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Real-time determination of aggregated alpha-synuclein induced membrane disruption at neuroblastoma cells using scanning ion conductance microscopy

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Abstract

Parkinson's disease (PD) is recognized as the second most common neurodegenerative disorder and has affected approximately one million people in the United States alone. A large body of evidence has suggested that deposition of aggregated alpha-synuclein (α -Syn), a brain protein abundant near presynaptic termini, in intracellular protein inclusions (Lewy bodies) results in neuronal cell damage and ultimately contributes to the progression of PD. However, the exact mechanism is still unclear. One hypothesis is that α -Syn aggregates disrupt the cell membrane's integrity, eventually leading to cell death. We used scanning ion conductance microscopy (SICM) to monitor the morphological changes of SH-SY5Y neuroblastoma cells and observed dramatic disruption of the cell membrane after adding α -Syn aggregates to the culturing media. This work demonstrates that SICM can be applied as a new approach to studying cytotoxicity of α -Syn aggregates.

Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorder after Alzheimer's disease (AD), affecting more than 1% of the people over the age of 65 in the United States.¹ PD is linked to the malfunction and death of neurons in the *substantia nigra*, which results in a gradual decline of dopamine production.² As the detailed mechanism is still unclear, no effective treatment for the neurodegeneration in PD has been developed. Alpha-synuclein (α -Syn), a 140-residue, water-soluble protein normally abundant in presynaptic termini of the brain, is believed to play a key role in PD development and progression³ due to its discovery in Lewy bodies (LBs)⁴ and abundant genetic evidence; this includes the presence of point mutations in α -Syn in familial PD cases⁵ and the expression of human α -Syn in mice resulting in PD symptoms (e.g. dopaminergic neuron loss, motor dysfunctions and inclusion body formation).^{6,7}

As native unfolded α -Syn is non-toxic and likely to have physiological functions, such as regulating vesicle trafficking and neurotransmission⁸, the misfolding and aggregation of α -

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Conflicts of interest

There are no conflicts to declare.

Syn is believed to lead to high neurotoxicity⁹. Early hypotheses have assumed α -Syn fibrils to be the toxic form of α -Syn on the basis that they are the major component of LBs,¹⁰ while recent evidence suggests that intermediate forms such as soluble oligomers might be most cytotoxic^{11,12}. One hypothesis of the cytotoxic mechanism is that the aggregates insert into the lipid bilayer and disrupt the cell membrane's integrity, leading to the permeabilization of lipid bilayers and eventually cell death.^{13–16} Demonstration of membrane disruption on living cells have been studied previously via fluorescent imaging.^{15–17} For example, Fusco *et al.* reported their observation of membrane integrity loss at human neuroblastoma cells upon incubation of the cells with toxic α -Syn oligomers through confocal scanning microscopy. While fluorescent based imaging techniques are highly sensitive and efficient in determining the permeability of the cell, it is still difficult to obtain direct evidence of membrane disruption, i.e. the morphological alteration of the cell membranes. A high-resolution surface imaging technique is needed to provide a more complete understanding of the behaviour of α -Syn aggregates on cell membranes.

Scanning ion conductance microscopy (SICM) is a scanning probe microscopy (SPM) technique that utilizes distance-sensitive ionic current flowing through a glass pipette to image the topography of a surface and is powerful due to its high spatial and temporal resolution and non-invasive mode of detection.¹⁸ SICM is capable of imaging cell surfaces^{19,20}, proteins in membranes of living cells with nanometer resolution²¹ and individual ion channels^{22,23}. Therefore, it can “visualize” the possible α -Syn induced formation of pores that are believed to be a few nanometers in diameter²⁴. Compared to atomic force microscopy (AFM), which is a more widely used SPM technique, the scanning probe in SICM is less likely to get contaminated, much cheaper, and easier to make/replace. Moreover, SICM can be employed for localized delivery/excitation and detection^{25,26} and has been applied to study individual aggregates of amyloid beta inducing temporary calcium influx through the cell membrane of neuronal cells.²⁷

In this project, SICM was used to study the effect of α -Syn aggregates on membranes of SH-SY5Y neuroblastoma cells, a cell line commonly used to mimic neuronal cells for studies of neurological disorders.²⁸ The initial test was done with cells pre-cultured with α -Syn aggregates; dramatic disruptions of cell membranes were observed with SICM. To further demonstrate that the disruption is due to α -Syn aggregates interacting with membranes, real-time monitoring of the cell topography was carried out after adding α -Syn aggregates to the cells to reveal the interaction between the aggregates and the cell membrane.

