Published in final edited form as:

J Mol Biol. 2007 May 11; 368(4): 1132–1144.

Isolation of a human single chain antibody fragment against oligomeric α -synuclein that inhibits aggregation and prevents α -synuclein induced toxicity

Sharareh Emadi, Hedieh Barkhordarian, Min S. Wang, Philip Schulz, and Michael R. Sierks

Department of Chemical and Materials Engineering, Arizona State University, Tempe, AZ 85287-6006.

SUMMARY

Protein misfolding and aggregation are pathological aspects of numerous neurodegenerative diseases. Aggregates of α -synuclein are major components of the Lewy bodies and Lewy neurites associated with Parkinson's Disease (PD). A natively unfolded protein, α -synuclein can adopt different aggregated morphologies, including oligomers, protofibrils and fibrils. The small oligomeric aggregates have been shown to be particularly toxic. Antibodies that neutralize the neurotoxic aggregates without interfering with beneficial functions of monomeric α -synuclein can be useful therapeutics. We were able to isolate single chain antibody fragments (scFvs) from a phage displayed antibody library against the target antigen morphology using a novel biopanning technique that utilizes Atomic Force Microscopy (AFM) to image and immobilize specific morphologies of α -synuclein. The scFv described here binds only to an oligomeric form of α -synuclein and inhibits both aggregation and toxicity of α -synuclein in vitro. This scFv can have potential therapeutic value in controlling misfolding and aggregation of α -synuclein in vivo when expressed intracellularly in dopaminergic neurons as an intrabody.

Keywords

Parkinson's Disease; AFM; scFv; α -synuclein; oligomer; phage display

INTRODUCTION

Parkinson's Disease (PD) is the second most common neurodegenerative disorder affecting about 1% of population over the age of 65¹. PD is characterized clinically by resting tremor, slowness of movement, muscular rigidity and impairment of postural reflex. Pathologically, PD is characterized by the progressive loss of dopaminergic neurons in the substantia nigra and formation of fibrillar cytoplasmic inclusions known as Lewy bodies and Lewy neurites^{2; 3}. α -synuclein was identified as the major component of Lewy bodies (LBs) and Lewy neurites found in PD and other neurodegenerative disorders including the LB variant of Alzheimer's disease, dementia with LBs, as well as the glial and neuronal cytoplasmic inclusions of multiple system atrophy^{4; 5; 6}. Three point mutations in the α -synuclein gene (A53T, A30P and E46K), as well as dosage effects caused by gene triplication, have been

Corresponding Author: Michael R. Sierks, Chemical and Materials Engineering, Box 876006, Tempe, AZ 85287-6006, Phone: 480-965-2828, Fax: 480-965-0037, Email: sierks@asu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

linked to familial PD^{7; 8; 9}. In addition, overexpression of α -synuclein in neuronal cell lines¹⁰, transgenic mice¹¹ and drosophila¹² has been shown to lead to the formation of intracellular LB-like inclusions and other PD-like symptoms. Thus α -synuclein has become a main target for understanding and controlling the progression of PD.

α -synuclein is a small protein of 140 amino acids expressed primarily at presynaptic terminals in the central nervous system¹³. The N-terminal region of α -synuclein encompasses three mutation sites associated with familial PD (A53T, A30P, E46K) and contains six imperfectly conserved repeats (KTKEGV), that may facilitate protein-protein binding. This repeat section acquires a secondary α -helical structure upon binding to acidic phospholipid membranes^{14; 15}. The central region, known as the non-amyloid component (NAC) is extremely hydrophobic and appears to be essential for aggregation¹⁶. The acidic C-terminal region is responsible for the chaperone function of α -synuclein^{17; 18}. Although the exact role of α -synuclein in normal cell function is still unknown, ample evidence suggests that overexpression of α -synuclein interrupts normal cell functions¹⁹ resulting in decreased neurite outgrowth and cell adhesion²⁰.

α -synuclein normally exists in an unfolded conformation, but it can adopt different folded conformations, including small aggregates or oligomers, spherical and linear protofibrils as well as the fibrillar structure found in LBs^{21; 22; 23}. Several environmental factors such as low pH, high temperature, metal cations²⁴, pesticides²⁵, heparin and other GAGs²⁶ have also been shown to play a role in conversion of α -synuclein into its β -sheet conformation. While misfolding and aggregation of α -synuclein are required for LB formation, the exact role of different morphologies of α -synuclein aggregates in PD is still unclear. High-molecular weight α -synuclein species including small oligomers have been identified in brains of humans with PD and other related disorders²⁷. Several studies have suggested that the prefibrillar oligomers, rather than the mature fibrils, are the neurotoxic species^{28; 29}. This assumption is also supported by the fact that annular-shaped protofibrils (ring, spherical or tubular) that developed in the presence of artificial or brain-derived vesicles, can penetrate membranes to form pore-like channels and cause membrane damage^{30; 31}. Moreover, in most cell culture models, toxicity is seen without extensive aggregated α -synuclein³² and cell death occurs prior to α -synuclein aggregation and deposition into insoluble fraction *in vitro*³³.

Since misfolding of α -synuclein into specific toxic morphologies is essential in the progression of PD and other related diseases, identification of the toxic forms of α -synuclein and prevention of their accumulation are important for understanding the progression of this disease and for developing a therapeutic strategy. Here we utilize a novel biopanning technology combining phage display technology and Atomic Force Microscopy (AFM) to isolate individual single chain antibody fragments which bind to a specific target morphology of α -synuclein³⁴. AFM is used to visualize the target morphology and to monitor the panning process. Using only a minimal amount of the target antigen, we were able to isolate an scFv that specifically binds to the oligomeric form of α -synuclein after only two rounds of selection. The scFv was able to inhibit α -synuclein cytotoxicity when co-incubated with α -synuclein and also when added to performed oligomeric aggregates.

The successful selection of the recombinant antibody expressed on the surface of bacteriophage by this approach has potential therapeutic value since the scFvs are based on human gene sequences that can be expressed intracellularly (termed intrabodies) to prevent formation of toxic aggregates *in vivo* or to facilitate their clearance. This approach has been used to block toxic effects of various pathogenic agents with high selectivity³⁵. It has been shown that an anti-huntingtin intrabody can successfully inhibit aggregation and neurotoxic properties of mutant huntingtin protein^{36; 37}. Recently, this approach has also been used successfully to counteract the pathogenic effects of overexpressed α -synuclein, thereby providing precedent

for the use of intrabodies in Parkinson's Diseases³⁸. Furthermore, oligomeric species of α -synuclein have been reported extracellularly in plasma and CSF³⁹ and immunization studies in mouse models of PD show that extracellular antibodies against α -synuclein can reduce accumulation of intracellular aggregates⁴⁰. These studies suggest morphology specific scFvs can be valuable both as a diagnostic tool to identify toxic species of α -synuclein in plasma and CSF and also in potential passive vaccination strategies for treating PD.

