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Interaction of α-Synuclein and a Cell Penetrating Fusion Peptide with Higher Eukaryotic Cell Membranes Assessed by ^{19}F NMR

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Abstract

We show that fluorine NMR can be used to monitor the insertion and change in conformation of a ^{19}F-labeled cell-penetrating peptide upon interacting with the cellular plasma membrane. α-Synuclein and a construct comprising a cell-penetrating peptide covalently attached to its N-terminus were studied. Important information about the interaction of the proteins with CHO-K1 cells was obtained by monitoring the diminution of ^{19}F resonances of 3-fluoro-L-tyrosine labeled proteins. For α-synuclein, a decrease in the resonance from position 39 was observed indicating that only the N-terminal third region of the protein interacts with plasma membrane. However, when the fusion construct was incubated with the cells, a decrease in the resonance from the fusion peptide region was noted with no change in the resonances from α-synuclein region. Longer incubation, studied by using confocal fluorescence microscopy, revealed that the fusion construct translocates into the cells, but α-synuclein alone did not cross the membrane in significant amounts.

Keywords

cell-penetrating peptide; delivery system; eukaryotic cell; ^{19}F NMR; α-synuclein

INTRODUCTION

Several groups have used nuclear magnetic resonance spectroscopy (NMR) to study the interaction of proteins and peptides with artificial membranes[1] and lipid bilayers,[2] but understanding the interaction, insertion, and conformational dynamics of proteins and peptides with native membranes remains challenging. The greatest barrier to delivery of proteins into cells is the plasma membrane. A series of delivery systems has been developed to overcome this problem. Cell-penetrating peptides cross the plasma membrane in an energy independent manner.[3–5] These peptides can be covalently attached to the cargo,[6] overexpressed as a fusion protein in bacteria,[7, 8] or bound through noncovalent (charge-charge and/or hydrophobic) interactions.[9] Several cell-penetrating peptides have been

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Supporting Information

MALDI-MS mass spectra of CTP-α-synuclein and ^{19}F spectra of Y125F α-synuclein at pH 6 and Y125F α-synuclein after 12 h of incubation in Ficoll. This material is available free of charge via the Internet at http://pubs.acs.org.

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developed.[7, 8] One of the most popular is the trans-acting activator of transcription (TAT) from the human immunodeficiency virus, which can deliver proteins,[10] oligonucleotides,[11, 12] liposomes,[13] and nanoparticles.[14] TAT targets the fused proteins to the nucleus.[15] The N-terminal TAT-α-synuclein construct was delivered to astrocytes[16] and PC12[17] cells. Kim et al.[7] designed a cytoplasmic transduction peptide (CTP) derived from TAT that has increased transduction potential and delivers biomolecules to the cytoplasm rather than the nucleus. Among the sequences tested, the eleven amino-acid version, YGR_2AR_6, proved most efficient in translocating β-galactosidase into several cell lines. After transduction, the peptide is cleaved by cytoplasmic enzymes, releasing the cargo.

Fluorine NMR (^{19}F NMR) is highly sensitive to chemical environment. It is particularly useful for in-cell NMR because there is no background signal. The utility of ^{19}F NMR for assessing biologically important interactions relies on the broadening of resonances from a small molecule when it is immobilized by binding to a large one. For instance, ^{19}F resonances from a labeled protein like α-synuclein are broadened into the baseline when the protein, or part of it, is immobilized upon binding to cells, vesicles, or micelles.[18–23] Under these conditions, the fraction bound can be easily quantified by measuring the decrease in area under each resonance. ^{19}F NMR can also be used to determine which part of a protein interacts with the larger molecule because resonances from bound but internally mobile regions remain sharp.

^{19}F NMR has been used to study protein-ligand interactions,[18] fibril formation,[19] protein interaction with lipid vesicles,[20] and sodium dodecyl sulfate micelles.[21] It has also been used to study the interaction of peptides with small unilamellar vesicles, bicelles[22] and living cells[23] as well as the physicochemical properties of antimicrobial peptides[24] and the biological activity of different peptides.[25, 26] Here, we used ^{19}F NMR to study the interaction of wild-type α-synuclein and a CTP-α-synuclein fusion protein with the plasma membrane of Chinese hamster ovary (CHO-K1) cells.

