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Carbon Nanotubes as Potential Agents against Fibrillation of α-Synuclein, a Parkinson's Disease-Related Protein

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ABSTRACT

Parkinson's disease (PD), is the second most common neurodegenerative disease in the world. The pathogenesis of PD is associated with α-Synuclein (αS) fibrillation. Previous works have indicated that blocking αS aggregation could be an effective strategy for the treatment of PD. Carbon nanotubes (CNTs) have specific properties that make them potentially useful in biomedicine and biotechnology. CNTs can access the brain, but no investigation has been done to survey the effect of single walled carbon nanotubes (SWNTs) or multi walled carbon nanotubes (MWNTs) on αS fibrillation. Through the use of Thioflavin T (ThT) fluorescence spectroscopy, transmission electron microscopy (TEM) and MTT assay, we found for the first time that both type of CNTs can significantly inhibit αS aggregation and subsequently change its neurotoxicity. While a complete mechanistic understanding remains to be elucidated, these data indicate that CNTs may have high therapeutic potential for the use against neuropathological features of PD.

Keywords: Parkinson's disease (PD), α-Synuclein (αS), Carbon nanotubes (CNTs), Fibrillation, Nanostructures

INTRODUCTION

Amyloid fibrillation of many proteins (such as Aβ peptide, the tau protein, and the αS protein) is associated to neurodegenerative and other diseases, including Alzheimer's disease [1], Huntington's disease [2] and PD [3]. A common theme is the formation of amyloid fibrils with cross-β-stacking structures through a nucleation-dependent polymerization process, which involves a lag phase followed by elongation into fibrils. However the exact mechanism of amyloid fibril formation rests unknown, previous studies show that conformational shifts/structural instabilities of proteins cause their aggregation.

The aggregation of αS protein is an important factor leading to PD and the aggregates of αS protein comprising soluble oligomers and mature fibrils are neurotoxic to brain cells [4]. Today, the key therapeutic strategy for PD is the modulation of αS protein aggregation. Hence, many αS protein aggregation inhibitors were considered as potential drug candidates. Often traditional inhibitors consist of peptides or peptide mimetics [5-7] and small organic molecules [8], but now a day, some nanomaterials with high cell permeability, high *in vivo* stability, high efficacy and great biocompatibility have been investigated as inhibitor of protein aggregation [9].

In recent years, special biomedical properties of CNTs with their hydrophobic tubular nanostructures have attracted a lot of attention. CNTs are chemically stable allotropes of carbon with unique physical, electronic and mechanical properties which have been exploited by many researchers in different applications, ranging from sensors to tissue scaffolds and artificial muscles [10,11]. Particular physicochemical characteristics of CNTs cause their biological effects widely unforeseeable. They are highly effective delivery systems for a variety of diagnostic and therapeutic agents [12,13]. Considering the growing body of research in biological applications of CNTs, investigation of their effects on biological macromolecules such as proteins seems necessary. As mentioned earlier protein and peptide aggregation into characteristic amyloid fibrils is a major cause of various neurodegenerative diseases. In such diseases, there is a conversion of proteins or peptides from their soluble functional states to highly organized fibrillar aggregates. CNTs were demonstrated to accelerate the

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fibrillation of β2 microglobulin by decreasing the lag phase for fibrillation nucleation [14]. In contrast, the aggregation of human acidic fibroblast growth factor (hFGF-1) was prevented by CNTs [15]. Also carbon-based nanomaterials like CNTs [16,17], Graphene quantum dots [9] Graphene oxide [18], have been applied to modulate the Aβ peptide aggregation.

In this work, we explored the effects of SWNTs and MWNTs on the aggregation of αS protein. Both SWNTs and MWNTs have been able to strongly inhibit the aggregation of αS protein which in turn diminished the protein toxicity on SH-SY5Y cell line. The results suggest that CNTs can be considered as potential inhibitors for αS protein aggregation in PD models.

MATERIALS AND METHODS

Materials

MWNT (10-20 nm diameter, ~30 μm length, and >95 wt% purity) and a carboxyl-modified SWNT (1-3 nm diameter, ~1 μm length, 2.56 wt% carboxyl content, and >95 wt% purity), manufactured by a thermal chemical vapor deposition process, was purchased from Sigma-Aldrich (Munich, Germany). Isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin and Thioflavin T (ThT) was purchased from Sigma-Aldrich Company.

Oxidation of CNTs

MWCNT and SWCNT with carboxyl content 2.56 wt% (30 mg) were separately dispersed into a flask containing 10 ml nitric acid. The mixture was then refluxed at 120 °C for 48 h. After cooling, the mixture was centrifuged at 9000 g for 5 min, and the solid was washed with deionized water. The solid was then dried in a vacuum oven.