Note that it is critical to distinguish the effects of intracellular and extracellular α -Syn.²⁹ Although α -Syn is believed to be originally present and aggregated intracellularly into LBs, it can also be secreted from neuronal cells³⁰. Extracellular α -Syn and its aggregates may also be related to the progression of PD throughout the brain³¹. The role of extracellular α -Syn has been supported by the observation of α -Syn oligomers in the CSFs of PD patients.³² In addition, a recent electrophysiological study at hippocampal neurons has shown that extracellular α -Syn has a stronger effect on membrane integrity.¹⁷ Thus, only the effects of extracellular α -Syn were highlighted in this work.

Experimental

Chemicals and Materials

All chemicals were used as received without further purification. Phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, Fisher Scientific, Chino, CA) (PBS) was used as the SICM imaging buffer. All aqueous solutions were prepared from deionized water (resistivity = 17.9M Ω -cm at 25°C, Barnstead Nanopure Diamond Water Purification System, APS Water, Lake Balboa, CA). PBS was filtered through a 0.02 μ m syringe filter (Whatman Anotop, Pittsburgh, PA) before use. Other chemicals used are described in detail in the following sections.

Cell Preparation

SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA) were grown under standard culture conditions (37 °C and 5% carbon dioxide) in Dulbecco's Modified Eagle's Medium (DMEM/F-12 1:1 Mixture) with L-glutamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (15mM, Fisher) supplemented with fetal bovine serum (10% (v/v), 35011CV, Fisher) and penicillin-streptomycin (1%) (Research Product International, Mt Prospect, IL). Fresh trypsin (0.75%, Fisher) was used to detach cells from the culture plate and fresh culturing medium was added every 3–4 d. In preparation for SICM, the cells were fixed by replacing the culturing media with paraformaldehyde (4%, Fisher) for 30 minutes. The fixing agent was then replaced with PBS prior to scanning.

α -Syn Preparation

E. coli BL21 (DE3) were transfected with pRK172/ α -Syn plasmids shared by Prof. Feimeng Zhou (California State University Los Angeles) and originally donated by Prof. Peter Lansbury (Harvard University); the E. coli were cultured for 16–18 h (37 °C) before further incubation in lysogeny broth (Fisher) and ampicillin (0.1mg/mL, Fisher). Ultraviolet-visible spectrophotometry (UV-Vis) was used to check the optical density of the bacteria. α -Syn plasmid expression was induced once bacteria reached an optical density of 0.5–0.6 at 600 nm using isopropyl β -D-1-thiogalactopyranoside (Fisher). The solution was centrifuged after agitation for 4 h and the pellet was collected. To isolate α -Syn, cells were lysed via sonication and boiled for 10 min. The resultant was centrifuged and the supernatant was collected. Streptomycin sulfate (10%, Fisher) and glacial acetic acid (Fisher) were mixed with the supernatant and centrifuged; the supernatant was collected and washed with supersaturated ammonium sulfate (Fisher). The resultant was centrifuged again; the pellet was collected and washed with ammonium acetate (100mM, Fisher) and ethanol (anhydrous, Fisher). This mixture was centrifuged again; the pellet was then dissolved in 100mM ammonium acetate (Fisher) and freeze-dried (BenchTop Pro, SP Scientific, Warminster, PA). For further purification, double-deionized water was added to the freeze-dried α -Syn and the solution was dialyzed with a dialysis tube (10 kDa, Thermo Scientific, Waltham, MA) and shaken for 4 h. α -Syn was then removed from the tube and centrifuged with an Amicon ultra 100kDa filter (Millipore Sigma, Darmstadt, Germany), collected, and freeze-dried for later use. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm successful expression and purification of α -Syn at 14kDa.

