Supporting Information

Real-time Monitoring of Cell Membrane Disruption by α-Synuclein Oligomers in Live SH-SY5Y Neuroblastoma Cells

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METHODS

Materials. All solutions were prepared from double-deionized water (resistivity = 17.9 Ω at 25 °C, Barnstead Nanopure Diamond Water Purification System, APS Water, Van Nuys, CA). Ammonium acetate, ammonium sulfate, ampicillin, calcium chloride, D-glucose, dialysis membranes (10 kDa cutoff), ethanol (anhydrous), fetal bovine serum, glacial acetic acid, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), lysogeny broth, magnesium chloride, nickel (II) chloride, potassium chloride, sodium chloride, streptomycin sulfate, and trypsin were purchased from Thermo Fisher Scientific (Waltham, MA). In-vitro aggregation of α-synuclein (α-Syn) was conducted in phosphate-buffered saline (PBS, 10 mM phosphate buffer, pH 7.40, 137 mM sodium chloride, 2.7 mM potassium chloride) filtered through a 0.02 μm syringe filter (Whatman Anotop, Pittsburgh, PA). SICM experiments were conducted in artificial cerebrospinal fluid (ACSF, 25 mM HEPES, pH 7.40, 145 mM sodium chloride, 10 mM D-glucose, 3 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM magnesium chloride) filtered through a 0.02 μm syringe filter.

α-Synuclein Expression, Purification, and Aggregation. *E. coli* were cultured for 16-18 h at 37 °C in lysogeny broth with ampicillin (0.1 mg/mL). α-Syn plasmid expression was induced using IPTG (1 mM) once an optical density of 0.5-0.6 when measured at a wavelength of 600 nm had been reached. The cell culture was incubated for 4 h with shaking at 150 rpm in a MaxQ SHKE4000-7 Benchtop Orbital Shaker (Thermo Fisher Scientific). Cells were collected via centrifugation at 5000 × *g* for 10 min (Eppendorf 5417R Centrifuge, F-45-30-11 Rotor, Eppendorf, Hamburg, Germany) and the resulting pellet was resuspended in double-deionized water, sonicated for 1 min, boiled for 10 min, and centrifuged. The supernatant was washed with streptomycin sulfate (10%) and glacial acetic acid and centrifuged to precipitate nucleic acid content. The protein content was precipitated by washing the supernatant with supersaturated ammonium sulfate and centrifuging. The protein pellet was washed with ammonium acetate (100 mM) and ethanol and centrifuged to precipitate α-Syn. The α-Syn pellet was resuspended in ammonium acetate (100 mM) and lyophilized (BenchTop Pro Lyophilizer, SP Scientific, Warminster, PA). Lyophilized α-Syn was resuspended in PBS, dialyzed against PBS for 4 h (10 kDa cutoff), and filtered through an Amicon Ultra centrifugal filter (100 kDa cutoff, Millipore-Sigma, Burlington, MA) to remove any aggregates. All samples were concentrated to a monomer concentration of approximately 800 μM (CentriVap Benchtop Centrifugal Vacuum Concentrator, Labconco, Kansas City, MO), flash-frozen in liquid nitrogen, and stored at -80 °C. α-Syn monomer-equivalent concentrations were determined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific) at a wavelength of 280 nm using a molar extinction coefficient of 5120 M^{-1} cm^{-1}, as estimated from ExPASy ProtParam by amino acid sequence.[S1] Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the purity of produced α-Syn.

α-Syn was aggregated using a protocol adapted from Chen et al.[S2] α-Syn (800 μM) was incubated in PBS at 37 °C for 22-24 h. The sample was filtered through an Amicon Ultra centrifugal filter (100 kDa cutoff, Millipore-Sigma)

to remove monomers; oligomers were backwashed off the filter with PBS and stored at room temperature for no longer than 14 d.[S3]

