuclein, containing 1 molar % A90C α-synuclein labelled with Alexa Fluor 647 prior to each experiment. The assay mixture, which included 50 mM Tris-HCl, pH 7.4, 10% PEG 10,000, was pipetted onto a 35 mm glass bottom dish and imaged on a Leica Stellaris Will inverted confocal microscope using a 40×/1.3 HC PL Apo CS2 oil objective. The excitation wavelength 633 nm and 488 nm were used for Alexa Fluor 647 labelled α-synuclein and ThT, respectively. Images were acquired every min for ~40 mins. Images were processed on ImageJ:

an area adjacent to the edge of the droplet was cropped and analysed, thus allowing for time-dependent measurement of amyloid formation at the droplets.

α-Synuclein preformed fibril seeds preparation

α-Synuclein pre-formed fibrils were formed from recombinant α-synuclein wild-type monomers diluted in buffer (50 mM Tris-HCl at pH 7.4) to concentrations of approximately 500 μM. Monomers in were incubated at 40 °C, with constant stirring speed (1,500 rpm) with a PTFE micro stirrer bar and left to aggregate on an RCT Basic Heat Plate (RCT Basic, model no. 0003810002; IKA, Staufen, Germany) for up to 72 h. Samples were centrifuged at 4 °C at 14,000 rpm for 15 min. Once fibrils were isolated from supernatant, they were suspended in buffer equivalent to discarded supernatant. The fibril concentration was measured by dissociating a small aliquot of fibrils in a total solution of 4 M guanidinium hydrochloride (GndHCl). After a 30 min incubation period, the fibril concentration was measured using UV-vis on a Cary 100 system (Agilent Technologies). The fibrils were aliquoted and stored at room temperature before experimentation. Before each experiment, fibrils were pre-treated by 15 sec (15 pulses) sonication at 10% power with 50% duty cycle using a Microtip sonicator (Bandelin Sonopuls HD2070) to disperse lumped fibrils.

Fourier-transform infrared spectroscopy (FTIR)

Product from protein phase separation assay in the presence of DMSO (1%) and claramine (75 μM) upon condensate and amyloid formation were washed off glass slide with 50 mM Tris-HCL. Product was centrifuged at 12,000 rpm for 10 min, at RT. Supernatant containing soluble α-synuclein was discarded, and pellets were washed twice with H_2O. Pellets were resuspended in 3 μL H_2O and subjected to attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy. 2 μL of solution were applied to a Perkin-Elmer Spectrum100 FTIR with an ATR diamond attachment and dried. Prior to scanning, sample and electronics chambers were purged with a constant flow of dry air. 100 replicate scans were averaged from 4000 to 800 cm^{-1}, normalized to amide I intensity (~ 1630 cm^{-1} peak), and second derivatives were taken with 9 points for slope analysis.

Estimation of the α-synuclein concentration required for phase separation in water-in-oil droplets

Fabrication of microfluidic devices. The fabrication process of the microfluidics was taken from a previously established protocol[1-3]. In brief, a soft photolithographic process was used to fabricate the master through which microfluidic devices were made. A 50 μm photoresist (SU-8 3050, MicroChem) was spin-coated onto a silicon wafer. This was soft baked at 95 °C for 3 min. A film mask was placed on the wafer and the whole system was exposed in UV light to induce polymerization. The wafer was then baked at 95 °C for 30 min. Finally, the master was placed into a solution of propylene glycol methyl ether acetate (PGMEA, Sigma-Aldrich), which helped in the development process. Elastomer polydimethylsiloxane (PDMS) with curing agent (Sylgard 184, DowCorning, Midland, MI) was mixed at a ratio of 10:1 to fabricate the devices. This mixture was then incubated at 65 °C and cured for a total of 3 h. Once hardened, the PDMS was peeled off the master, and holes were punched into the PDMS, which acted as inlets and outlets. Finally, the PDMS slab was bound to a glass slide by treatment with a plasma bonder (Diener Electronic, Ebhausen, Germany).