Expression and Purification of αS

For protein expression, recombinant pNIC28-Bsa4 vector containing gene of αS was transferred to *E. coli* C41 (DE3): starter cultures were prepared by inoculating a single colony of the bacteria to LB medium containing kanamycin (50 μg ml^{-1} final concentration). After reaching OD 600 nm = 0.4, 0.1 mM IPTG was added, and the culture was incubated for another 5 h at 37 °C [19]. The induced cells were harvested by centrifugation at 5000 g for 15 min. The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8) and 1mM PMSF, and then the cells were lysed by sonication on ice. The cell lysate was centrifuged at 16 000 g for 30 min at 4 °C. The supernatant was applied to nickel-nitrilotriacetic acid (Ni-NTA)-Sepharose column (Qiagen) and the purification was done according to the manufacturer recommendations (Qiagen, Inc.). The purified protein was analysed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Fibrillation Experiment

Purified αS was dialyzed against sterile PBS buffer (pH 7.4) and was immediately incubated at final concentration of 50 μM containing 0.02% NaN3 as an antiseptic agent, in the absence or presence of different CNTs concentrations (50, 30, 15, 5 and 2 μg ml^{-1}, respectively) in at 37 °C in Turbo Thermo Shaker (TMS-200, China) shaking at 850 rpm. Fibrils formation was monitored by fluorescence enhancement of ThT at different time points. 6 μl of each sample were taken and diluted into 94 μl of PBS containing 25 μM ThT to reach the final protein concentration of 3 μM. Fluorescence was measured in clear black 96-well plates using a Cytation3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) with excitation wavelength at 440 nm and emission at 485 nm. Each sample was assayed in triplicates, and each experiment was repeated at least three times.

Electron Microscopy

The samples to be analyzed by TEM were prepared by incubation of αS with or without SWNTs and MWNTs (50 μg ml^{-1}) for 100 h. Then each sample was placed on 300-mesh Formavar/carbon-coated grids for 2 min before removing excess solution. Then the samples were stained with 1% uranyl acetate solution for another 2 min. Finally, excess fluid was removed and the all samples were viewed by a Zeiss-EM10C-80 KV TEM.

Cell Toxicity Assays

For cell toxicity assays of CNTs, human SH-SY5Y neuroblastoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) F-12 supplemented with 10% fetal bovine serum in a 5% CO_2 humidified atmosphere at

37 °C. Then, the cells were transferred to 96-well plates at a density of 10,000 cells/well and 3 wells per treatment. The next day, the medium was changed and the cells were treated with different concentrations (0.5, 1, 2, 3 and 5 μg ml^{-1}) of SWNTs and MWNTs and incubated for 24 h. The viability of the cells was measured by MTT assay and the color intensity of formazan solution was determined at 570 nm with ELISA Microplate Reader (μQuant, Biotek, USA).

The inhibitory effect of CNTs on αS toxicity was surveyed as follows. Briefly, 10,000 cells were seeded in 96-well plates, the next day, the medium was changed and the cells were treated by the aliquots of the aggregation products of αS (50 μM), which were taken after 36 h (the end of the lag phase) incubated with or without (2 μg ml^{-1}) of SWNTs and MWNTs. This time point (36 h) of incubation, where the ThT fluorescence emission starts to increase, is supposedly rich in oligomers [20,21]. The toxicity of these samples was assessed by adding them to the cells at a final concentration of 7.5 μM followed by incubation for 24 h. The viability of the cells was then measured by MTT assay.

RESULTS

Effect of SWNTs and MWNTs on Amyloid Fibrillation of Purified αS Protein

αS protein was over expressed and then purified by metal affinity chromatography and analyzed by gel electrophoresis (Fig. 1).

ThT fluorescence is served as a highly sensitive marker for the amyloid formation of various aggregating proteins and peptides because it has strong fluorescence when bound to amyloid fibrils. The aggregation of 50 μM of αS protein was monitored in the presence of 50, 30, 15, 5 and 2 μg ml^{-1} of SWNTs (Fig. 2) and MWNTs (Fig. 3). In all of SWNTs and MWNTs concentrations, the lag time of the αS protein aggregation was extended and the transition time for amyloid formation has been delayed. An increased lag time suggests that the CNTs inhibited nucleation, and the increased transition time suggests that the CNTs inhibited subsequent growth of these nuclei into amyloids. At a concentration of 50 μg ml^{-1} CNTs, the aggregation of the αS protein into amyloid fibrils was completely inhibited.