RESULTS

Biopanning against human monomeric/oligomeric α -synuclein

The Tomlinson I and J antibody libraries were used to pan against a sample of monomeric/oligomeric α -synuclein immobilized on a mica surface. Three rounds of panning were performed. Polyclonal phage ELISA indicated an increase in bound phage from the second to the third round of panning (data not shown). The presence of positive binding phage after each round was verified by incubating an aliquot of eluted phage with α -synuclein and imaging by AFM. After two rounds of panning, only bound phage from the α -synuclein sample (data not shown) and not from the control sample without α -synuclein was observed. The eluted phage obtained from the second and third rounds of panning were used to infect *E. coli* TG1 and 48 individual clones from each round were tested for binding to antigen. As indicated by monoclonal phage ELISA, 26 and 13 clones from the second and third rounds of panning respectively, showed positive binding to monomeric/oligomeric α -synuclein. Furthermore, PCR analyses showed the presence of full-length scFvs in 11 out of the 21 clones from round 2 and 3 out of the 13 clones from round 3. We selected two full-length scFvs for further studies based on phage ELISAs that indicated a preferential binding for the oligomeric form of α -synuclein. DNA sequencing indicated that both clones contained an amber stop codon (TAG) in one of the randomized positions of the heavy chain (data not shown). We replaced the amber stop codon with a glutamine codon (CAG) in the stronger binder clone (D5 scFv) using site-directed mutagenesis as described⁴¹. The binding of the corrected D5 clone to the oligomeric form of α -synuclein was verified by monoclonal phage ELISA (data not shown) as well as AFM imaging (Figure 1c).

Expression and purification of soluble scFv

We purified soluble scFv from the corrected D5 clone for further characterization. Purified protein showed a single protein band with molecular mass of 29 kDa, corresponding to expression of a full-length scFv by SDS-PAGE and Western-blot (Figure 2).

Binding specificity of selected purified scFv to oligomeric α -Synuclein

The D5 scFv specifically recognized the oligomeric form of α -synuclein as indicated by soluble ELISA against three different α -synuclein morphologies, while no binding was observed with a non-specific control scFv (anti-phosphorylaseB) (Figure 3). Western blot analysis showed binding of D5 scFv to the aggregated α -synuclein samples at 29 kDa and 56 kDa bands, which correspond to the approximate sizes of dimeric and tetrameric forms of α -synuclein, but no binding to monomeric α -synuclein (Figure 4). Binding studies using a Biacore X biosensor also did not indicate any binding between the D5 scFv and monomeric α -synuclein (data not shown).

In order to further demonstrate that the D5 scFv recognizes an oligomeric morphology of α -synuclein, we incubated a 50 μ M sample of α -synuclein and removed aliquots at different time points during aggregation. We then tested each sample for reactivity with scFv using a nitrocellulose membrane and visualized the aggregate morphologies on mica using AFM. Immunoreactivity of D5 with α -synuclein was only detected with the 4 and 8 days samples (Figure 5A) and no reactivity was observed with a non-specific control protein (BSA). At the

early time points, small aggregates of α -synuclein are visible, while at later time points, elongated protofibrils and fibrils are present (Figure 5B). These results indicate that the D5 scFv reacts specifically with an oligomeric form and does not react with either monomeric or the fibrillar forms of α -synuclein.

scFv inhibits α -synuclein aggregation

To determine if purified scFv could alter α -synuclein aggregation, we co-incubated α -synuclein (50 μ M) with D5 scFv (15 μ M) and followed aggregation kinetics using Thioflavin T (ThT) and AFM imaging. Incubation of α -synuclein alone shows a time dependent increase in fluorescence, as the α -synuclein begins to aggregate (Figure 6A). Co-incubation of α -synuclein (50 μ M) with scFv (15 μ M) inhibits aggregation over the 21-day period, as determined by ThT fluorescence. Co-incubation of 50 μ M α -synuclein with a similar 15 μ M concentration of a non-specific control scFv (anti-phosphorylase B) did not alter aggregation kinetics as determined by ThT fluorescence (data not shown).

Further evidence that the scFv inhibits α -synuclein aggregation is shown via AFM imaging. From the AFM scans, α -synuclein incubated without scFv shows extensive protofibril or filament formation at day 18. The sample incubated with scFv however, did not show any formation of protofibrils or filaments, but did show an increase in formation of small amorphous aggregates during the same time period (Figure 6B).

scFv blocks cytotoxicity of aggregated α -synuclein

We examined the cytotoxicity of aggregated α -synuclein samples over a 21-day period toward the human neuroblastoma cell line SH-SY5Y using an MTT assay to measure toxicity. When incubated alone, α -synuclein exhibited cytotoxicity to SH-SY5Y cells only at the earlier incubation times with maximal reduction of MTT activity at 4 days (~ 20%). Co-incubation of α -synuclein (final concentration, 2 μ M) with scFv (final concentration, 0.3 μ M) shows protection from toxicity as indicated by MTT activity over the entire time span studied (Figure 7). Soluble oligomers have been identified as the primary toxic species of α -synuclein in numerous studies^{28; 29}. In order to more accurately determine the effects of the scFv on cytotoxicity of oligomeric α -synuclein samples, we measured the toxicity of a 4 day pre-aggregated α -synuclein sample containing high concentrations of oligomeric forms toward SH-SY5Y cells after 2 hours of preincubation at room temperature with or without D5 scFv using both a blue trypan staining assay, which distinguishes dead cells from live ones, and an LDH assay. When incubated alone, the preaggregated 4-day α -synuclein sample exhibited substantial cytotoxicity to SH-SY5Y, causing 34.7 % cell death as indicated by blue trypan staining, and a 27 % increase in LDH activity (Table 1). Addition of 1 μ M D5 scFv to the α -synuclein sample shows complete protection from toxic effects in the 4 day sample as indicated by both the blue trypan staining and LDH levels (Table 1). Similarly, addition of 1 μ M D5 scFv alone to the cells showed comparable blue trypan staining and LDH level to that of the control sample (Table 1). Addition of 1 μ M control scFv (anti-phosphorylase B) to the α -synuclein sample did not affect the level of toxicity of α -synuclein. Similarly, addition of 1 μ M control scFv to the cells showed comparable LDH level to that of the control sample (data not shown).

DISCUSSION

Human neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's Diseases among others, have been correlated with the misfolding and aggregation of an underlying protein⁴². In each case a specific protein or peptide aggregates in a specific part of the brain causing the progressive degeneration of neurons associated with these diseases. Recently, neuropathologic and genetics studies and transgenic animal models have provided

strong evidence for the involvement of misfolding and aggregation of α -synuclein in the progression of PD 11; 12; 43 and other related diseases 5; 44; 45. Despite the compelling evidence of misfolded α -synuclein in PD, the exact role and mechanism by which α -synuclein is involved in neurodegeneration remains unclear. A number of different α -synuclein aggregate morphologies such as small aggregates, oligomers, spherical and linear protofibrils as well as fibrillar structure have been identified and shown to be neurotoxic 21; 22; 28; 29. While all these morphologies occur *in vitro*, their relevant role in the development of PD is unknown. Therefore, in order to better understand the progression of this disease and to develop effective therapeutic strategies, it is important to identify the toxic forms of α -synuclein and the means to prevent their accumulation. Several different strategies have been attempted to control aggregation of α -synuclein. Transition metals such as iron and magnesium have been shown to change the conformation of α -synuclein, resulting in either inhibition or promotion of α -synuclein aggregation 46. Other factors including lipid binding, phosphorylation and the C-terminal domain of α -synuclein have also been shown to modulate the conformation of α -synuclein 47; 48; 49.