Characterization of α-Syn Aggregates by Atomic Force Microscopy. Atomic Force Microscopy (AFM) was used to characterize the size distribution of the α-Syn aggregates. A muscovite mica substrate (V-1 quality, Electron Microscopy Sciences, Hatfield, PA) was cleaved, rinsed with double-deionized water, incubated with nickel (II) chloride (10 mM) for 20 min, and dried under a stream of nitrogen. The negatively charged mica surface[S4] was incubated with nickel (II) chloride to improve the adhesion of α-Syn, which has a net negative charge at pH 7.40. The substrate was incubated with a 20 μL aliquot of α-Syn aggregate solution for 20 min, gently rinsed with double-deionized water, and dried under a stream of nitrogen. The substrate was imaged in noncontact air mode with a Park NX12 multifunctional microscopy platform (Park Systems, Seoul, South Korea) equipped with an AFM head and mounted on a Nikon Ti-U inverted optical microscope (Nikon Inc., Tokyo, Japan). Images were acquired using a PPP-NCHR noncontact cantilever (force constant 42 N/m, Park Systems) at a scan rate of 0.5 Hz and a set point of approximately 10.71 nm using SmartScan (Park Systems). The acquired images were linearly flattened and aligned with Gwyddion version 2.51 (http://gwyddion.net/).[S5] Aggregate heights were measured using the "Grains" function in Gwyddion with a minimum height threshold of 3 nm. A 95% confidence interval of the mean aggregate z-height was calculated by bias-corrected and accelerated bootstrapping (2000 bootstrap samples) in MATLAB release R2019b (MathWorks, Inc., Natick, MA).

Characterization of α-Syn Aggregates by Circular Dichroism Spectroscopy. For characterization by circular dichroism spectroscopy, α-Syn aggregates were diluted to a monomer concentration of 15 μM in 5 mM phosphate buffer (pH 7.40). A J-1100 Circular Dichroism Spectrophotometer (JASCO, Inc., Easton, MD) was used to obtain all measurements. Circular dichroism spectra were obtained between 190-240 nm in duplicate scans with a pitch of 0.1 nm and a scan rate of 100 nm/min. Raw data were collected in ellipticity units (deg) and converted to per-residue molar ellipticity units ($deg \cdot cm^2 \cdot dmol^{-1}$) by dividing by the path length, the protein concentration, and the number of peptide bonds in one α-Syn monomer (139). The circular dichroism spectrum obtained for the α-Syn aggregates was compared to those of 56 known proteins with the CONTIN/LL algorithm in CDPro to estimate secondary structure composition.[S6]

Neuroblastoma Cell Culture. SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA) – cells commonly used to mimic neurons for the study of Parkinson's disease[S7] – were grown under standard culture conditions (37 °C and 5% carbon dioxide) in Dulbecco's Modified Eagle Medium with Ham's F-12 nutrient mixture, L-glutamine, and HEPES supplemented with fetal bovine serum (10% v/v) and penicillin-streptomycin (1%, Research Product International, Mount Prospect, IL). Trypsin (0.25%) was used to detach cells from culture plates for sample splitting.

Scanning Ion Conductance Microscopy of Live SH-SY5Y Neuroblastoma Cells. Nanopipette SICM probes were pulled from quartz capillaries (1.0 mm outer diameter, 0.50 mm inner diameter, 7.5 cm length, Sutter Instruments, Novato, CA) using a P-2000 laser-based micropipette puller (Sutter Instruments). Pulling parameters were varied to give tip radii of approximately 30-50 nm, although a typical protocol was as follows: heat=780, filament=4, velocity=16, delay=120, pull=115. The nanopipettes were filled with ACSF solution and fitted with Ag/AgCl electrodes. All images were acquired with a Park NX12 multifunctional microscopy platform (Park Systems) equipped with a detachable SICM head, mounted on a Nikon Ti-U inverted optical microscope (Nikon Inc.), and operated with SmartScan (Park Systems). A CCD camera (Pike F-032B, Allied Vision, Exton, PA) was connected to the optical microscope to assist in probe positioning.

SH-SY5Y neuroblastoma cell media was exchanged with ACSF solution warmed to 37 °C. The pipette probe was immersed in the ACSF solution, and a potential bias was applied between the pipette and bath electrodes to give an electrolytic current of 1 nA; most probes reached this current at potentials of 0.070 to 0.100 V. After the probe was positioned above a target cell, it was approached incrementally with a set point of 98% of the baseline current. Images were acquired in approach-retract-scan (ARS) mode. ARS parameters were varied to optimize image quality without sacrificing imaging time. A typical set of parameters was the following: threshold=1%, control height=5 µm, retract step=0.03 µm, approach step=0.01 µm, number of measurements averaged=3.