α-Synuclein is a 140-residue intrinsically-disordered protein comprising a positively-charged N-terminus, a hydrophobic middle region, and a negatively charged C-terminus (Figure 1). It is the main component of Lewy bodies found in the cytoplasm of neurons in the *substantia nigra pars compacta* of patients with Parkinson's disease.[27] The N-terminal region of α-synuclein forms an α-helix upon interaction with vesicles of different lipid composition.[20]

MATERIALS AND METHODS

Site-directed Mutagenesis

The CTP-α-synuclein construct was created in two steps with a Stratagene site-directed mutagenesis kit. First, YGR_2A was inserted at the N-terminus of the wild-type α-synuclein gene by using the primers:

Forward 5′-3′:
GCAGGAGATATACATATGTATGGCCGTCGTGCGGATGTATTCATGAAAGG

Reverse 5′-3′:
CCTTTCATGAATACATCCGCACGACGGCCATACATATGTATATCTCCTGC

Second, the R_6 fragment was inserted using the primers:

Forward 5′-3′:
GTATGGCCGTCGTGCGCGTCGTCGTCGTCGTCGTGATGTATTCATGAAAGG

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Reverse 5′-3′:
CTTTCATGAATACATCACGACGACGACGACGACGCGCACGACGGCCATAC′

The insertions were confirmed with the sequencing primer, 5′-GGGAGACCACAACGGTTTCCCTCTAG-3′.

Expression and Purification of α-Synuclein Variants

V3C CTP-α-synuclein was expressed in *Escherichia coli* and purified as described by Ruf et al.[28] The insertion of YGR_2AR_6 was confirmed by using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) of Lys C digested samples (Figure S1). 3-Fluoro-L-tyrosine labeled variants were expressed and purified as described by Li et al.[19] Purified α-synuclein was lyophilized and stored at −80 °C.

Alexa Fluor Labeling

Twelve mg of V3C CTP-α-synuclein were dissolved in sterile degassed H_2O to a final concentration of 2 mg/mL. Tris(2-carboxyethyl)phosphine and $NaHCO_3$ were added in a ten fold molar excess over protein. The mixture was incubated at room temperature with shaking for 30 min. Next, Alexa Fluor 488 C5-maleimide (Invitrogen) was added in a ten fold molar excess over protein. The mixture was incubated at room temperature with shaking for 2 h. The labeled protein was purified by gel filtration chromatography on a Superdex 75 column with 20% acetonitrile in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) as an eluent. The labeled protein was dialyzed against water, and the labeling efficiency was determined as described in the Alexa Fluor 488 C5-maleimide labeling protocol (Invitrogen). The absorbance at 494 nm along with the extinction coefficient of 71,000 $M^{-1}cm^{-1}$ for Alexa Fluor 488 was used to quantify the labeled α-synuclein. The Lowry method was used to quantify the total amount of protein (Lowry protein assay kit, Pierce). The labeling efficiency for V3C CTP-α-synuclein with the dye was 84%. Aliquots of 1 mg labeled protein were lyophilized and stored at −80 °C.

Cell Culture

CHO-K1 cells were obtained from the UNC Lineberger Cancer Center. The cells were seeded in 6-well glass plates (Corning Life Sciences) at a density of ~2×10^5 cells/well in F-12 media supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO_2.