Formation and confinement of droplets. neMESYS syringe pumps. Syringe pumps (Cetoni, Korbussen, Germany) were used to control the flow rates within the microchannels. Protein solution was mixed with PEG at a ratio of 1:1 at the first junction. At the second junction, the oil phase, which consisted of fluorinated oil (Fluorinert FC-40, Sigma-Aldrich) and 2% w/w fluorosurfactant (RAN biotechnologies) intersected the aqueous phase resulting in water-in-oil droplets being formed. Following droplet generation, droplets were confined within a microfluidic trap[1-3]. In brief, droplets are directed towards

an array of traps whereby once a droplet is driven within the microfluidic confinement, it is unable to escape unless a pressure is applied from the outlet. Droplets were then incubated at room temperature to allow for shrinkage. This resulted in an increase of the local concentration of protein and PEG within the droplets which led to protein phase separation. The water-in-oil droplets and the protein phase separation was monitored using fluorescence microscopy.

Calculation of protein concentration during phase separation. The concentration of the protein was obtained by calculating the ratio of the droplet volume just after trapping (V_1) and at the point of phase separation (V_2), i.e. at the point at which condensates start appearing within the water-in-oil droplet. By multiplying the initial monomeric protein concertation by the value of this ratio, the actual protein concentration at the point of phase separation could be determined.

Transmission electron microscopy (TEM)

α-Synuclein samples from the ThT-based aggregation assay that contained either DMSO (1%) or claramine (75 μM) were obtained after fibril formation. The obtained sample (5 μL) was deposited on a carbon film of 400 mesh 3 mm copper grid. The grids were washed once with Milli-Q water, then incubated with 1% (w/v) uranyl acetate for 2 min and washed twice again with Milli-Q water before being air-dried at room temperature. Samples were imaged using a Tecnai G2 transmission electron microscope operating at 80–200 keV.

Liquid-chromatography mass spectrometry (LC-MS)

Monomeric α-synuclein was diluted in storage buffer to a concentration of 5 μM in the absence and presence of claramine (1:1 protein drug molar ratio). Protein liquid-chromatography mass spectrometry (LC-MS) was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPC system using an Acquity UPLC BEH300 C4 column. The mobile phase was composed of H2O with 0.1% formic acid (solvent A) and 95 % MeCN and H2O with 0.1% formic acid (solvent B) at a flow rate of 0.2 mL/min. The electrospray source was operating with a capillary and cone voltage of 20 kV and 40 C, respectively. Nitrogen was applied as desolvation gas at a total flow rate of 850 L/h. The mass spectra were reconstructed using the MaxEnt algorithm on the MassLynx software according to the manual.

AmyloFit data analysis

The aggregation of α-synuclein within droplets was monitored as described above. The experimental ThT fluorescence readout was uploaded on the free online platform AmyloFit[4] (https://www.amylofit.ch.cam.ac.uk). Next, the software normalised the data to 0% and 100% by averaging the values at the baseline and the plateau of the reaction. Upon data normalisation, the concentration of aggregate mass could be observed as a function of time. The minimum, maximum fluorescence intensity and aggregation half-time were calculated from the time at which half the protein that is present, initially as monomer, has aggregated, i.e., the time at which the normalized intensity reaches 0.5. An advanced basin-hopping algorithm was applied to fit the experimental data to a model of protein aggregation. Each experiment was repeated four times and then averaged before fitting, while a primary nucleation dominated model was assumed. The number of basin hops was set to 40 for each fit. The applied model was only considered suitable if it was able to match the experimental data well. The AmyloFit user manual can be consulted for more in-depth information on the fitting procedure [4].