Both β and γ -synuclein are potent inhibitors of α -synuclein fibril formation 50; 51, and short peptides directed at the central portion of α -synuclein were shown to inhibit aggregation and reduce toxicity 52. We previously reported inhibition of α -synuclein aggregation by scFv fragments isolated against monomeric α -synuclein 53. However, an effective therapeutic for PD should target only the toxic aggregate species, while leaving other forms of α -synuclein free to carry out their cellular functions. Here we utilize a novel technique that combines phage display technology with AFM, to isolate antibody fragments that bind specifically to the oligomeric form of α -synuclein. After only two rounds of selection, we were able to isolate several scFvs that bind the oligomeric form of α -synuclein. We then selected one of the scFvs for further study and demonstrated that soluble D5 scFv specifically recognized only the oligomeric form of α -synuclein by different assays including ELISA, time course dot blots and western blot analyses. ELISA and time course dot-blot show that the D5 scFv binds to an oligomeric form of α -synuclein, while Western-blot analysis shows that the scFv binds to the SDS-stable oligomers with molecular weight of about 29 kDa and 56 kDa corresponding to dimer and tetramer, respectively. The D5 scFv, when incubated at a 1:4 scFv to α -synuclein molar ratio *in vitro*, completely inhibited α -synuclein fibril formation as determined by both Thioflavin T staining and AFM studies (Figure 6). The scFv also decreased extracellular toxicity when co-incubated with α -synuclein compared to a sample where α -synuclein was incubated alone with the human neuroblastoma cell line SH-SY5Y (Figure 7). Without addition of scFv, α -synuclein exhibited cytotoxicity to SH-SY5Y cells only during early time points (0-4 days) in the aggregation process, showing a maximum reduction in MTT activity at 4 days (Figure 7). AFM image analyses indicate an increasing concentration of oligomeric α -synuclein at shorter time periods such as the 4 day sample, followed by an increase in the amount of fibrillar α -synuclein at the later time points such as the 18 day sample (Figure 6B). These results are consistent with other studies which identified the soluble oligomeric α -synuclein forms as the toxic species responsible for neurodegeneration and neuronal cell death 21; 28; 29. The 4 day sample consisting of predominantly oligomeric α -synuclein forms were shown to be highly toxic toward SH-SY5Y cells, resulting in over 30% cell death as indicated by blue trypan staining, and causing a 27% increase in LDH activity (Table 1). When the D5 scFv was co-incubated with α -synuclein before adding to the SH-SY5Y cells, no toxic effects were observed (Figure 7). In particular, the day 4 sample with scFv added completely protects against α -synuclein induced toxicity as opposed to the day 4 α -synuclein alone sample (Table 1).

We postulate that the D5 scFv inhibits α -synuclein toxicity and formation of fibrils not by inhibiting nucleation sites or protein folding conformation as seen with other aggregation inhibitors but rather by specifically binding to an oligomeric form of α -synuclein preventing

further aggregation to fibrils, and most importantly blocking interactions of oligomers with the cell membrane thereby preventing membrane damage.

In summary, we demonstrate that scFv antibody fragments that bind to a specific morphology of α -synuclein can be isolated from a phage display human antibody library using a novel combination of phage display and AFM technologies. The scFv isolated here specifically recognizes the highly toxic oligomeric morphology of α -synuclein and can be used to control α -synuclein aggregation and toxicity. While a number of different strategies including β -synuclein, hsp70, magnesium, truncated forms of α -synuclein, small peptides and vaccination ^{40; 46; 49; 51; 52; 54} have been developed to control α -synuclein aggregation and toxicity *in vivo* and *in vitro*, the major advantage of the present approach over other techniques is that the scFv will only target the toxic oligomeric morphology of α -synuclein, leaving any monomeric forms untouched and allowing them to function as needed. Furthermore, the isolated scFvs can be subjected to affinity maturation protocols to improve the specificity further as previously demonstrated ⁵⁵. Recently, it was shown that polyclonal antibodies that bind to a toxic oligomeric conformation of β -amyloid that has been implicated in Alzheimer's Disease, also bind to oligomeric structures of α -synuclein and other oligomeric proteins that are involved in Huntington's Disease, Type II diabetes and prion-related diseases. This suggests that all the above oligomeric forms of proteins share a common structural motif ⁵⁶. Recently, monoclonal antibodies that bind to an oligomeric conformation of β -amyloid have been developed to control β -amyloid aggregation and toxicity ^{57; 58}. A significant advantage of the human recombinant antibody fragments isolated here is that the scFvs can be well characterized and expressed intracellularly (intrabodies) for potential *in vivo* therapeutic applications or the treatment of diseases like PD and Huntington's Disease where specific toxic aggregates accumulate intracellularly ⁵⁹. In addition, this approach has been used successfully to neutralize the pathogenic effects of the huntingtin and α -synuclein proteins in cell models ^{36; 38} and huntingtin in drosophila models ⁶⁰. Furthermore, when expressed as intrabodies in dopaminergic neurons, the scFvs isolated here will specifically bind only to the toxic oligomeric morphologies of the target protein, while leaving any other beneficial protein forms untouched, thereby providing great potential value as part of a therapeutic for controlling PD. Recent studies have shown that oligomeric α -synuclein is found extracellularly in CSF ³⁹ and that vaccination of transgenic mice overexpressing α -synuclein with α -synuclein can reduce intracellular aggregates ⁴⁰. Therefore an scFv against oligomeric α -synuclein may also have application in passive vaccination strategies for treating PD. These potential therapeutic strategies need to be tested in animal models of PD.

MATERIALS AND METHODS

The Tomlinson I and J phage libraries, helper phage KM13, *E. coli* TG1 and HB2151 were obtained from MRC Center for Protein Engineering (Cambridge, England) (<http://www.hgmp.mrc.ac.uk>). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Phage Display Antibody Library

Both phage libraries have a diversity of greater than 10^8 scFvs, comprised of a single polypeptide with the V_H and V_L domains connected by a flexible $(Gly_4Ser)_3$ linker. The scFv genes are contained in an ampicillin resistant phagemid vector and expressed in *E. coli* TG1 cells. The CDR3 of the heavy chain was designed to be as short as possible while maintaining an antigen binding surface. Libraries are in phagemid/scFv format and have been prescreened for binding to Protein A and Protein L. Libraries were grown separately and mixed in equal titers for the panning experiments.