Fluorine NMR

Spectra of ^{19}F-labeled α-synuclein were acquired at 37 °C on a Varian Inova 600 MHz NMR spectrometer at a frequency of 564.5 MHz using a 5 mm triple-resonance probe (Varian 600 H-F(C,X)). Each spectrum comprised 2048 transients with a 1 s delay between transients. Each transient was acquired using a 60 kHz sweep width and an acquisition time of 1.9 s. The CHO-K1 cells were incubated with ^{19}F-labeled CTP-α-synuclein in media at 37 °C. Aliquots were combined with equal volumes of Ficoll dissolved in cell media. The final sample contained 100 μM ^{19}F CTP-α-synuclein and ~1.5×10^7 cells/mL in media with 20% (w/v) Ficoll and 10% D_2O. The interaction of CHO-K1 cells with ^{19}F-labeled wild-type and Y125F α-synucleins were studied under the same conditions. Cell viability was tested after each NMR experiment by using the trypan blue exclusion assay. The viability was always greater than 90%.

Fluorescence Image Acquisition

The cells were treated with fluorescently-labeled CTP-α-synuclein for 20 h. For imaging, four washing steps with phosphate buffered saline were performed after translocation. The

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cells were imaged in their complete media using a Zeiss confocal microscope equipped with LSM 5 software and a 40x oil objective. Mitochondria were stained with Mito Tracker Red CMX Ros (Invitrogen) immediately prior to imaging. The measurements were acquired with multichannel detection using 488 nm excitation (Ar laser) for Alexa Fluor 488 and 543 nm excitation (HeNe1 laser) for Mito Tracker Red. Untreated cells were tested under the same conditions and no autofluorescence was noted.

RESULTS AND DISCUSSION

CTP-α-Synuclein Expression, Purification and Labeling

The fused construct (Figure 1) was expressed in *E. coli*. The CTP-α-synuclein expression level is ~2/3 that of the wild-type protein under the same conditions. The purity was confirmed by SDS-PAGE, and the presence of the CTP was confirmed by matrix-assisted laser desorption/ionization mass spectroscopy analysis (Figure S1). V3C CTP-α-synuclein was labeled with Alexa Fluor 488 maleimide dye with labeling efficiencies of ~80%. The presence of a single fluorescent band at the appropriate molecular weight on the SDS-PAGE indicated purity.

Cell Suspensions for NMR Experiments

Studying the interaction of wild-type and CTP-α-synuclein with the plasma membrane by using NMR requires that the cells remain in suspension during data acquisition. CHO-K1 cells tend to settle quickly to the bottom of the NMR tube. This settling removes the bound α-synuclein from the NMR detection zone, degrading accuracy.

Several devices,[29] gels,[30] and natural biodegradable polymers[31] have been used to facilitate suspension and prolong cells viability during NMR experiments. We overcame the settling problem by using a hydrophilic polysaccharide, Ficoll, and adjusting its concentration so as to keep the CHO-K1 cells suspended (Figure 2). After 3 h in the NMR tube, the cells settled in 10% (w/v) Ficoll and exhibited low viability. At 20% (w/v) Ficoll the cells remained suspended with a viability higher than 90%. Ficoll has also been used to prevent settling of *Xenopus laevis* oocytes during NMR data acquisition.[32]

Interaction of Wild-type and Y125F α-Synucleins with the Plasma Membrane

In the absence of cells, the middle peak in the spectrum of wild-type α-synuclein represents an overlap of the resonances from the 3-fluoro-tyrosines at positions 39 and 125.[19] Upon incubating ^{19}F-labeled wild-type α-synuclein with the cells a 35% decrease in the area under the middle peak was noted (Figure 3*A*), but the resonances from the 3-fluoro-tyrosines at positions 133 and 136 decreased by only 10% and 6%, respectively. The decrease could arise from increased heterogeneity, but several studies show that higher eukaryotic cells in the media do not dramatically interfere with the NMR spectra of intracellular proteins and extracellular peptides.[8, 23, 33] Studies conducted in detergent micelles and synthetic lipid vesicles demonstrate that the decreases in peak intensities arise from binding, without interference from the heterogeneity of the environment.[1, 20] Instead, we interpret the decrease in the middle peak as arising from the restricted motion experienced by that part of the protein interacting with the plasma membrane.