C. elegans strains and maintenance

The *C. elegans* AM134 ((rmIs126[P(unc-54)Q0::YFP]), (YFP)) strain was the control strain used in this study, other strain used was, α-synuclein transgenic strain OW40 ((zgIs15 [P(unc-54)αsyn::YFP]), in which α-synuclein is expressed in the body wall muscle cells and fused to YFP[5,6]. Synchronised

axenic nematodes were acquired by bleaching gravid hermaphrodite adult nematodes with sodium hyochlorite. Axenised eggs were resuspended in minimal media buffer (M9) (0.3% KH2PO4, 0.6% Na2HPO4, 0.5% NaCl and 1 mM/L MgSO4), incubated overnight at 20 °C to hatch. Nematodes were maintained on OP50 *Escherichia coli* bacterial strain seeded on nematode growth medium (NGM) (0.3% NaCl, 0.75% casein, 1 mM MgSO4, 1 mM CaCl2, 250 mM KH2PO4 (pH 6) and 5 µg/mL mM cholesterol) agar (1.7%) plates. For experimental purposes, 5-fluoro-2'deoxyuridine (FUDR) (75 µM) were added to agar plates to inhibit generation of offspring. 1% of DMSO was seeded onto bacteria lawn of seeded plates for control and 5 µM of claramine was added to experimental plates. Experiments were conducted with L4 stage nematodes at 20 °C and plates were incubated at 20 °C.

Confocal microscopy inclusions quantification in C. elegans

At indicated time points, nematodes were washed with M9, and palleted in 1 mL of M9 solution containing anaesthetic levamisole (10 mM) (Sigma) to induce paralysis. Nematodes were mounted onto a glass petri plate (MatTek) with the aid of CyGEL™ (Biosatus, Ltd), a thermoreversible hydrogel which is liquid when cold and a gel when warmed at room temperature. High-magnification images were acquired with a Leica TCS SP5 inverted confocal microscope scope with an 20x/1.3 HC PL Apo CS2 oil objective. YFP was detected using 512 nm as excitation and an emission range from 549-550 nm. At least 4 nematodes were imaged per condition. Representative confocal images of nematodes displaying the head (between the tip of nose and the pharyngeal bulb) were analysed using FIJI. Inclusions were defined as having an area greater than 10 square pixels (0.2 µm/pixel) and a circularity of 0.5-1.

Automated C. elegans motility assay

L4 Nematode nematodes were cultured at 20 °C on either DMSO (1%) or claramine (5 µM), motility assay was carried out on NGM agar plates, at indicated time points, and the nematodes were washed off the plates with M9 buffer and spread over an unseeded NGM agar plate, after which their movements were recorded at 20 fps, using a lab-developed microscopic setup between 30 sec to 1 min. At least 100 nematodes were counted in each experiment unless stated otherwise. Videos were analysed using a custom-made tracking code.

C. elegans fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed on nematodes at indicated timepoints using a Leica Stellaris Will inverted stage scanning confocal microscope. To conduct FRAP experiments, a 63x magnification oil objective (63x/1.4 HC PL Apo CS oil) was used. Nematodes were paralysed and immobilised using levamisole (10 mM) and CyGEL respectively on a glass bottom petri plate. Bleaching was done using the 488 nm laser at 20% intensity for 5 sec following a 2 sec pre-bleach sequence. Immediate post-beach images were captured at ~1300 ms per frame rate, for ~25 sec. Intensity traces of bleached area were background corrected and normalised to reference signal and FRAP time which is defined by the time to half maximal signal recovery. This was determined using the Leica TCS Stellaris FRAP wizard system on the confocal microscope.