Alpha synuclein

Lyophilized human recombinant α -synucleins were a generous gift from Dr. V. Uversky (University of Indiana, School of Medicine) and Dr. P. Lansbury (Brigham and Women's Hospital, Harvard Medical School). A mixture of monomeric and oligomeric α -synuclein was prepared directly from the lyophilized stock by dissolving in PBS 1x (10 mM phosphate, 150 mM NaCl) pH 7.4 and filtered through a 0.2 μ m pore membrane. Size exclusion chromatography was used to separate monomeric from all other oligomeric species of alpha synuclein. Fibrillar α -synuclein was prepared by incubating 70 μ M of α -synuclein in a buffer containing glycine (0.2 M) and NaCl (150 mM) (pH 2.7) at 56 °C for 12 days, then at 37 °C for 7 days, and stored at 4 °C for 10 days, without shaking.

Aliquots (10 μ l) of different α -synuclein morphologies (mixture monomer/oligomer, oligomer, and fibrillar) (Figure 1a and 1b) as well as a 6-day preincubated solution of aggregated α -synuclein (0.7 μ M) with purified D5 phage (10^{12} pfu/ml) (Figure 1c), were deposited on freshly cleaved mica and fixed for 5 minutes. Then the substrate was washed three times with 1 mL of ultra pure water. The sample was then dried under a gentle stream of nitrogen gas and imaged by AFM (Figure 1).

Phage production

Production of phage was performed essentially as described (<http://www.hgmp.mrc.ac.uk>). Briefly, bacterial cultures in the log phase ($A_{600} = 0.5$ – 0.6) were infected with helper phage KM13 at a ratio of 1:20 (number of bacterial cells/phage particles) for 30 minutes at 37 °C without shaking. Media was changed to 2 $\times$ YT with 100 μ g/mL ampicillin and 25 μ g/mL kanamycin, and grown overnight at 30 °C for phage production. Phage were purified from the supernatant by polyethylene glycol (PEG) precipitation and resuspended in PBS (phosphate-buffered saline) and used for panning.

AFM panning against monomeric and oligomeric α -synuclein

Aggregation of α -synuclein is a complex process that involves oligomeric intermediates of various sizes and morphologies, rather than a simple two-state transition from monomer to fibrils. We utilized a mixture of monomeric/oligomeric morphologies of α -synuclein to pan against the antibody library in order to isolate potential therapeutic antibodies. Selection of phage against the α -synuclein mixture was performed by incubating a 6 μ M sample of α -synuclein with an aliquot of 4×10^{12} pfu/mL of purified phage for 2 minutes. Then a 10 μ L aliquot of the incubated solution was deposited on freshly cleaved mica and fixed for 5 minutes before it was rinsed three times with 1 mL of ultra pure water. The sample was subsequently washed 10 times with 1 mL PBS-Tween 0.1% and 15 times with PBS in order to remove the non-specifically bound phage. After several rounds of washing, the sample was dried under nitrogen gas and imaged by AFM to verify the presence of bound phage on the mica surface. The bound phage were then eluted using 800 μ L of trypsin (1 mg/mL) and incubated for 10 minutes with continuous rocking at room temperature. To determine the eluted phage titers, 500 μ L of eluted phage were added to 1.2 mL of TG1 (OD_{600} of 0.4) and incubated for 30 minutes at 37 °C. The infected *E.coli* cells were plated in serial dilutions on agar plates containing ampicillin (100 μ g/mL). Eluted phage were amplified by infection of fresh *E. coli* TG1 cells in the presence of helper phage KM13 (5×10^{10}) and purified according to standard protocols (<http://www.hgmp.mrc.ac.uk>).

Selection by phage EIISA

a) Polyclonal ELISA—High binding polystyrene microtiter plates were coated with 50 μ g/mL of monomeric/oligomeric α -synuclein in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) at 37 °C for 2 hours. Non-specific binding was blocked by incubation with 3% BSA at 37 °C

for 2 hours. An aliquot of 10^{10} titer unit of PEG precipitated phage in 100 μ L of 2% PBS-milk (PBSM) was added to the wells and incubated for 90 min at room temperature. Bound phage were detected after one hour incubation with a 1:5000 dilution of anti-M13 antibody horseradish peroxidase (HRP) conjugate. A 100 μ L aliquot of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) was added and the reaction was stopped after 20 min with 2M H_2SO_4 (VWR). The activity was determined by subtracting OD₆₅₀ from OD₄₅₀ using a Wallac 1420 plate reader (Perkin Elmer).

b) Monoclonal ELISA—48 individual clones obtained from panning against the monomeric/oligomeric α -synuclein mixture were grown as described (<http://www.hgmp.mrc.ac.uk>). The high binding microtiter plates (Corning, USA) were coated with 50 μ g/mL of human recombinant α -synuclein as described above. Non-specific binding was blocked by incubation with PBS+2% non-fat milk (2% PBSM) for 2 hours at room temperature. Bacterial supernatant containing antibody fragments was added to each well (100 μ L/well). Bound phage were detected as described above.

Soluble scFv ELISA

Soluble scFv was produced by expressing recovered phagemid samples in the non-suppressor *E.coli* strain HB2151⁶¹. Individually selected clones were grown essentially as described (<http://www.hgmp.mrc.ac.uk>), and scFv production was induced by addition of 1mM isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma) and incubated overnight at 30°C. Supernatant of samples were separated by centrifugation (1500 $\times$ g, 30 min, 4°C) and periplasmic fractions were prepared as described⁶². Both the supernatant and periplasmic fractions were assayed for antigen binding by ELISA as described above. High affinity microtiter plates were coated with 50 μ g/mL of human recombinant oligomeric α -synuclein. After blocking and washing, an aliquot (100 μ L) of the supernatant or periplasmic fractions containing antibody fragments was added to each well and the plate was incubated for 2 hours at room temperature. After that the plates were incubated with an anti-C-myc tag (9E10) mouse monoclonal antibody (1:500 dilution) (Santa Cruz Biotechnology, USA). Bound antibodies were detected after a 1-hour incubation using a 1/500 dilution of goat anti-mouse IgG HRP conjugated (Santa Cruz Biotechnology).

Production and Purification of scFv

Soluble scFv from selected individual clones was produced as described above. The supernatant and periplasmic fractions from a 1-L culture were combined, passed through a 0.2 μ m filter (Whatman, Clifton, NJ), and then concentrated in a tangential flow filter (Millipore) using a 10 kDa filter membrane (Millipore, Billerica, MA). Concentrated samples were applied to a protein A-Sepharose column (GE healthcare, NJ) which was equilibrated in PBS 1x, pH 7.4, at 4 °C. After washing the column in the same buffer, bound scFv was eluted from column with 0.2 M glycine, pH 3. Fractions containing scFv were pooled, adjusted to neutral pH, dialyzed and stored at -20 °C. The purity of the scFv was estimated by electrophoresis on 12 % (W/V) SDS-polyacrylamide gels.

