



## TiO<sub>2</sub> Nanoparticles as Potential Promoting Agents of Fibrillation of $\alpha$ -Synuclein, a Parkinson's Disease-Related Protein

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**Background:** In recent years, nanomaterials have been widely used in large quantities which make people be more frequently exposed to the chemically synthesized nanoparticles (NPs). When NPs are introduced into an organism, they may interact with a variety of cellular components with yet largely unknown pathological consequences.

**Objective:** It was found that NPs enhance the rate of protein fibrillation in the brain by decreasing the lag time for nucleation. Protein fibrillation is implicated in the pathogenesis of the several neurodegenerative diseases such as Parkinson's disease (PD).  $\alpha$ -Synuclein ( $\alpha$ S) is natively an unfolded protein which is involved in the pathogenesis of PD. In the present study, we have analyzed the effects of three different NPs on  $\alpha$ S fibrillation.

**Materials and Methods:**  $\alpha$ S protein expression and purification was done and fibrils formation was induced in the absence or presence of the three types of NPs (i. e., TiO<sub>2</sub>, SiO<sub>2</sub>, and SnO<sub>2</sub>). The enhancement of the fluorescence emission of Thioflavin T (ThT) and transmission electron microscopy (TEM) were used to monitor the appearance and growth of the fibrils. The adsorption of  $\alpha$ S monomers on the surface of NPs was investigated by tyrosine fluorescence emission measurements.

**Results:** We found that TiO<sub>2</sub>-NPs enhances  $\alpha$ S fibril formation even at a concentration of 5  $\mu$ g.mL<sup>-1</sup>, while the two other NPs show no significant effect on the kinetics of the fibrillation. Intrinsic tyrosine emission measurement has confirmed that the TiO<sub>2</sub>-NPs interact with  $\alpha$ S fibrillation products. It is suggested that TiO<sub>2</sub>-NPs may enhance the nucleation of  $\alpha$ S protein that leads to protein fibril formation.

**Conclusion:** The fibrillization process of  $\alpha$ S protein is profoundly affected by the presence of TiO<sub>2</sub>-NPs. This finding unveils the neurotoxicity potential of the TiO<sub>2</sub>-NPs, which may be considered as a probable risk for PD.

**Keywords:**  $\alpha$ -Synuclein ( $\alpha$ S); Nanoparticles (NPs); Parkinson's disease (PD); Titanium Dioxide Nanoparticles (TiO<sub>2</sub>-NPs)

### 1. Background

Nanoparticles (NPs) have existed in the environment since the beginning of the Earth's and its evolution. They are found naturally in the atmosphere, most natural waters, soils, and sediments (1). In recent years, nanomaterials have been widely used in the large quantities in consumer products, which makes people be frequently exposed to such particles (2). Also, with the advent of the nanoscience, there has been much interest about the ways through which NPs interact with the biological systems, due to their potential applications in different aspects of nanotechnology, and effects on human health (3, 4). However, few studies have dealt

with the toxicological and environmental effects of the exposure to the nanomaterials; direct or indirect effects. Over the past two decades, there has been considerable research interest in the use of NPs in the study of protein and peptide aggregation, and amyloid-related diseases, as well. Amyloid fibrils consist of protein molecules folded into anti-parallel stacked cross- $\beta$ -sheet structures, resulting in their anisotropic structure. When NPs are introduced in a living organism they may interact with a variety of cellular components with yet largely unknown pathological consequences. The influence of NPs on amyloid formation is of great interest due to their small sizes and high surface area-

to-volume ratio. Targeting NPs dependent amyloid structure formation, the nucleation stage in this process is one of the most investigated ways for controlling or inducing this phenomenon (5). The observed effect of NPs on the nucleation phase is determined by the composition and the amount of particles as well as the nature of the particle's surface (6). Various thermodynamic parameters influence the interaction of proteins with the NPs in solution, regulate the protein assembly into fibrils, as well as disaggregation of the preformed fibrils (6).

A number of naturally occurring proteins play role in the fibrillar aggregation formation in body among which are Amyloid  $\beta$  peptide in Alzheimer's disease (AD) (7),  $\alpha$ S in PD (8), huntingtin in Huntington's disease (9), amylin in type II diabetes,  $\beta$ 2-microglobulin in dialysis-related amyloidosis (10), and prion protein in bovine spongiform encephalopathy (11) is believed to play an important role in the pathogenesis of the mentioned clinical disorders.

The 140-residue  $\alpha$ S (14.4 kDa) is a presynaptic neuronal protein with an unknown normal function which its amyloidogenic propensity is associated with the several neurodegenerative disorders. These disorders include PD, multiple system atrophy, and dementia with Lewy bodies, collectively called synucleinopathies (12, 13), where  $\alpha$ S is the major fibrillar component of intracellular inclusions, also known as Lewy bodies and Lewy neuritis. In vitro studies have demonstrated that  $\alpha$ S can spontaneously form amyloid fibrils under typical physiological conditions (ionic strength, temperature, and pH). The presence of foreign surfaces in the system, for instance in the form of engineered NPs, has been shown to profoundly affect the formation of amyloids (14-16).