Two types of α -Syn aggregates were prepared through incubation. Type I aggregates were prepared by incubating 10 μ M α -Syn monomers with phosphate buffer (pH 7.4) in a temperature-controlled shaker at 37 °C for 4 d. Type II aggregates were prepared by incubating 100 μ M α -Syn for 3 d until visible aggregates appeared. The products were immobilized onto mica sheets (Electron Microscopy Sciences, Hatfield, PA) for characterization with AFM prior to usage. Clean mica sheets were modified with 10mM NiCl_2 (Fisher) for 20 min, rinsed with deionized water, and air-dried with nitrogen gas. The α -Syn aggregate solution (15 μ L) was dropped on the mica sheet and left to rest for 30 min before it was washed with deionized water and air-dried with nitrogen gas. The mica sheet was then mounted on to the AFM sample stage for imaging.

Nanopipette Fabrication

Nanopipette probes for SICM imaging were pulled from quartz capillaries (1.0 mm outer diameter, 0.50 mm inner diameter, 7 cm in length, Sutter Instruments, Novato, CA) using a P-2000 laser-based micropipette puller (Sutter Instruments). Pulling parameters can be varied, although a typical program is: heat=780, filament=4, velocity=16, delay=120, pull=135. The nanopipettes were filled with PBS solution and a Ag/AgCl electrode was inserted into the pipette prior to usage.

Instrumentation for Imaging

All images were acquired with a Park NX12 multifunctional microscopy platform (Park Systems, Seoul, South Korea) equipped with detachable heads for SICM and AFM and a Nikon Ti-U inverted optical microscope. A CCD camera (Pike, F032B, Allied Vision) was connected to the optical microscope to assist in probe positioning. The instrument was operated with Smart Scan software (Park Systems). The acquired images were first-order flattened with XEI (Park Systems) or Gwyddion (© David Neas and Petr Klapetek).

All cell images were acquired with SICM mode, using a quartz nanopipette (diameter of 30–50 nm) as the scanning probe. A potential bias was applied between the pipette electrode and a second Ag/AgCl bath electrode placed in the electrolyte solution. Ideal nanopipettes for SICM imaging gave ionic currents around 1 nA at a bias of 0.1 V. Prior to imaging, the nanopipette was brought close to the sample surface via monitoring using the CCD camera connected to the inverted microscope. Once the nanopipette was visible at the sample focus plane, the scanning area was selected by moving the sample stage. An incremental approach of the nanopipette was then carried out with the set-point current as 98% of the baseline current. All images were acquired in approach-retract-scanning (ARS) mode.³³ In ARS, the parameters can be varied to adjust the imaging quality and overall scanning time. A typical set of parameters were as following: threshold = 2%, control height = 6 μ m, retract step = 0.02 μ m, approach step = 0.02 μ m, averages (the number of signal averaging) = 3. AFM mode was used for characterizing the prepared α -syn aggregates. A non-contact cantilever (PPP-NCHR 5M, Park Systems) was used for imaging in tapping mode.

Results and discussion

α -Syn preparation and characterization

Two types of α -Syn aggregates were prepared and characterized with AFM. As described earlier, type I was prepared from a 10 μ M α -Syn monomer solution, while type II was from a 100 μ M α -Syn monomer solution. The resulting aggregates differed significantly in size, as indicated by AFM images. As shown in Fig. 1A, type I was mainly composed of spherical aggregates with an average height of 3–5 nm and a small number of short fibrils that are <1 μ m in length. The dimension of spherical aggregates fit the reported values for α -Syn oligomers.^{10,16} In contrast, type II was dominated by long fibrils of about 10 nm in height and a few micrometers in length, similar to previously reported values for fibrils.¹⁰ Note that the concentrations of oligomers or fibrils were not accurately defined as the aggregates exist as dynamic mixtures that are difficult to purify. Recent studies tend to suggest that only certain types of oligomers are cytotoxic, rather than fibrils.^{11,16,34} However, in our investigation, we noticed that both forms could lead to cell membrane disruption.