Size exclusion chromatography

Size exclusion chromatography was used to separate monomeric and oligomeric α -synuclein forms. A Suprose 12 HR prepacked column (GE Healthcare, USA), 30 cm length with 1 cm diameter, was washed and run with PBS (pH 7.4) at a flowrate of 0.25 mL/min. A mixture of monomeric and oligomeric α -synuclein (1.5 mg/mL) was prepared directly from lyophilized stock by dissolving in PBS 1x, pH 7.4 and applied to the column equilibrated in PBS 1x, pH 7.4 at 4 °C. After washing the column in the same buffer, 0.5 mL fractions were collected and analyzed by electrophoresis on a 10 % Tris-Tricine SDS-PAGE⁶³.

Tris-Tricine SDS-PAGE and Western-blot

Lyophilized stock of α -synuclein was dissolved directly in PBS 1x (pH 7.4), filtered using a 0.2 μ m filter (Millipore, Billerica, MA), and passed through a size exclusion column (Suprose 12, GE Healthcare) to separate the monomeric and oligomeric α -synuclein forms. The concentration of the eluted samples (monomeric and oligomeric) was determined using a BCA Protein Assay Kit (Pierce, IL, USA). A 50 μ g aliquot of each sample was analyzed by 10% SDS-PAGE using Tris/Tricine buffer system as described previously⁶³, and then transferred to nitrocellulose membrane (Bio-Rad, CA) by blotting at 100 V for 90 min in transfer buffer (Tris 25 mM; Glycine 190 mM; Methanol 20 %). The membrane was blocked with 5 % PBS-milk for 2 hours. An aliquot of 0.3 mg/mL scFv was added and incubated for 24 hours at 4 °C, followed by a 2 hour incubation with 1/500 dilution of 9E10 antibody. The immunoreactivity was detected by 1 hour incubation with 1/500 dilution of goat-anti-mouse IgG HRP conjugated, followed by staining with 3,3'-Diaminobenzidine Tetrahydrochloride (DAB, Sigma).

Dot Blot Assay

Lyophilized α -synuclein was dissolved to a final concentration of 50 μ M in 25 mM Tris-HCl and 150 mM NaCl (pH 7.4), filtered through 0.2 μ m filter (Millipore, Billerica, MA), and incubated at 37 °C for 18 days. An aliquot (5 μ L) of incubated solution was applied to nitrocellulose membranes (Bio-Rad, CA) and air dried. The membrane was blocked with PBS 1x+0.5% milk and probed with 0.3 mg/mL of scFv antibody as described above.

Plasmid preparation and PCR amplification

Plasmid was isolated from *E.coli TGI* using a Qiagen Plasmid Miniprep Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The presence of a full length 935 bp scFv insert was confirmed by polymerase chain reaction (PCR) using 24 cycles of amplification utilizing the phagmid DNA as template and the backward primer, LMB3 (5'-CAGGAAACAGCTATGAC), and forward primer, pHEN seq (5'-CTATGCGGCCCCATTCA) as described⁶⁴. PCR analysis was performed using 1.5% (w/v) agarose gels (Merck).

BIAcore study

Antigen-scFv binding was performed using a BIAcore X biosensor (BIAcore Inc., NJ). A CM5 sensor chip (BIAcore Inc) was activated as recommended by the manufacturer using an equimolar mixture of NHS (N-hydroxysuccinimide) and EDC (N-ethyl-N'-(dimethylaminopropyl)carbodiimide), and coupled with 20 μ g/mL of human recombinant monomeric α -synuclein in pH 4.0 Sodium acetate buffer, and then blocked with ethanolamine. The final coupling of monomeric α -synuclein was 180 RU. The scFv concentration was between 1.5 μ M – 3.4 μ M with HBS-EP (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA), 0.005 % (v/v) surfactant P20, (pH 7.4) (BIAcore Inc.) as the running buffer at a flow rate of 10 μ L/min. The sensor surface was regenerated using Na₂CO₃/NaHCO₃, (pH 9.9). The final values were obtained by subtracting the readings from the sample cell (with α -synuclein) by the readings from the control cell (without α -synuclein).

Thioflavin T aggregation assay

α -synuclein was dissolved to a final concentration of 50 μ M in 25 mM Tris-HCl and 150 mM NaCl (pH 7.4), passed through 0.2 μ m filter, and incubated at 37 °C either with or without the addition of 15 μ M scFv. Aggregation of α -synuclein was measured in triplicate at various time points by adding 10 μ L aliquots of the above solution into 2 mL of 5 μ M thioflavin T solution in 25 mM Tris-HCl and 150 mM NaCl (pH 7.4). The fluorescence intensity of the samples were measured at an excitation wavelength of 450 nm and an emission wavelength of 482 nm

with a Shimadzu RF-551 spectrofluorophotometer using 1 cm light-path quartz cuvettes with both excitation and emission bandwidths of 5 nm.

Cell culture and viability assay

Human neuroblastoma cells (SH-SY5Y) were maintained in medium containing 50% minimal essential medium (MEM), 50% Ham's modification of F-12, 10% fetal bovine serum (FBS), 1% L-glutamine (3.6mM), and 1% penicillin/streptomycin antibiotic and grown in a 5% CO₂ atmosphere at 37 °C. Cells were harvested from flasks and plated in 96-well polystyrene plates (Corning Inc., Corning, NY) with 2×10^4 cells per 100 μ L of medium per well. Plates were incubated at 37 °C for 24 h to allow cells to attach. After 24h, the medium was exchanged with 100 μ L serum free media, followed by the addition of the preincubated mixtures of α -synuclein with or without scFv to individual wells. The final concentrations of α -synuclein and scFv were 2 μ M and 0.3 μ M, respectively. The same volume of medium was added to the control cultures. Plates were then incubated at 37 °C for an additional 48 h. Cell viability was measured by both LDH (Lactic Dehydrogenase Based, Sigma) and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assays essentially as described previously⁶⁵. Briefly, after the cells were incubated with the various α -synuclein samples, MTT was added to the culture medium to a final concentration of 0.5 mg/mL. After incubation at 37 °C for 3 h, the plates were centrifuged and the media was aspirated from each well. A 100 μ L aliquot of MTT dissolvent (0.1 N HCl in isopropanol) was added to each well. The plates were agitated at room temperature for 15 min to dissolve crystals. The absorbance was measured by a Victor Wallac multi-well assay plate reader (PerkinElmer, Gaithersburg, MD) at 560 nm. Averages from four replicate wells were used for each sample and control, and each experiment was repeated three times. Cell viability was calculated by dividing the absorbance of wells containing samples (corrected for background) with the absorbance of wells containing media alone (corrected for background).

LDH activities of each of the SH-SY5Y culture samples were measured following the protocol from the manufacturer (Sigma-Aldrich, St Louis, MO). Briefly, aliquots (50 μ L) of the media from each well were transferred to a 96-well plate. The remaining media was removed, the supplied lysis buffer was added, and the plates were incubated at 37 °C for 1 h. The supplied buffer and substrate were then added to both the supernatant and lysed cell samples as described by the manufacturer. The plates were incubated in the dark at room temperature for 30 min. The reaction was stopped and the absorbance was measured by subtracting OD₆₅₀ from OD₄₉₀ using a Wallac 1420 plate reader (Perkin Elmer). The percentage of LDH release was calculated by dividing the LDH activity in culture media by the total LDH value. The data are reported as percentage of control value obtained from three independent experiments.