Morphological Analysis

The overall morphology of the αS incubated under amyloid forming conditions in the presence of CNTs was analyzed by TEM. By comparing Fig. 4A (αS alone) with Fig. 4B (αS-SWNTs) and Fig. 4C (αS-MWNTs), the differences between αS protein amyloid are remarkable. It can be clearly observed that αS by itself formed extensive filaments (Fig. 4A), while αS samples incubated under amyloid forming conditions in the presence of CNTs (Fig. 4B, 4C) showed oligomers and very few short filaments.

CNTs Change αS Cytotoxicity

The ability of CNTs to inhibit αS protein aggregation proposed that they might be suitable in blocking αS cellular toxicity. MTT assay was used to assess the ability of CNTs to hinder αS-mediated cellular toxicity on human neuroblastoma SH-SY5Y cells.

Briefly, SH-SY5Y cells were incubated with aliquots taken at time point 36 h of αS aggregation reaction which are supposed to be rich in oligomers. It was supposed that at the beginning of the incubation period, the majority of αS proteins are monomers, while at the end of the lag phase that the ThT fluorescence emission starts to increase the largest population of oligomers exists [20,21]. The toxicity of different concentrations of CNTs by itself on SH-SY5Y cells was also evaluated using MTT assay as described above. No significant decrease in cell viability was observed upon treatment of the cells with CNTs up to 2 μg ml^{-1} (data not shown).

Significant decrease in cell viability can be seen by treatment of the cells with the samples taken at the end of lag phase (36 h). This could be due to the toxic oligomers which are abundant at the end of lag phase [22,23]. When the same experiments were conducted by treating the cells with the aliquots taken from αS aggregation reaction at 36 h in the presence of SWNTs or MWNTs, toxicity decreased. This may suggest that CNTs at 2 μg ml^{-1} concentration are able to block formation of the toxic species (oligomers) in the beginning of aggregation pathway (Fig. 5, dark blue bar, light blue bar).

DISCUSSIONS

Over the past two decades, there have been many

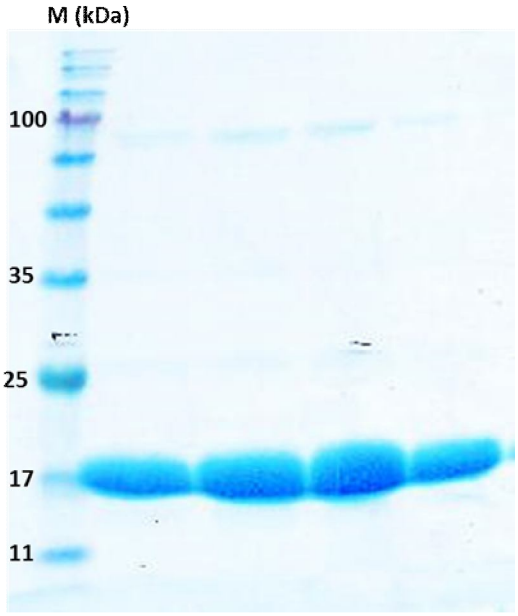

Fig. 1. SDS-PAGE analysis of the purified recombinant αS protein using Ni-NTA sepharose column.

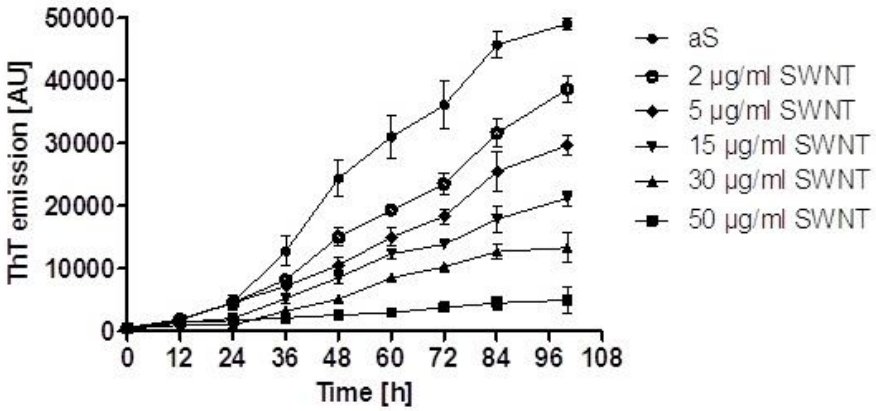

Fig. 2. Aggregation kinetics of the 50 µM αS protein in the absence and presence of 50, 30, 15, 5 and 2 µg ml^{-1} SWNTs at pH 7.6.