Optimization of imaging conditions for neuroblastoma cells

Imaging conditions were optimized for obtaining images of SHSY5Y neuroblastoma cells to improve the spatial and temporal resolution. Being non-invasive is the most essential consideration in applying SPM in studying surface topographical alterations. As reported previously, the SICM hopping mode, in which the pipette is retracted to a safe height before moving to a new pixel, is a non-invasive and label-free technique that can efficiently resolve nanoscale structures on live-cell membranes.³⁵ The mechanism of ARS mode (Park Systems) is slightly different in that the pipette is retracted by a certain distance away from the sample before moving to the next pixel. In this way, the control height (the distance by which the pipette is lifted up) does not have to be larger than the maximum height of the sample, but only dependent on how steep the sample is. For example, a 50 μ m $\times$ 50 μ m scan over a group of cells with a 64 $\times$ 64 pixel-resolution is shown in Fig. 2A; a cross-line height profile along the red dashed line with the highest feature at 14 μ m is shown in Fig. 2C. The change in height per pixel can be obtained by differentiating the height profile; the steepest change observed was about 5 μ m, as shown in Fig. 2D. Therefore, a 6 μ m-control height is sufficient.

Another consideration in optimization is how to minimize the imaging time while maintaining high spatial resolution and making no contact with the sample. The overall scanning time depends on the number of pixels and the scanning rate per pixel, which is controlled by the ARS parameters, e.g., the control height as mentioned, retract and approach steps (how fast the nanopipette is moved away and toward the sample at every pixel) and the times of signal averaging. We noticed that other ARS parameters didn't affect the quality of images as much as control height. Thus, the imaging condition was mostly maintained with "limited quality" option: retract step = 0.02 μ m, approach step = 0.02 μ m, average number = 3. In a typical approach, the nanopipette was brought to the center of a cell with the help of the optical microscope and then used to image the cell with a low resolution (e.g. 64 $\times$ 64) and a large control height (typically 6 μ m); this was used to obtain an overview of the targeted cell(s), as shown in Fig. 2A. During the scanning process, the

parameters can be adjusted based on the morphology of the surface. A typical scanning time for obtaining a SICM image with 64×64 pixels is about 5 to 10 min. Fig. 2B shows a zoomed-in $5 \mu\text{m} \times 5 \mu\text{m}$ image with a 256×256 pixel-resolution (i.e., a theoretical spatial resolution of 20 nm). In this case, the control height was decreased to $3 \mu\text{m}$ since the surface is relatively flat. Note that the resolution is also dependent on the size of the scanning pipette. Therefore, the actual spatial resolution of Fig. 2B was around 30 nm. Theoretically, a higher resolution can always be obtained by using a smaller pipette.

SICM imaging of SH-SY5Y cells incubated with α -Syn aggregates

End-point structural changes were investigated by imaging SHSY5Y cells incubated with α -Syn aggregates. The cells were incubated in the culturing media (6 mL), in which 100 μL of α -Syn aggregate solution (either type I or type II) was added two days before imaging. Pore-like defects were observed for a large number of cells that have been imaged; representative images are shown in Fig. 3A–C. The observation indicated a possible cell-membrane disruption caused by α -Syn aggregates. Cross-line height profiles are plotted in Figs. 3D–F showing that the disruption ranged from 100 nm to a few micrometers in diameter, and 0.15 to $1 \mu\text{m}$ in depth. The disruption could be as individual pores (Figs. 3B–C), or a spreading of disruption over a large area (Fig. 3A). In Fig. 3A, it seemed that part of the membrane was peeled off and the cell's contents prevented the scanning pipette from scanning deeper, while Fig. 3B shows that under the disrupted membrane, further penetration into the underlying structure was in process. The smallest pore that was observed was around 100 nm in diameter (Fig. 3C).

Even though cell membrane disruption was observed at most cells cultured with α -Syn aggregates, a similar phenomenon was occasionally observed with untreated cells, indicating that the disruption might be caused by the fixing agent.³⁶ Thus, real-time determination of membrane disruption was carried out by adding α -Syn aggregates in situ, as discussed below.

Real-time SICM imaging of disruption caused by α -Syn aggregates added in-situ

To demonstrate that the disruption observed in Fig. 3 was caused by α -Syn aggregates, cell membrane structural changes were measured in real-time upon in-situ addition of α -Syn aggregates. For this test, cells with good integrity were selected and monitored before and after treatment with α -Syn aggregates. To achieve this, 10 μL of α -Syn aggregate solution were added in-situ to the PBS buffer during imaging. Continuous imaging of the cells was carried out to monitor the possible disruption caused by α -Syn aggregates.