Blue Trypan assay

Human neuroblastoma cells (SH-SY5Y) were maintained in media as described previously. After 24 hours, the media was exchanged with 100 μ L serum free medium and an aliquot of a mixture α -synuclein (50 μ M) preincubated for 4 days with or without scFV (25 μ M) was added to individual wells. The plates were then incubated at 37°C for an additional 48 hours. Cells were harvested and washed twice in 1x PBS and stained with trypan blue (0.2 %). The viable and dead cells were enumerated using a Malassez homocytometer. Values are reported as % of dead cells. Experiments were repeated in triplicate.

Atomic Force Microscopy (AFM) Imaging

Mica was used as an AFM substrate⁶⁶. An aliquot (10 μ L) of each sample was placed on freshly cleaved mica for 5 minutes, rinsed and dried as described in the panning protocol. AFM images were acquired in air using a tapping mode AFM with a Bioscope extender and a

NanoScope IIIa controller (Veeco/Digital Instruments, Santa Barbara, CA). The data was analyzed using Femtoscan software (Advanced Technologies Center, Moscow, Russia).

Statistical analysis

Data are reported as mean $\pm$ standard deviation. Differences were tested for significance using one-way ANOVA followed by the Fisher Least Squares Difference (L.S.D) and Tukey analysis. The standard errors and *P* value were analyzed with Excel and Minitab software. A *P* value of less than 0.01 denotes statistical significance.

Acknowledgements

This work was supported in part by grants from the NIH (AG17984), Michael J Fox Foundation, and the Arizona Biomedical Research Commission. We thank Dr. Warren Marcus, Dr. Andleeb Zameer and Srinath Kasturirangan for their help.

References

1. Hoehn MM, Yahr MD. Parkinsonism: onset, progression, and mortality. *Neurology* 1998;1967;50:318 and 16 pages following. [PubMed: 9484345]
2. Schapira AH. Pathogenesis of Parkinson's disease. *Baillieres Clin Neurol* 1997;6:15–36. [PubMed: 9426866]
3. Gomez-Tortosa E, Newell K, Irizarry MC, Albert M, Growdon JH, Hyman BT. Clinical and quantitative pathologic correlates of dementia with Lewy bodies. *Neurology* 1999;53:1284–91. [PubMed: 10522886]
4. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature* 1997;388:839–40. [PubMed: 9278044]
5. Wakabayashi K, Hayashi S, Kakita A, Yamada M, Toyoshima Y, Yoshimoto M, Takahashi H. Accumulation of alpha-synuclein/NACP is a cytopathological feature common to Lewy body disease and multiple system atrophy. *Acta Neuropathol (Berl)* 1998;96:445–52. [PubMed: 9829807]
6. Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, Trojanowski JQ, Iwatsubo T. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am J Pathol* 1998;152:879–84. [PubMed: 9546347]
7. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 1997;276:2045–7. [PubMed: 9197268]
8. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol* 2004;55:164–73. [PubMed: 14755719]
9. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muentner M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K. alpha-Synuclein locus triplication causes Parkinson's disease. *Science* 2003;302:841. [PubMed: 14593171]
10. Zhou W, Hurlbert MS, Schaack J, Prasad KN, Freed CR. Overexpression of human alpha-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. *Brain Res* 2000;866:33–43. [PubMed: 10825478]
11. Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A, Mucke L. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 2000;287:1265–9. [PubMed: 10678833]
12. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. *Nature* 2000;404:394–8. [PubMed: 10746727]
13. Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A, Saitoh T. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 1995;14:467–75. [PubMed: 7857654]

14. Davidson WS, Jonas A, Clayton DF, George JM. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J Biol Chem* 1998;273:9443–9. [PubMed: 9545270]
15. Bussell R Jr, Eliezer D. A structural and functional role for 11-mer repeats in alpha-synuclein and other exchangeable lipid binding proteins. *J Mol Biol* 2003;329:763–78. [PubMed: 12787676]
16. Giasson BI, Murray IV, Trojanowski JQ, Lee VM. A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J Biol Chem* 2001;276:2380–6. [PubMed: 11060312]
17. Kim TD, Paik SR, Yang CH. Structural and functional implications of C-terminal regions of alpha-synuclein. *Biochemistry* 2002;41:13782–90. [PubMed: 12427041]
18. Park SM, Jung HY, Kim TD, Park JH, Yang CH, Kim J. Distinct roles of the N-terminal-binding domain and the C-terminal-solubilizing domain of alpha-synuclein, a molecular chaperone. *J Biol Chem* 2002;277:28512–20. [PubMed: 12032141]
19. Eriksen JL, Dawson TM, Dickson DW, Petrucelli L. Caught in the act: alpha-synuclein is the culprit in Parkinson's disease. *Neuron* 2003;40:453–6. [PubMed: 14642269]
20. Takenouchi T, Hashimoto M, Hsu LJ, Mackowski B, Rockenstein E, Mallory M, Masliah E. Reduced neuritic outgrowth and cell adhesion in neuronal cells transfected with human alpha-synuclein. *Mol Cell Neurosci* 2001;17:141–50. [PubMed: 11161475]
21. Conway KA, Harper JD, Lansbury PT. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat Med* 1998;4:1318–20. [PubMed: 9809558]
22. Giasson BI, Uryu K, Trojanowski JQ, Lee VM. Mutant and wild type human alpha-synucleins assemble into elongated filaments with distinct morphologies in vitro. *J Biol Chem* 1999;274:7619–22. [PubMed: 10075647]
23. Serpell LC, Berriman J, Jakes R, Goedert M, Crowther RA. Fiber diffraction of synthetic alpha-synuclein filaments shows amyloid-like cross-beta conformation. *Proc Natl Acad Sci U S A* 2000;97:4897–902. [PubMed: 10781096]
24. Uversky VN, Li J, Fink AL. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. *J Biol Chem* 2001;276:44284–96. [PubMed: 11553618]
25. Uversky VN, Li J, Fink AL. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. *FEBS Lett* 2001;500:105–8. [PubMed: 11445065]
26. Cohlberg JA, Li J, Uversky VN, Fink AL. Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. *Biochemistry* 2002;41:1502–11. [PubMed: 11814343]
27. Campbell BC, Li QX, Culvenor JG, Jakala P, Cappai R, Beyreuther K, Masters CL, McLean CA. Accumulation of insoluble alpha-synuclein in dementia with Lewy bodies. *Neurobiol Dis* 2000;7:192–200. [PubMed: 10860784]
28. Volles MJ, Lansbury PT Jr. Zeroing in on the pathogenic form of alpha-synuclein and its mechanism of neurotoxicity in Parkinson's disease. *Biochemistry* 2003;42:7871–8. [PubMed: 12834338]
29. Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury PT Jr. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* 2002;418:291. [PubMed: 12124613]
30. Volles MJ, Lee SJ, Rochet JC, Shtilerman MD, Ding TT, Kessler JC, Lansbury PT Jr. Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* 2001;40:7812–9. [PubMed: 11425308]
31. Ding TT, Lee SJ, Rochet JC, Lansbury PT Jr. Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. *Biochemistry* 2002;41:10209–17. [PubMed: 12162735]
32. Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* 2002;8:600–6. [PubMed: 12042811]
33. Gosavi N, Lee HJ, Lee JS, Patel S, Lee SJ. Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. *J Biol Chem* 2002;277:48984–92. [PubMed: 12351643]