Supplementary References

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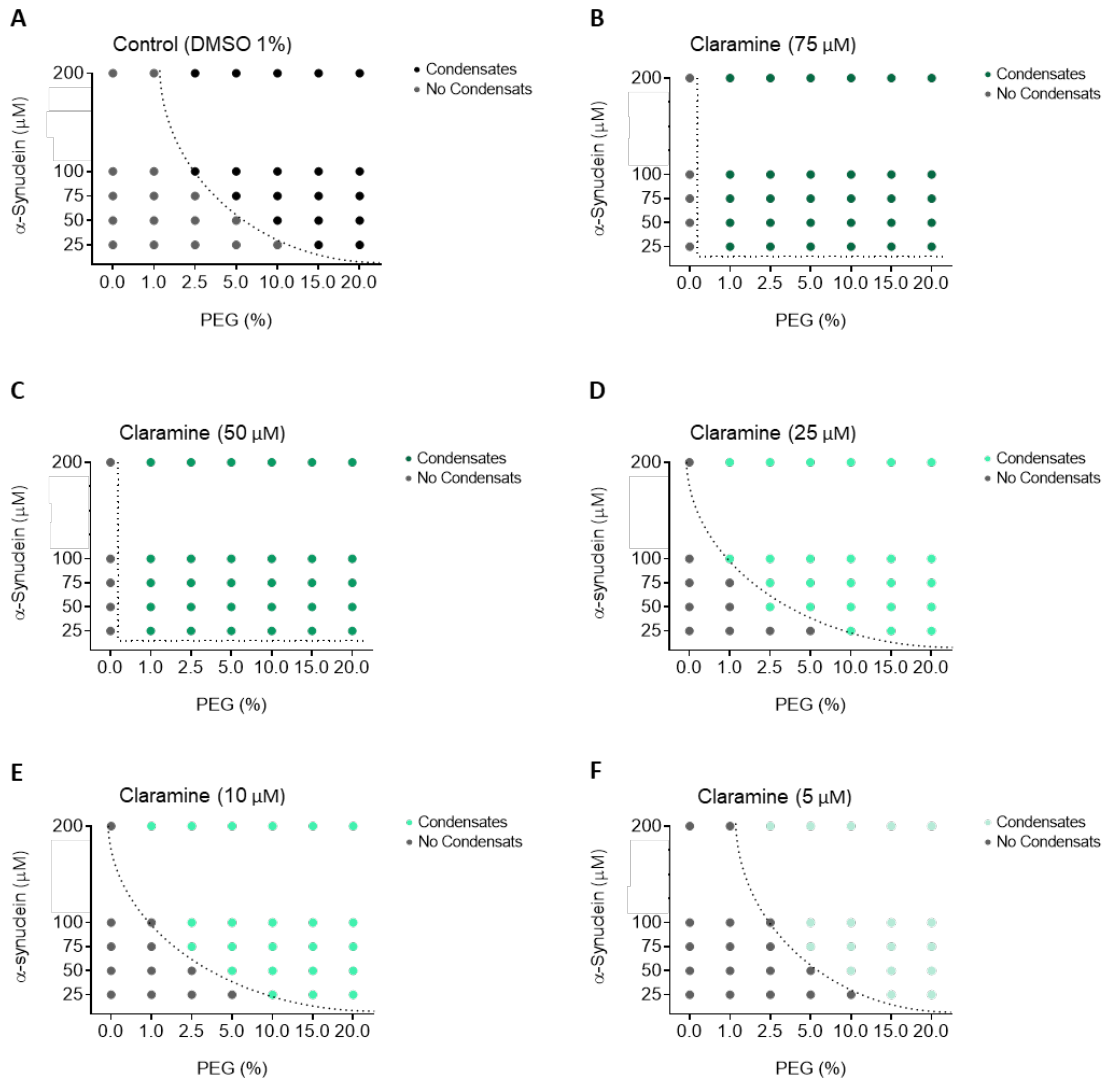
4 Meisl, G. *et al.* Molecular mechanisms of protein aggregation from global fitting of kinetic models. *Nat. Protoc.* **11**, 252-272 (2016).

5 Perni, M. *et al.* A natural product inhibits the initiation of α-synuclein aggregation and suppresses its toxicity. *Proc. Natl. Acad. Sci. USA* **114**, E1009-E1017 (2017).