We used Y125F α-synuclein to determine which of the two resonances changed upon interaction with the cells. For this variant, the middle peak corresponds only to the resonance from position 39. A significant decrease in the resonance from position 39 was noted upon incubating Y125F α-synuclein with the cells, but the resonances from positions 133 and 136 remained almost unchanged (Figure 3*B*). Control experiments (Figure S2) show that the decrease observed in the resonance from position 39 is not caused by a change in pH

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or the presence of Ficoll. Furthermore, no significant change in peaks' intensities was noted when Y39F α-synuclein was used (Figure 4). The slight decrease in the area under resonances from samples containing cells (Figures 3A and 4) arises from dilution by the cells themselves and by residual media remaining after collecting the cells. Based on our findings, it appears that the N-terminal region interacts with plasma membrane, while the C-terminal tail retains its motional freedom. Although high concentrations of polysaccharides (ca. 300–400 g/L) can induce protein aggregation[34], this is not a concern for the work reported here because no changes in NMR spectra were observed for a control sample where the protein was stored for 12 h in 20% Ficoll (Figure S2).

These data suggest a model wherein at least the N-terminal third of α-synuclein, but not its C-terminal region, lie on or in the membrane surface. This observation is based on the fact that the Y39F resonance decreases, but the resonances from the C-terminal region remain nearly constant. Also, no significant translocation of the protein was noted when fluorescently-labeled V3C α-synuclein was used (data not shown) indicating that the bound protein does not cross the plasma membrane. The interaction of ^{19}F-labeled α-synuclein with SDS micelles[20] and LUVs[35] has also been studied, and a similar binding pattern was noted. Our findings agree with NMR data on α-synuclein binding to small unilamellar vesicles where signal attenuation was noted for different segments of the protein with uniform, narrow widths for all resonances.[1] Furthermore, changes in chemical shifts were absent and NOE experiments excluded contributions from fast exchange. Also, higher molecular weight biomolecules are not as prone to changes in chemical shift as are smaller ones, such as peptides.[22]

Interaction of CTP-α-Synuclein with Plasma Membrane

^{19}F-labeled CTP-α-synuclein was used to study the early stage insertion of the CTP into the plasma membrane. CTP-α-synuclein has five tyrosines, one in the CTP at position -11, one at the position 39 in the N-terminal region, and three in the C-terminal region (Figure 1). Figure 5*A* shows the spectrum of the ^{19}F-labeled fusion protein in cell media containing 20% (w/v) Ficoll and 10% D_2O. A significant change was noted immediately after adding CHO-K1 cells (Figure 5*B*). After 1 h, the most downfield resonance, from the 3-fluoro-tyrosine at position -11, disappeared leaving a spectrum resembling that of wild-type α-synuclein (Figure 5*C*). The disappearance of this resonance is attributed to the interaction of the CTP in the fused construct with the plasma membrane. The resonance from 3-fluoro-tyrosine at position 133 partially overlaps the resonance from the 3-fluoro-tyrosine in the CTP. The CTP loses motional freedom upon interacting with the cell membrane, diminishing its intensity. The partial overlap makes it appear that the intensity of the resonance from 3-fluoro-tyrosine 133 also decreases. From these data we conclude that most of the fusion construct bound the cells in the first hour.

The data for CTP-α-synuclein suggest a different type of binding compared with that of the wild-type α-synuclein. The CTP inserts into the lipid bilayer while the α-synuclein portion does not interact with plasma membrane after 1 h of incubation, as suggested by the observation that the resonance at position 39 is unaffected. To confirm that the fusion protein interacts with plasma membrane and to verify that CTP-α-synuclein can be translocating into the cells, we used a different approach.