The first set of images (Figs. 4A–D) were focused on the edge area of an SH-SY5Y cell near the dendrites. The scanning time for each image was about 30 min. Two dendrites underwent structural changes and were eventually “cut” 2.5 h after α -Syn aggregates were added to the buffer, as circled in Figs. 4A–C. The third dendrite was not fully cut off but became thinner. To clarify the fact that the changes in dendrites structure were not caused by SICM scanning itself, control tests were carried out by imaging cells without being exposed to α -Syn aggregates. The results showed that the cell structure was stable and remained unchanged in the small features (Figs. 4E and G). To further demonstrate the scanning is non-invasive, a

more intensive scan was carried out by continuously doing a line scan along the same line (indicated by the red line in Fig. 4E). A comparison between the height profile at 33 min and the one at 60 min in Fig. 4F does not show any obvious change in the height or width of those dendrite structures. Note that in Figs. 4A–C (2D scan), each line was only scanned twice in every half hour, while in the line scan it was scanned 20 times per minutes. The fact that a much intensive line didn't affect the cell structure demonstrates that scanning itself is non-invasive.

Therefore, the disruption observed in Figs. 4A–C was mostly likely caused by added α -Syn aggregates. Note that the exact location of each dendrite did drift slightly due to the movement of dendrites in buffer solution.

The effect of type I α -Syn aggregates was also studied. As shown in Fig. 5A and B, a $10\ \mu\text{m} \times 10\ \mu\text{m}$ area within a cell was scanned before and after adding $50\ \mu\text{L}$ of α -Syn aggregate solution near the scanning area.

An obvious spreading of disruption was observed one hour after adding the type I α -Syn aggregate solution in-situ. The height profiles across the top dashed line show a relatively good fit between two images, while the lower one indicates and the overall height decreased, which was likely due to loss of cytoplasm following the membrane disruption.

We also noticed that the effects of both types of aggregates varied. In another case with type II α -Syn aggregates, no severe membrane disruption was observed after six-hour imaging. However, the surface roughness increased after treatment with type II α -Syn aggregates (dominated by fibrils), as shown in Fig. 6E–F, where the cross-line height profiles (at locations marked as red dashed lines in Fig. 6A) before (black) and after (red) treatment were compared. The alteration of surface roughness can be explained by α -Syn fibrils binding to cell membranes.³⁷ A zoom-in of a $5\ \mu\text{m} \times 5\ \mu\text{m}$ area in Fig. 6C shows that the surface became less rough after a few hours and only one particle (likely a remained α -Syn fibril) was caught. A further zoom-in of the spot marked with a red dashed box revealed more detailed information of the fibril and the underlying cell membrane. The membrane dented in the area surrounding the fibril and underwent small alteration, although the structural change was not as disruptive as previous cases.

Conclusions

In this work, SICM was used to monitor the effect of α -Syn aggregates on the integrity of cell membranes. Disruption was detected in cells pre-incubated with α -Syn aggregates and observed in-situ upon addition of the aggregates, although the interaction varied among different cells. Overall, these data suggest that SICM can be used to directly observe in situ cell damage induced by α -Syn aggregates. This observation can also be extended to the studies of other neurodegenerative diseases such as AD, as this may be a fundamental mechanism of cell death in this type of diseases.

There are still steps that can be taken to further enhance our methods. The current SICM mode only provides structural information, rather than chemical information for understanding the mechanism behind the disruption. A possible improvement would be

combining SICM with surface plasmon resonance microscopy (SPRM)^{38,39}, as the latter is an efficient method for quantitatively determining kinetic information of the interaction between α -Syn aggregates and cell membranes. SPRM can be used to determine the active binding sites at cell membranes and the binding kinetics, while SICM can be continuously carried out to monitor the morphological changes and to correlate any physical damages to the binding information from SPRM.

Another concern is that in the current system, cells were fixed with paraformaldehyde, which killed the cells. Although real-time changes after delivering α -Syn aggregates were observed, it is still possible that the disruption might be due to delayed damage from the fixing agent. Moreover, the normal cell functions were inhibited; live cells are likely to respond to α -Syn interaction and undergo certain processes to prevent the damage. The least ambiguous experiment would still be imaging live cells without applying fixing agent. With our current setup, SICM images of live SH-SY5Y cells could be acquired for around 1 h. We are currently working on optimizing the imaging environment to maintain the cell viability for long-term studies.