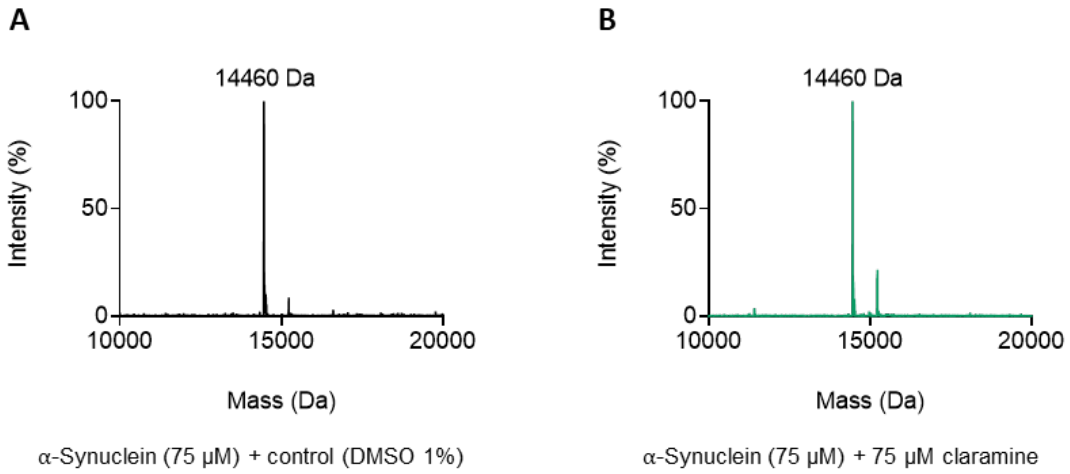
6 Van Ham, T. J. *et al.* C. Elegans model identifies genetic modifiers of α-synuclein inclusion formation during aging. *PLoS Gen.* **4**, e1000027 (2008).

Table S1. Rate constants of the microscopic processes involved in α-synuclein aggregation within liquid condensates. We used 75 μM α-synuclein in the absence (1% DMSO) and presence of claramine at 25, 50 and 75 μM. The rates obtained from kinetic traces are displayed in **Supplementary Figure 3**).

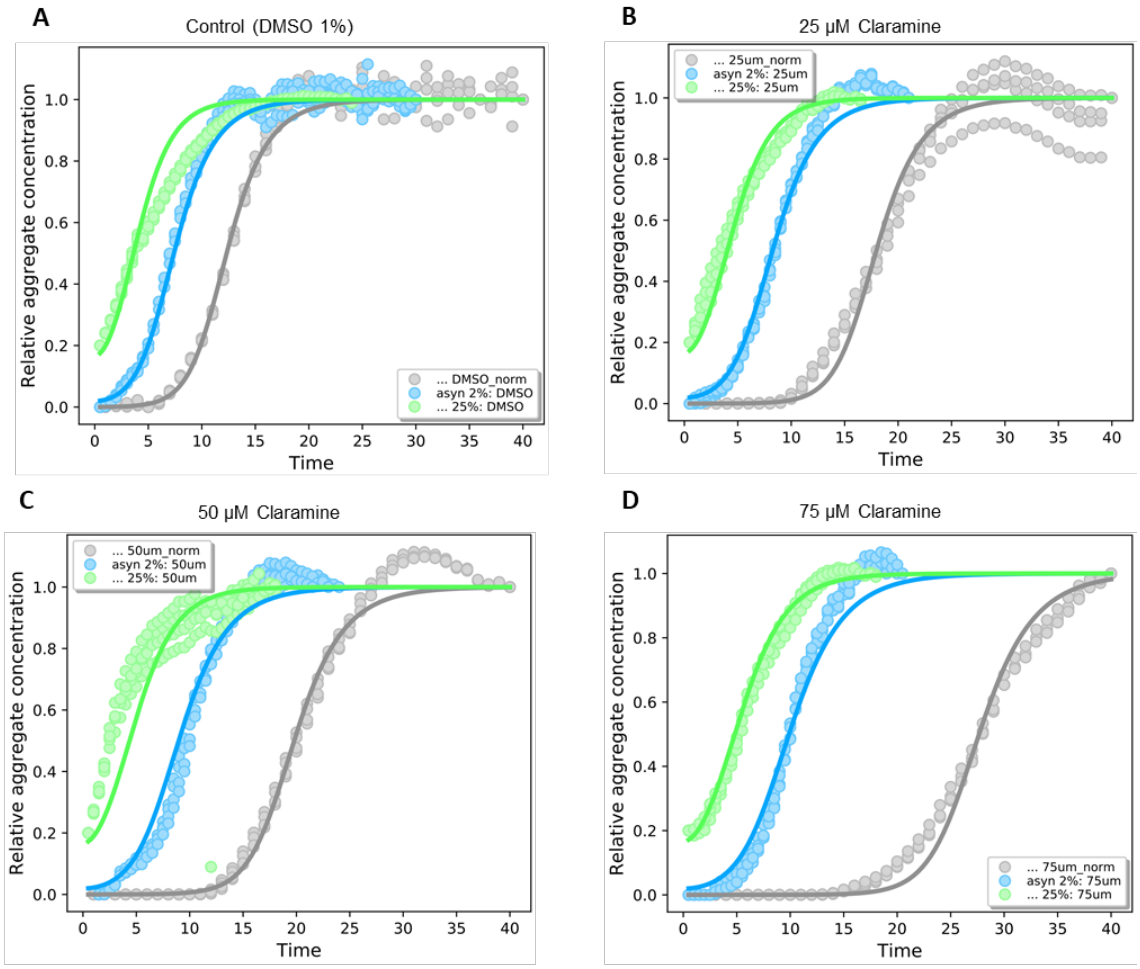
Conditions	Primary nucleation K_n ($M^{-1}min^{-1}$)	Secondary nucleation K_2 ($M^{-2}min^{-1}$)	Elongation K_+ ($M^{-1}min^{-1}$)
Control	0.825	1.97×10^7	8.73×10^4
25 μM Claramine	0.0201		
50 μM Claramine	0.00603		
75 μM Claramine	0.0000351		