Data Processing and Statistical Analysis. The acquired SICM images were flattened according to a two-dimensional polynomial profile and aligned with Gwyddion version 2.51, setting the average z-height to 0 µm. This process accounts for the overall curvature of the cell membrane by fitting the z-height data to polynomials in both the x and y directions. The residuals between the actual data and the fitted data are used to construct the flattened image, as shown in Figure S1; as a result, the flattening process preserves local features on the membrane while eliminating overall membrane curvature.

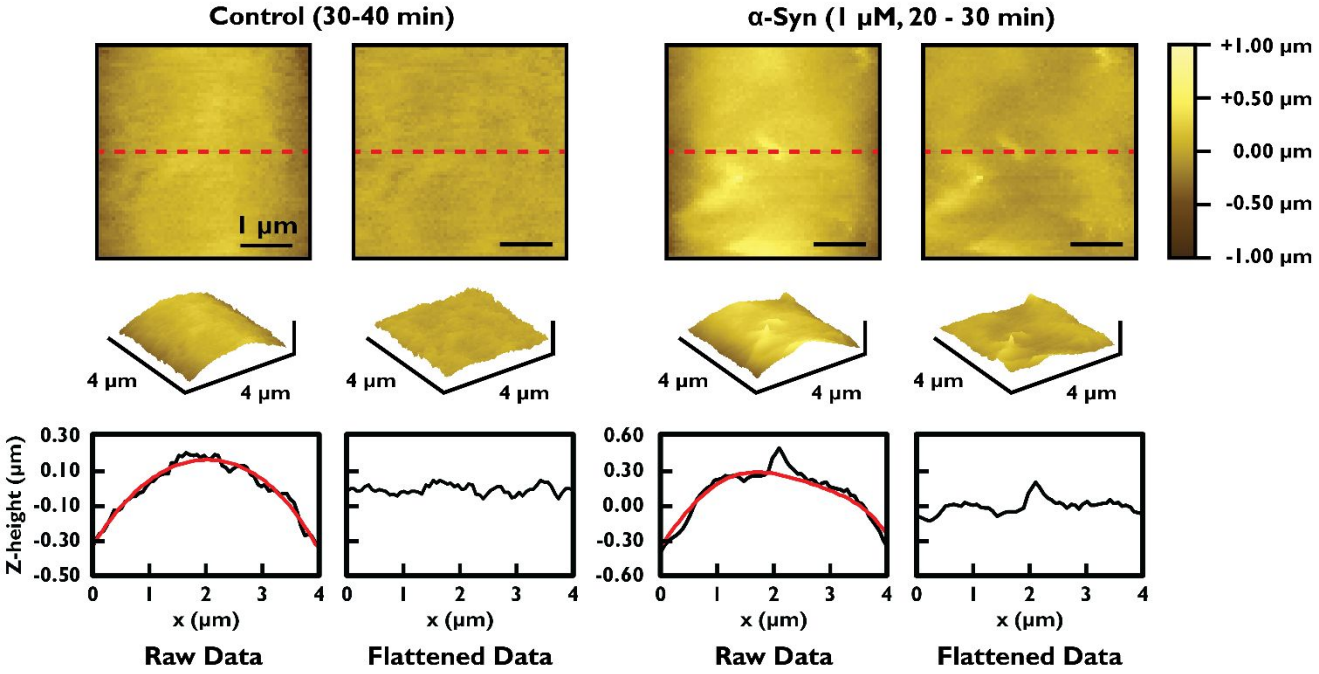

Figure S1. SICM image flattening in Gwyddion. Images (top), orthographic projections (middle), and line profiles (bottom; black lines show experimental data, while red lines show fitted data) are shown to demonstrate that the flattening process preserves the local features and roughness of the membrane while eliminating overall membrane curvature.