Confocal Microscopy Studies of CTP-α-Synuclein Translocation

Translocation of CTP-α-synuclein into mammalian cells was assessed by incubating the fluorescently-labeled construct with CHO-K1 cells for 20 h (Figure 6). Mitochondrial staining confirmed localization of the protein to the cytoplasm with no fluorescence present in the nucleus. Some co-localization of α-synuclein and mitochondria was noted, in

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agreement with other studies.[36–40] Control experiments using fluorescently-labeled α-synuclein under the same conditions showed no significant intracellular fluorescence. These observations indicate that α-synuclein does not cross the plasma membrane in significant amounts over this time period in the absence of CTP. These data support our models where, in the absence of CTP, the N-terminal region of α-synuclein is located on the plasma membrane, but CTP disrupts this structure as a result of its penetration into the lipid bilayer.

CONCLUSIONS

Fluorine NMR, combined with fluorescence microscopy, provides information on the interaction of α-synuclein and CTP-α-synuclein with the plasma membrane of higher eukaryotic cells. This study also shows the utility of ^{19}F NMR for investigating the interactions between proteins and the plasma membrane as well for monitoring the insertion of cell-penetrating peptides. The results advance our understanding of the interaction of proteins and peptides with the plasma membrane and indicate that ^{19}F NMR will be a valuable tool for studying the translocation of other carriers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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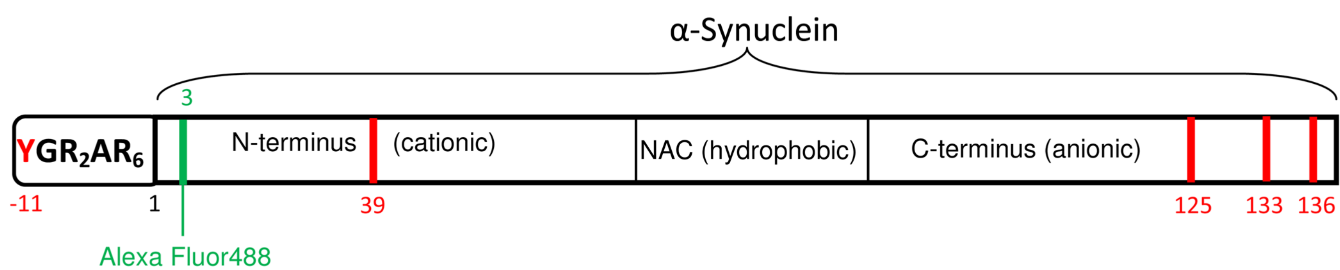

Figure 1.
Schematic representation of the cytoplasmic transduction peptide (CTP), YGR_2AR_6, covalently attached to the N-terminus of α-synuclein. Red indicates the positions of tyrosines. Green shows the position of the fluorescent label.

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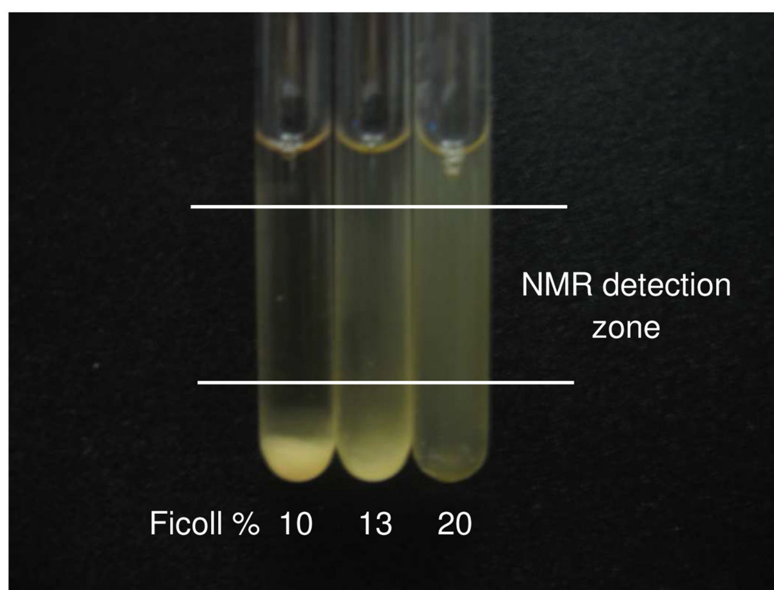

Figure 2.
NMR tubes containing an initial uniform suspension of ~1.5×10^7 CHO-K1 cells/mL in F-12 media with 10% D_2O and various (w/v) concentrations of Ficoll at 37 °C after 3 h. The cells viability was greater than 90% for 20% Ficoll.