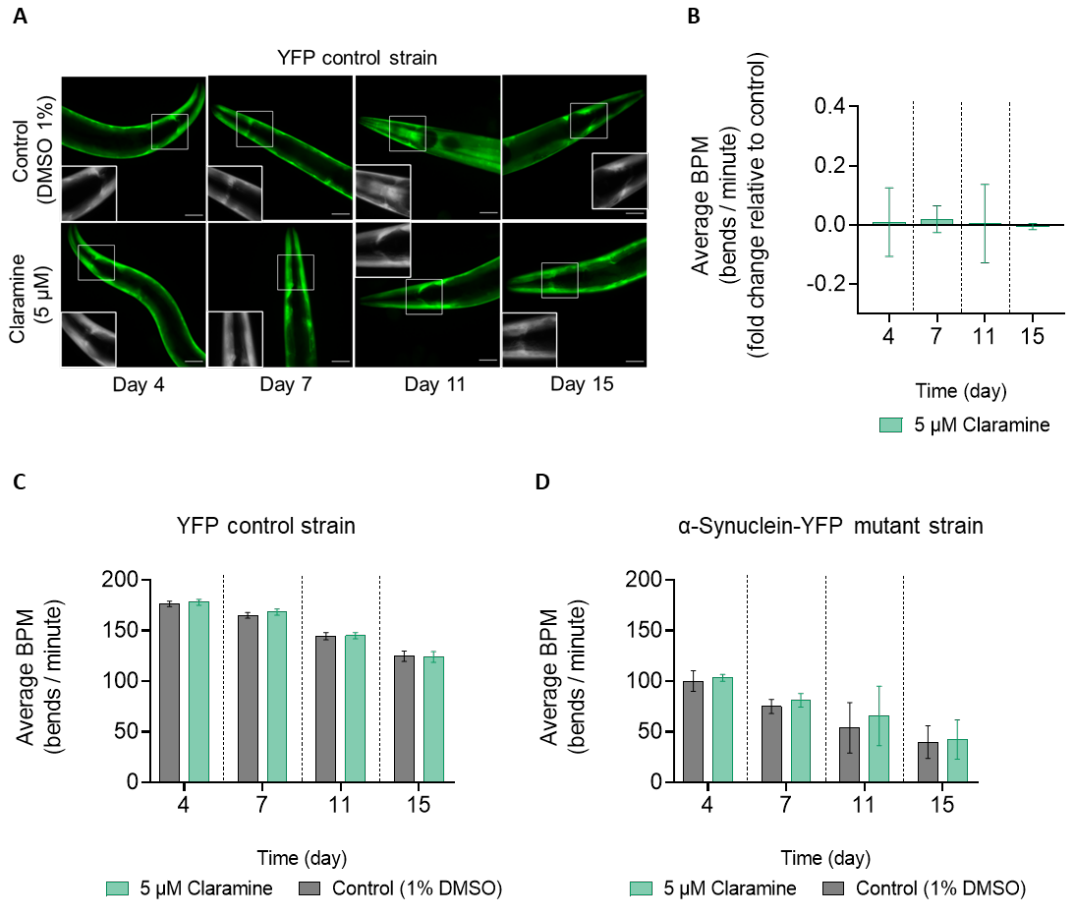
Supplementary Figure 1. Claramine shifts the phase boundary of α-synuclein. (A-E) Phase diagram for different PEG and α-synuclein concentrations (1% DMSO) in the absence **(A)** and presence of claramine at concentrations of 75 µM **(B)**, 50 µM **(C)**, 25 µM **(D)**, 10 µM **(E)** and 5 µM **(F)** at which phase separation was observed after a 10 min incubation period. The dots indicate the tested conditions, the grey dots for lack of phase separation, and the coloured dots for condensates. The dotted black lines represent the phase boundary. All experiments were performed in 50 mM Tris-HCl at pH 7.4. The data shown are representative of experiments repeated at least three times.