studies on the effect of nanomaterials on protein and peptide aggregation and also amyloid-related diseases. Intensive researches were reported that the aggregation of protein and peptide into amyloid fibrils is a main factor of various neurodegenerative diseases. Previous reports showed that some nanomaterials can be easily the olfactory bulb to the striatum and hippocampus of brain, causing striatal damage and dopaminergic toxicity [24]. Nanomaterials by targeting nucleation kinetics could control or induce amyloid formation. It has been reported that protein folding and aggregation could be affected by binding of proteins to nanoparticle surfaces [25,26]. Experimental researches demonstrate that fullerene by specific binding to the hydrophobic (KLVFF) motif of Aβ [27], CNTs [14,15], polymeric [28] and fluorinated nanoparticles [29] depending on their surface physicochemical properties, prevent/induce

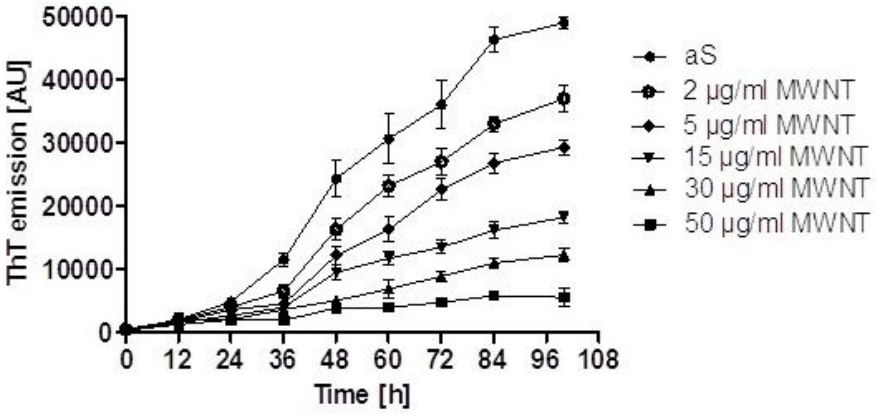

Fig. 3. Aggregation kinetics of the 50 µM αS protein in the absence and presence of 50, 30, 15, 5 and 2 µg ml^{-1} of MWNTs at pH 7.6.

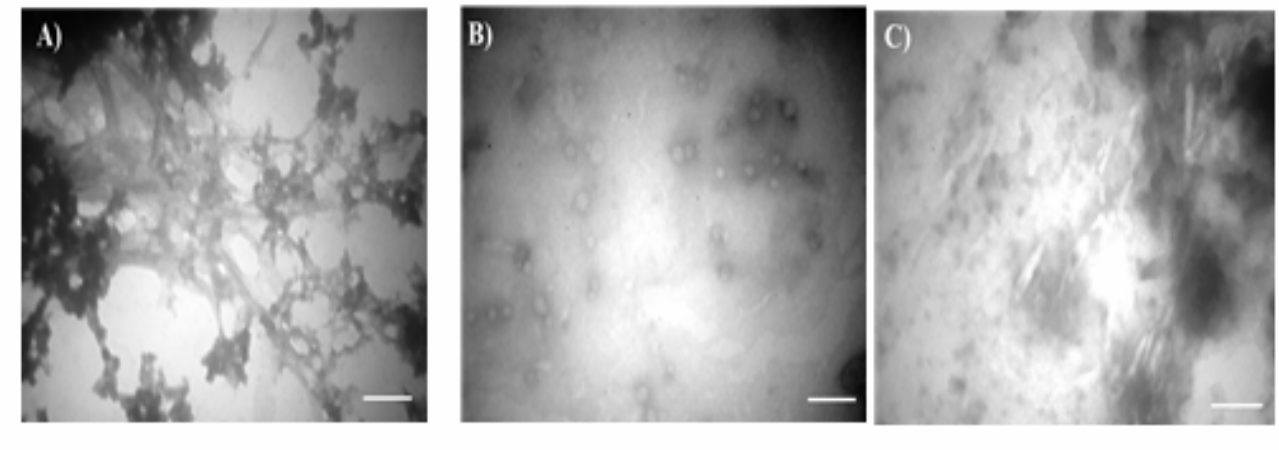

Fig. 4. Fibrillation-blocking effects of CNTs, as monitored by TEM. The αS protein (50 µM) was incubated either alone (A) or with 50 µg ml^{-1} SWNTs (B) or with 50 µg ml^{-1} MWNTs (C) at 37 °C for 100 h.

amyloid formation by increasing/decreasing the lag phase time for nucleation; although, they all leave the elongation phase invariant, proposing a surface-modulated nucleation mechanism [14,28]. In addition, the protein's intrinsic stability may influence the surface-mediated nucleation process. Linse *et al.* [14] indicated that fibrillation of the β2-microglobulin protein, which is β-sheet-rich in its native form, was accelerated by hydrophobic copolymeric NiPAM:BAM nanoparticles, whereas Cabaleiro *et al.* [28] demonstrated that these nanoparticles retard the fibrillation of intrinsically disordered Aβ peptide. They suggested binding of monomers of amyloid forming proteins to the surface of nanomaterials may block the active sites of oligomerization and also decrease protein concentration in solution.