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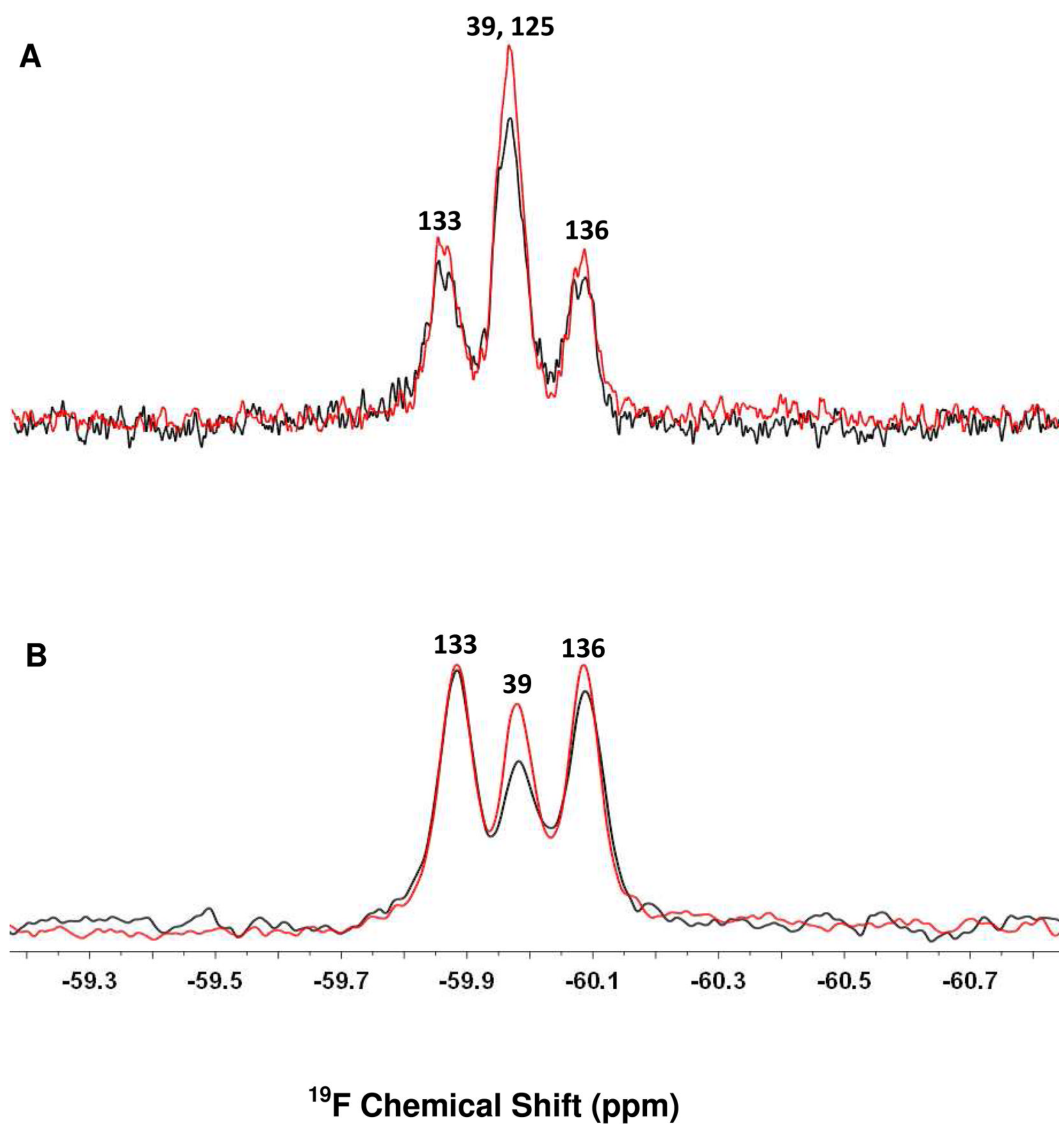

Figure 3.
^{19}F-labeled α-synuclein spectra in the presence (black) and absence (red) of CHO-K1 cells. (A) wild-type α-synuclein (B) Y125F α-synuclein. The protein concentration was 100 μM. Residue assignments[19] are shown above the resonances.