34. Barkhordarian H, Emadi S, Schulz P, Sierks MR. Isolating recombinant antibodies against specific protein morphologies using atomic force microscopy and phage display technologies. *Protein Eng Des Sel* 2006;19:497–502. [PubMed: 16984950]
35. Wheeler YY, Chen SY, Sane DC. Intrabody and intrakine strategies for molecular therapy. *Mol Ther* 2003;8:355–66. [PubMed: 12946308]
36. Murphy RC, Messer A. A single-chain Fv intrabody provides functional protection against the effects of mutant protein in an organotypic slice culture model of Huntington's disease. *Brain Res Mol Brain Res* 2004;121:141–5. [PubMed: 14969746]
37. Lecerf JM, Shirley TL, Zhu Q, Kazantsev A, Amersdorfer P, Housman DE, Messer A, Huston JS. Human single-chain Fv intrabodies counteract in situ huntingtin aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A* 2001;98:4764–9. [PubMed: 11296304]
38. Zhou C, Emadi S, Sierks MR, Messer A. A human single-chain Fv intrabody blocks aberrant cellular effects of overexpressed alpha-synuclein. *Mol Ther* 2004;10:1023–31. [PubMed: 15564134]
39. El-Agnaf OM, Salem SA, Paleologou KE, Curran MD, Gibson MJ, Court JA, Schlossmacher MG, Allsop D. Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. *Faseb J* 2006;20:419–25. [PubMed: 16507759]
40. Masliah E, Rockenstein E, Adame A, Alford M, Crews L, Hashimoto M, Seubert P, Lee M, Goldstein J, Chilcote T, Games D, Schenk D. Effects of alpha-synuclein immunization in a mouse model of Parkinson's disease. *Neuron* 2005;46:857–68. [PubMed: 15953415]
41. Marcus WD, Lindsay SM, Sierks MR. Identification and repair of positive binding antibodies containing randomly generated amber codons from synthetic phage display libraries. *Biotechnol Prog* 2006;22:919–22. [PubMed: 16739981]
42. Martin JB. Molecular basis of the neurodegenerative disorders. *N Engl J Med* 1999;340:1970–80. [PubMed: 10379022]
43. Narhi L, Wood SJ, Steavenson S, Jiang Y, Wu GM, Anafi D, Kaufman SA, Martin F, Sitney K, Denis P, Louis JC, Wypych J, Biere AL, Citron M. Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. *J Biol Chem* 1999;274:9843–6. [PubMed: 10092675]
44. Tu PH, Galvin JE, Baba M, Giasson B, Tomita T, Leight S, Nakajo S, Iwatsubo T, Trojanowski JQ, Lee VM. Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble alpha-synuclein. *Ann Neurol* 1998;44:415–22. [PubMed: 9749615]
45. Dickson DW, Lin W, Liu WK, Yen SH. Multiple system atrophy: a sporadic synucleinopathy. *Brain Pathol* 1999;9:721–32. [PubMed: 10517510]
46. Golts N, Snyder H, Frasier M, Theisler C, Choi P, Wolozin B. Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein. *J Biol Chem* 2002;277:16116–23. [PubMed: 11850416]
47. Jo E, McLaurin J, Yip CM, St George-Hyslop P, Fraser PE. alpha-Synuclein membrane interactions and lipid specificity. *J Biol Chem* 2000;275:34328–34. [PubMed: 10915790]
48. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T. alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* 2002;4:160–4. [PubMed: 11813001]
49. Crowther RA, Jakes R, Spillantini MG, Goedert M. Synthetic filaments assembled from C-terminally truncated alpha-synuclein. *FEBS Lett* 1998;436:309–12. [PubMed: 9801138]
50. Park JY, Lansbury PT Jr. Beta-synuclein inhibits formation of alpha-synuclein protofibrils: a possible therapeutic strategy against Parkinson's disease. *Biochemistry* 2003;42:3696–700. [PubMed: 12667059]
51. Hashimoto M, Rockenstein E, Mante M, Mallory M, Masliah E. beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor. *Neuron* 2001;32:213–23. [PubMed: 11683992]
52. El-Agnaf OM, Paleologou KE, Greer B, Abogrein AM, King JE, Salem SA, Fullwood NJ, Benson FE, Hewitt R, Ford KJ, Martin FL, Harriott P, Cookson MR, Allsop D. A strategy for designing inhibitors of alpha-synuclein aggregation and toxicity as a novel treatment for Parkinson's disease and related disorders. *Faseb J* 2004;18:1315–7. [PubMed: 15180968]

53. Emadi S, Liu R, Yuan B, Schulz P, McAllister C, Lyubchenko Y, Messer A, Sierks MR. Inhibiting aggregation of alpha-synuclein with human single chain antibody fragments. *Biochemistry* 2004;43:2871–8. [PubMed: 15005622]
54. Klucken J, Shin Y, Masliah E, Hyman BT, McLean PJ. Hsp70 Reduces alpha-Synuclein Aggregation and Toxicity. *J Biol Chem* 2004;279:25497–502. [PubMed: 15044495]
55. Boder ET, Midelfort KS, Wittrup KD. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci U S A* 2000;97:10701–5. [PubMed: 10984501]
56. Kaye R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 2003;300:486–9. [PubMed: 12702875]
57. Kaye R, Glabe CG. Conformation-dependent anti-amyloid oligomer antibodies. *Methods Enzymol* 2006;413:326–44. [PubMed: 17046404]
58. Lambert MP, Velasco PT, Chang L, Viola KL, Fernandez S, Lacor PN, Khuon D, Gong Y, Bigio EH, Shaw P, De Felice FG, Krafft GA, Klein WL. Monoclonal antibodies that target pathological assemblies of Aβeta. *J Neurochem* 2007;100:23–35. [PubMed: 17116235]
59. Marasco WA, Haseltine WA, Chen SY. Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. *Proc Natl Acad Sci U S A* 1993;90:7889–93. [PubMed: 8356098]
60. Wolfgang WJ, Miller TW, Webster JM, Huston JS, Thompson LM, Marsh JL, Messer A. Suppression of Huntington's disease pathology in *Drosophila* by human single-chain Fv antibodies. *Proc Natl Acad Sci U S A* 2005;102:11563–8. [PubMed: 16061794]
61. Carter P, Bedouelle H, Winter G. Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucleic Acids Res* 1985;13:4431–43. [PubMed: 2989795]
62. Tomlinson I, Holliger P. Methods for generating multivalent and bispecific antibody fragments. *Methods Enzymol* 2000;326:461–79. [PubMed: 11036658]
63. Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987;166:368–79. [PubMed: 2449095]
64. Hoogenboom HR, Winter G. By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J Mol Biol* 1992;227:381–8. [PubMed: 1404359]
65. Shearman MS, Hawtin SR, Tailor VJ. The intracellular component of cellular 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction is specifically inhibited by beta-amyloid peptides. *J Neurochem* 1995;65:218–27. [PubMed: 7790863]
66. Shlyakhtenko LS, Potaman VN, Sinden RR, Gall AA, Lyubchenko YL. Structure and dynamics of three-way DNA junctions: atomic force microscopy studies. *Nucleic Acids Res* 2000;28:3472–7. [PubMed: 10982865]