Nanomaterials composition and also the amount and nature of the nanomaterials's surface were shown to be effective on the nucleation phase of amyloid formation. Therefore, the interaction of proteins and nanomaterials in solution has influence on various thermodynamic parameters that regulate the protein assembly into fibrils, and also the disaggregation of preformed fibrils.

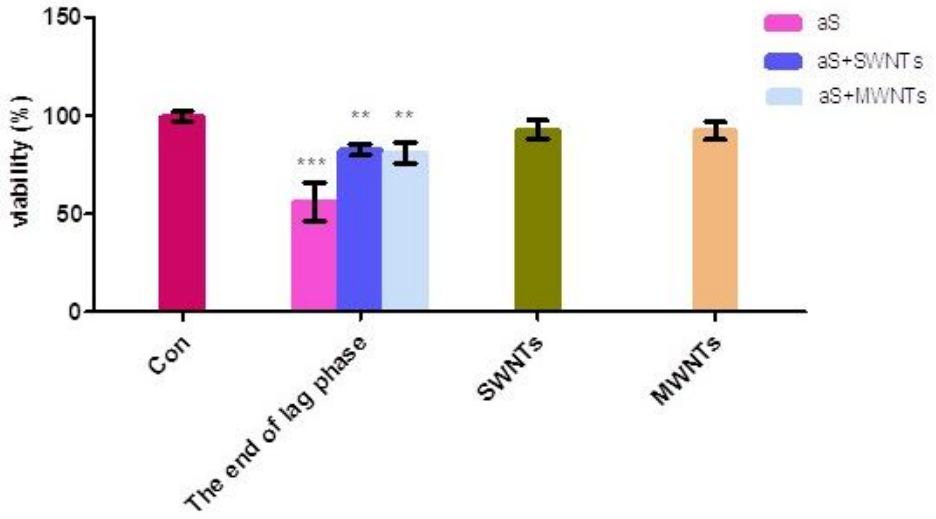

Fig. 5. The effect of CNTs on the cell toxicity of αS protein (50 μM). Cell viability was determined using the MTT method. Each experiment was conducted three times. *p < 0.05; **p < 0.01; ***p < 0.001, Unpaired t-test.

Pervious experimental researches had demonstrated that hydrophobic nanomaterials retard Aβ fibrillation by slowing down the nucleation process [9,16-18]; although, the effects of nanomaterials on Aβ oligomeric structures are unknown, but computational studies showed that β-sheet formation of Aβ peptide was prevented by SWNTs, probably by destabilizing the β-sheet structure through hydrophobic and π-stacking interactions [17]. Li *et al.* [17] hypothesize that these interactions between SWNTs and Aβ (16-22) peptide would interfere with the peptide-peptide interactions that are responsible for Aβ (16-22) aggregation, thus lead to slow down the nucleation process.

We have found both SWNT and MWNT have been able to inhibit αS amyloid formation strongly as confirmed by fluorescence emission of ThT (Fig. 2) and (Fig. 3) and TEM images (Fig. 4). In accordance to previous reports on Aβ proteins [17] we suggest that the hydrophobic region of αS (amino acids 60-95) which is involved in oligomerization of αS is the most probable site for CNTs interactions. These interactions may lead to the reduction of β-sheet formation by the hydrophobic residues and prolong the αS nucleation phase. The cytotoxicity assessments demonstrate the effective inhibitory role of CNTs in the formation toxic aggregates of αS protein. Nevertheless, further studies must be done to unravel the mechanism of inhibitory effects of CNTs on protein fibrillation.

CONCLUSIONS

In summary, inhibiting the αS aggregation has become one of the most essential strategies to treat PD. In this study, for the first time, CNTs are reported as efficient inhibitor for their application in inhibiting the aggregation of αS protein; give an advantage to the clinical research for PD. Therefore this research can be provided a novel insight into the development of Parkinson's drugs.

CONFLICT OF INTEREST STATEMENT

The authors state that they have no conflict of interest.

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