Roughness was measured across the membrane surface in 1 μm × 1 μm sections as the root-mean-square deviation of z-heights from the average. Membrane roughness for each timepoint was measured using the mean of all data points with a 95% confidence interval for the mean calculated by bias-corrected and accelerated bootstrapping (2000 bootstrap samples) in MATLAB release R2019b (MathWorks, Inc.). To compare roughness values among the different timepoints and treatments, a non-parametric rank-based ANOVA-like analysis was conducted in R version 3.6.3 (R Core Team, Vienna, Austria) using the nparLD package.[S8] Such an analysis was used because the underlying data were non-normally distributed and could not be assumed to be independent of time, precluding analysis by methods such as traditional ANOVA or the Mann-Whitney-Wilcoxon test. The method used compared the data for each timepoint to the data for every other timepoint; for each pair of timepoints, a p-value was calculated for the null hypothesis stating that the two timepoints gave identical roughness. Similarly, this method was used to compare the data for different treatments at individual timepoints, with the null hypothesis stating that the two treatments gave identical roughness. The measured p-values were then multiplied by the number of comparisons made (e.g., 45 for j=10 groups) to apply the Bonferroni correction for multiple comparisons.

RESULTS AND DISCUSSION

Characterization of α-Synuclein Monomers by AFM

Samples of freshly purified α-Syn monomers were characterized by AFM to ensure that the α-Syn oligomers were significantly larger than the α-Syn monomers. Noncontact air-mode AFM was used to assess the size of the monomers adhered to a flat mica substrate. A representative AFM image is shown in Figure S2A. AFM imaging determined that the z-heights of the aggregates ranged from 0.90 to 3.50 nm, as shown in Figure S2B. The arithmetic mean of monomer z-heights was 1.67 nm (n = 796), with a 95% confidence interval of (1.63 nm, 1.71 nm). A Mann-Whitney-Wilcoxon test was used to compare the distribution of z-heights between the monomer and aggregate samples, suggesting that the samples had significantly different z-heights ($p < 0.001$).

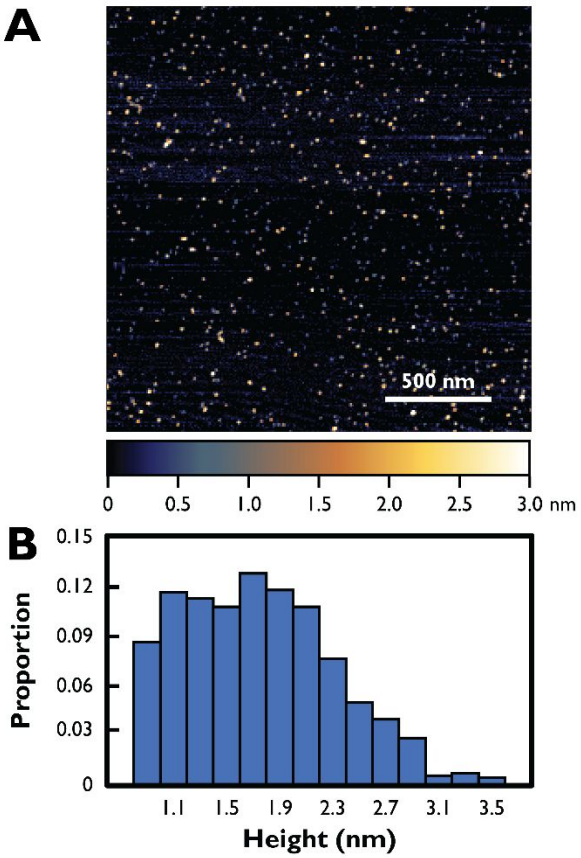

Figure S2. Characterization of α-Syn monomers by AFM. (A) AFM image of α-Syn monomers adhered to a mica substrate. Pixels are colored by z-height to show aggregate size. Images were first-order flattened using Gwyddion. (B) Histogram of the z-heights of aggregates imaged with AFM (n = 796).