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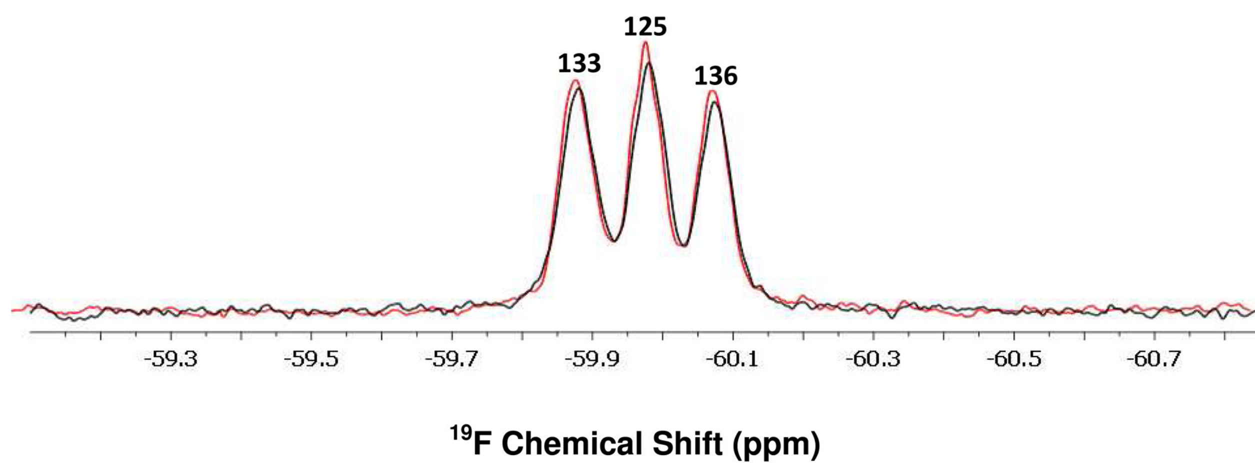

Figure 4.
^{19}F-labeled Y39F α-synuclein spectra in the presence (black) and absence (red) of CHO-K1 cells. Residue assignments[19] are shown above the resonances.