Finally, the quantification of the exact concentration of a certain form of α -Syn aggregates and the amount of aggregates being delivered to the cells is still lacking. For the first concern, in future investigation, the stage of aggregation will be strictly controlled and the final products will be purified to isolate only a certain form of aggregate. Moreover, in addition to AFM, the characterization of α -Syn aggregates will be supported by circular dichroism and MALDI TOF mass spectrometry. For the second concern, the exact number of α -Syn aggregates being delivered to the cell membrane can be controlled with resistive pulse sensing based delivery.^{27,40} It has been reported that individual α -Syn aggregates, e.g. oligomers, can be detected in resistive pulse sensing with solid nanopores. Utilizing the scanning pipette in SICM allows locally delivering the aggregates at a confined area of the cell membrane. In our preliminary approach, localized delivery via the scanning pipette that was filled with type I aggregates was carried out by applying a negative bias. No changes in the cell membrane were observed, likely due to an insufficient amount of delivered aggregates. Further investigation is needed to quantify the interaction between α -Syn aggregates and cell membranes.

To further deepen the understanding of the underlying mechanism, electrochemical detection or scanning electrochemical microscopy (SECM)^{41,42} can be integrated into the system. Oxidative stress, a disequilibrium between the levels of reactive oxygen species (ROS) produced and the ability of a biological system to detoxify the reactive intermediates, is believed to play an important role in degeneration of dopaminergic neurons in PD.⁴³ Overproduction of ROS could result from abnormal aggregation of α -Syn.⁴³ However, direct evidence is still limited. ROS, such as H₂O₂, can be detected electrochemically via amperometry, which is a label-free, highly-sensitive and quantitative technique, and has been widely used in biological studies.⁴⁴ Previously, fabricated metal and carbon nanoelectrodes have been used for intracellular electrochemical measurements of ROS production.^{45,46} On the other hand, multifunctional nanoprobe combining SECM and SICM have been developed for cellular imaging and measurement.^{26,47,48} We are currently fabricating multifunctional nanoprobe for intracellular determination of elevated ROS levels due to

locally delivered α -Syn aggregates. Once demonstrated, the protocol can be extended to studying other hypotheses.

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Figure 1.

AFM images of (A) type I and (B) type II α -Syn aggregates; (C) height profiles along the dashed lines labeled in A; (D) height profile along the dashed grey line in B. The inset in A is a high-resolution image of an oligomer, and the cross-line height profile labeled by the green solid line is shown as the inset in C. A and B were scanned at 0.5 Hz and 0.7 Hz per line, respectively, with 256×256 pixels in tapping mode. Original images were first-order flattened in XEI and adjusted in Gwyddion for obtaining line profiles.

Figure 2.

SICM imaging and height profiles of SH-SY5Y cells. (A) Fast scan of a group of cells with low resolution (0.78 μm per pixel); (B) high-resolution imaging (30 nm per pixel) of part of a neuron membrane; (C) height profile along the dashed line labeled in A; (D) height change per pixel from C. Original images were flattened with the align rows tool in Gwyddion.

Figure 3.

SICM images (A-C) of a group of SH-SY5Y cells that were incubated with type I α -Syn aggregates (A and B) and type II α -Syn aggregates (C), and cross-line height profiles (D-F) along the dashed lines. Original images were flattened with the align rows tool in Gwyddion.

Figure 4.

SICM images (A-C) of a portion of an SH-SY5Y cell before (A) and after (B-D) adding 10 μL type II $\alpha\text{-Syn}$ aggregate solution at the labelled time, and images (E, G) of a portion of another SH-SY5Y cell without adding aggregates. F is the overlapping of two height profiles during a continuous line-scan at red dashed line in E. Original images were flattened with the align rows tool in Gwyddion (A-D) or XEI (E and G).

Figure 5. SICM images from a portion of an SH-SY5Y cell before (A) and one hour after (B) adding 50 μL type I α -Syn aggregate solution. (C-D) cross-line height profiles along the upper (C) and lower line (D) marked in B. Original images were flattened with the align rows tool in Gwyddion.

Figure 6.

SICM images from an SH-SY5Y cell before (A) and after (B-D) adding 10 μL type II α -Syn aggregates and cross-line height profiles (E-G) at locations indicated by the red dashed lines in A from top to bottom. Original images were flattened with XEI (A and B) or Gwyddion (C and D).