SICM Line Profiles of Membrane Features

SICM imaging of neuroblastoma cell membranes treated with 6.0 μM α-Syn revealed the appearance of several features in the lipid membrane. Firstly, large pores in the membrane were observed; these pores were 0.5-1 μm in lateral size and displayed z-heights of -0.4 to -0.8 μm. Additionally, several small features could be observed on the membrane with z-heights ranging from +0.3 to +0.6 μm. Line profile plots of these features are shown in Figure S3.

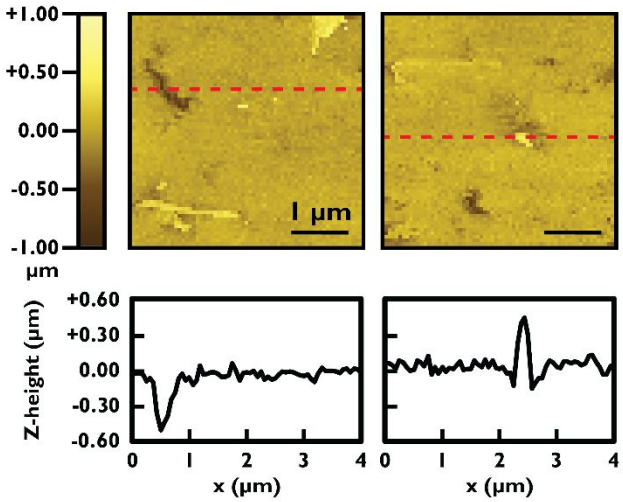

Figure S3. Representative SICM height profiles of a membrane pore (left) and a small positive feature (right). The height profiles plot the measured z-height along a single row (dashed red line) of the SICM image. The large, transient pores in the membrane had z-heights ranging from -0.4 to -0.8 µm, while the small positive features had z-heights ranging from +0.3 to +0.6 µm.

Influence of Surface Charge on SICM Imaging

Because SICM measures ionic current near a substrate, its measurements might be expected to be sensitive to the substrate surface charge.[S9] Approach curves measuring SICM current versus z-height were simulated in COMSOL Multiphysics (COMSOL, Inc., Burlington, MA) to substrates of varying surface charge to determine the influence of surface charge on measured z-height (Figure S3). Varying the surface charge of the substrate between -50 and +50 mC/m^2 did not significantly affect the z-height measured at a normalized current of 0.99 I_0, where I_0 is the current observed far from the substrate. Note that a surface charge of -50 mC/m^2 corresponds to 1*e* of charge assigned to approximately 30% of all phospholipids, a proportion much higher than what would be expected for a real neuronal membrane[S10] (assuming an area of 0.47 nm^2 per phospholipid and a cholesterol fraction of 0.5). These results suggest that, for the current threshold used in this work (0.99 I_0), changes to membrane surface charge would not significantly affect SICM imaging. This is consistent with previous work published by Chen et al.[S9] affirming that the substrate surface charge is not expected to affect SICM imaging for typical current thresholds (e.g., 0.99 I_0); intentional measurement of the substrate surface potential by SICM requires a dual-barrel open-circuit setup. Thus, the micrometer-scale distortions observed on neuronal membranes after treatment with α-Syn oligomers likely represent real topographical changes rather than changes to the membrane surface charge.

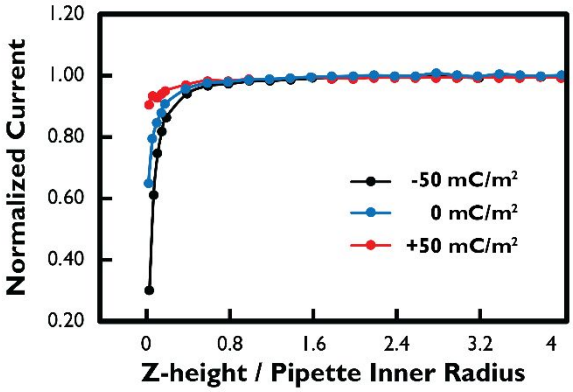

Figure S3. Simulated SICM approach curves with substrates carrying different surface charges (-50 mC/m^2, 0 mC/m^2, and +50 mC/m^2). All simulations were performed in COMSOL Multiphysics. The current was normalized to the baseline current measured far from the substrate (I_0), and the z-height was normalized to the pipette inner radius.

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