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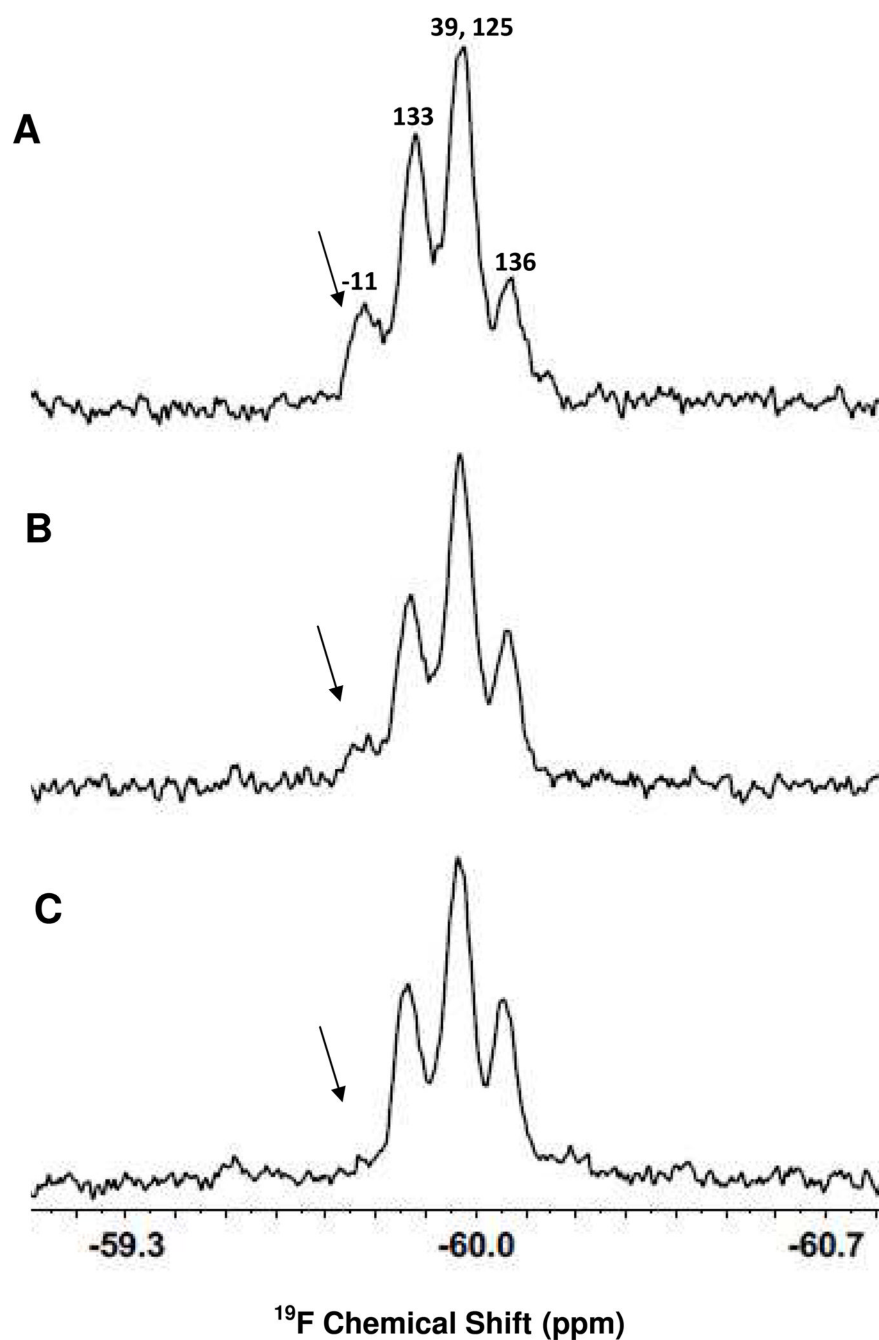

Figure 5.

Interaction of ^{19}F-labeled CTP-α-synuclein with CHO-K1 cells. (A) CTP-α-synuclein in cells media containing 20% (w/v) Ficoll and 10% D_2O (B) CTP-α-synuclein and CHO-K1 cells in the same media and (C) after 1 h incubation. The protein concentration was 100 μM. The arrow indicates the decrease in the resonance from Y-11.

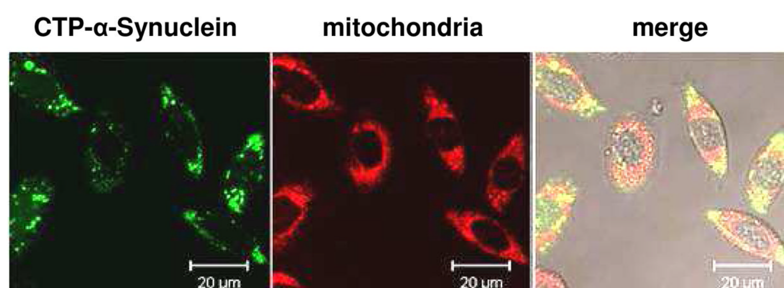

Figure 6.
CTP mediated delivery of fluorescently-labeled α-synuclein into CHO-K1 cells. From left to right: Alexa Fluor 488 fluorescence (green), Mito Tracker Red fluorescence (red), and merged images. No autofluorescence was noted.

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