ABBREVIATIONS

PD	Parkinson's disease
α-syn	α-synuclein
scFv	single chain antibody fragment
AFM	atomic force microscope
NAC	non-amyloid component

A30P	human A30P α -synuclein
A53T	human A53T α -synuclein
E46K	human E46K α -synuclein
ThT	Thioflavin T., GAG, Glycosaminoglycan

Figure 1. AFM images of α -synuclein morphologies and mixture with D5 phage

A 10 μ l aliquot of mixture of monomeric/oligomeric (1a), fibrillar (1b) and a 6-day aggregated solution of α -synuclein (0.7 μ M) preincubated for 2 minutes with purified D5 phage (10^{12} pfu/ML) (1c) were deposited on freshly cleaved mica and fixed for 5 minutes. After washing and drying, images were acquired in air using a tapping mode AFM. The scale bars represent 1 μ m. Arrows represent α -synuclein ($\rightarrow$) and D5 scFv ($\cdots\rightarrow$).

Figure 2. SDS-PAGE and WESTERN-BLOT analysis of scFv expression

Lane 1: molecular weight marker; Lane 2: concentrated supernatant and periplasmic fractions of isolated scFv; Lane 3: Flowthrough; Lane 4: eluted sample from protein A affinity chromatography column. Each sample was separated with 12% SDS-PAGE, followed by Coomassie blue staining (2a). For Western-blotting, eluted sample from protein A affinity column was transferred to a nitrocellulose membrane and probed with 9E10 antibody overnight at 4 °C. Bound antibodies were detected after a 1-hour incubation using a 1/500 dilution of goat-anti-mouse IgG HRP conjugated (2b).

Figure 3. Binding specificity of D5 scFv by ELISA analysis

The microtiter plate was coated for 2 hours at 37 °C with or without 50 µg/mL of 3 different morphologies of α -synuclein: monomer, oligomer and fibrillar. The oligomers were obtained by dissolving the lyophilized stock of α -synuclein in PBS 1x followed by size exclusion chromatography to separate monomer from other oligomeric species. The fibrillar α -synuclein samples were prepared as described in the methods section. After blocking, the plate was incubated for 2 hours at room temperature with 100 µg/mL of purified D5 scFv or control scFv (anti-phosphorylase B). Bound scFv was detected by an anti-c-myc tag (9E10) HRP conjugate. Activity was determined by subtracting OD₆₅₀ from OD₄₅₀ and comparing absorbance between control well (without immobilized α -synuclein) to the sample well (with α -synuclein) where the control value was normalized to 1. The data represent the average $\pm$ standard deviation (SD) of three separate experiments. A p value (**p < 0.01) was considered to be statistically significant (one-way ANOVA).

Figure 4. Binding specificity of D5 by SDS-PAGE analysis

Lane 1: Molecular weight; lane 2 and 3: monomeric and oligomeric fraction of α -synuclein isolated from size exclusion chromatography respectively. The samples were run on 10% polyacrylamid Tris-Tricine SDS-PAGE followed by Coomassie blue staining (4a). The sample was then transferred to nitrocellulose membrane and probed with 0.3 mg/mL of anti-scFv (D5) antibody. Bound antibodies were detected after a 2-hour incubation using a 1/500 dilution of 9E10 antibody, followed by 1 hour incubation with goat-anti-mouse IgG HRP conjugated (4b).

Figure 5. Binding specificity of D5 by time course immunoreactivity assay

A) Lyophilized α -synuclein (50 μ M) was incubated at 37 $^{\circ}$ C without stirring. At indicated time points, aliquots (5 μ l) were applied to a nitrocellulose membrane and probed with 0.3 mg/mL of D5 scFv. Immunoreactivity was detected after a 2-hour incubation using a 1/500 dilution of 9E10 antibody, followed by 1 hour incubation with goat-anti-mouse IgG HRP conjugated. B) AFM analysis of incubated sample after 4, 8 and 18 days. Images were acquired in air using a tapping mode AFM. The scale bars represent 1 μ m.

Figure 6. Effect of D5 scFv on aggregation of α -synuclein

A) The kinetics of α -synuclein fibril formation were monitored by thioflavin T (ThT) fluorescence in the presence (●) and absence (○) of scFv. α -synuclein (50 μ M) was incubated with purified scFv (15 μ M) at 37 °C without stirring and 10 μ L aliquots were added to 2 mL of 5 μ M ThT. Fluorescence intensity was measured at excitation wavelength 450 nm and emission wavelength 482 nm. The data represent the average $\pm$ standard deviation (SD) of two separate experiments with each experimental value being repeated in triplicate. B) AFM imaging of α -synuclein aggregation with or without scFv. AFM analysis of α -synuclein aggregates at 4 and 18 days when incubated alone (50 μ M) or with 15 μ M scFv. Images were acquired in air using a tapping mode AFM. The scale bars represent 1 μ m.

Figure 7.

Effect of scFv on α -synuclein induced cytotoxicity. Aliquots of α -synuclein (50 μ M) preincubated at indicated times with or without scFv (15 μ M) were added to wells coated with SH-SY5Y cells. After an additional 2 day incubation of cells and samples, the viability of the cells was measured using an MTT toxicity assay. The final concentration of α -synuclein and scFv were 2 μ M and 0.3 μ M, respectively. Data shown are expressed as percentage of control values from three independent experiments with each experimental value being repeated in quadruplicate.

Table 1

scFv suppresses cell death and toxicity induced by aggregation of α -synuclein. α -synuclein (50 μ M) was incubated in 25 mM Tris-HCl and 150 mM NaCl (pH 7.4) for 4 days at 37 °C without shaking. Samples were preincubated with or without scFv for 2 hours at room temperature and then assayed for cytotoxicity at a final concentration of 1 μ M. Cell viability was measured using blue trypan staining and LDH toxicity assay after an additional 2 days incubation of cells and aggregate samples. For blue trypan assay, the data are expressed as percentage of dead cell from one experiment repeated in triplicate. For LDH assay, the data are expressed as percentage of control values from three independent experiments with each experimental value being repeated in triplicate. A p value (**p < 0.01) was considered to be statistically significant (one-way ANOVA).

	% LDH release	% of Dead cell
Control	100	5.3 $\pm$ 2.7
α -synuclein alone	127 $\pm$ 10 **	34.7 $\pm$ 10 **
α -synuclein + scFv (D5)	91 $\pm$ 5	4.7 $\pm$ 1.5
D5 alone	105 $\pm$ 7	5 $\pm$ 2.3