Supplementary Figure 2. Claramine does not covalently bind to α-synuclein. (A,B) LC-MS spectra of α-synuclein in the absence **(A)** and presence of claramine **(B)**. The observed and calculated mass of 14460 Da is a confirmation of the presence of α-synuclein. All experiments were performed using 75 μM α-synuclein in 50 mM Tris-HCl at pH 7.4 in the presence of 10% PEG.



Supplementary Figure 3. The fit of the α-synuclein aggregation kinetics within liquid condensates in the presence of claramine indicates an increase in the primary nucleation rate. Global analysis of the kinetic traces to quantify the effect of claramine on α-synuclein aggregation within the liquid condensates. **(A-D)** traces of α-synuclein (75 µM (grey)) with 2% (blue) and 25% (green) seeds in the absence (control (1% DMSO) **(A)** and presence of claramine at 25 µM **(B)**, 50 µM **(C)** and 75 µM **(D)**. All experiments were performed using 75 µM α-synuclein with either 2% or 25% preformed fibrils in 50 mM Tris-HCl at pH 7.4 in the presence of 10% PEG unless otherwise stated. The data represent the mean ± SEM of n=4 individual experiments.



Supplementary Figure 4. Claramine administration did not lead to observable negative effects in YFP control *C. elegans*. (A) Microscopy images showing the effect of the administration of 1% DMSO or claramine (5 µM) to the YFP expression in the body wall muscle cells in the YFP control strain over time between days 4 and 15 of adulthood. The scale bar represents 20 µm. **(B)** Data from automated worm motility assay showing the relative fold change in average bends per minute of worms treated with 5 µM claramine over time between days 4 and 15 of adulthood in YFP control strain. **(C-D)** Raw data from automated nematode platform showing the average bends per minute for untreated nematodes and claramine-treated nematodes over time for YFP control strain **(C)** and α-synuclein-YFP mutant strain **(D)**. At least fifty worms were analysed in total per experiment. The data represent the mean ± SEM of at least 3 individual experiments.