As an example, Yanina *et al.* have shown that gold NPs induce a strong acceleration of  $\alpha$ S aggregation by influencing both the nucleation and growth phases of the overall pathway. The effects were observed to be dependent on the size and concentration of the NPs; being strongest for the 10 nm diameter NPs, which produced a 3-fold increase in the overall aggregation rate at concentrations as low as 20 nM (17).

TiO<sub>2</sub>-NPs have been widely used in many daily products such as cosmetics, antiseptic agents, coating flux, water treatment chemicals, and photocatalysts etc. Uversky *et al.* have shown the association between exposure to heavy metals and an enhanced risk for developing PD (18). Several possible mechanisms have been proposed for metal-stimulated fibrillation of  $\alpha$ S, the simplest of which would be involvement in the direct interactions between  $\alpha$ S and the metal, leading to the

structural changes in  $\alpha$ S which result in its enhanced propensity toward aggregation. For instance, it has been reported that  $\alpha$ S aggregation is facilitated in the presence of Cu<sup>2+</sup> (19) and Al<sup>3+</sup> which induce structural perturbations in the protein (20). Thus, both environmental and other non-inherited factors may be responsible for the more common sporadic forms of the PD (21).

## 2. Objectives

While nanotechnology, production, and application of the nanoparticles are exponentially growing, the knowledge regarding the toxicological and environmental effects of the direct and indirect exposure to the nanomaterials is still in its early stages. In this study, we aimed to investigate the *in vitro* effects of the three different NPs (TiO<sub>2</sub>, SiO<sub>2</sub>, and SnO<sub>2</sub>) on the fibrillation of the  $\alpha$ S protein; the major constituent of the protein clumps and the pathological hallmark of the PD.

## 3. Materials and Methods

### 3.1. Materials

TiO<sub>2</sub>-NPs, type P25, in anatase phase with the average primary particle size of 21±5 nm, and SiO<sub>2</sub>-NPs were purchased from Plasmachem, Germany. SnO<sub>2</sub>-NPs were kindly gifted to us by Dr. S. Sadeghi (Department of chemistry, Tarbiat Modares University, Tehran, Iran). Kanamycin was obtained from Sigma-Aldrich Company (USA). Ni-NTA sepharose column was purchased from Qiagen (Germany).

### 3.2. Expression and Purification of $\alpha$ S

For protein expression, the recombinant pNIC28-Bsa4 vector containing  $\alpha$ S gene was transformed into *E. coli* C41 (DE3); starter cultures were prepared by inoculating a single colony of the bacteria to LB medium containing kanamycin (50  $\mu$ g.mL<sup>-1</sup>). After reaching to an OD 600nm 0.4, IPTG (0.1 mM) was added, and the culture was incubated for another 5 hours at 37°C (22). The induced cells were harvested by centrifugation at 5000  $\times$ g for 15 min. The cell pellet was resuspended in the lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8) and 1mM PMSF, and then cells were lysed by sonication on ice. The cell lysate was centrifuged at 16000  $\times$ g for 30 min at 4 °C, the supernatant was applied to nickel-nitrilotriacetic acid (Ni-NTA) sepharose column (Qiagen, Germany), and the purification was done according to the manufacturer instructions (Qiagen, Inc. Germany). The purified protein was analyzed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### 3.3. Characterization of NPs

The average particle size and Zeta potential of NPs were determined by Zetasizer Nano ZS instrument (Malvern Instruments, UK) at 25° C. The NPs suspension was prepared by mixing the NPs with filtered PBS buffer (pH 7.4) and sonicating for 30 min at 600W, 20 kHz.

### 3.4. Fibrillation Experiment

Purified  $\alpha$ S was dialyzed against sterile PBS buffer (pH 7.4) containing 0.02% NaN<sub>3</sub> as an antiseptic agent, and was immediately incubated at a final concentration of 35  $\mu$ M, in the absence or presence of the different concentration of NPs at 37 °C in a Turbo Thermo Shaker ( TMS-200, China) while shaking at 1000 rpm for 100 hours. Fibrils formation was monitored by measuring the fluorescence enhancement of ThT (Sigma-Aldrich Company, USA). 10  $\mu$ L of each sample were taken at different time points and diluted with 100  $\mu$ L of PBS containing 25  $\mu$ M ThT to reach the final protein concentration of 3.5  $\mu$ M. Fluorescence was measured in a clear black 96-well plate using a Cytation3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) with excitation wavelength at 440 nm and emission at 485 nm. The samples were assayed in triplicates, and, each experiment was repeated at least three times.

### 3.5. Fluorescence Titrations

In order to analyze the interaction of  $\alpha$ S with NPs,

the prepared  $\alpha$ S proteins at the concentration of 50  $\mu$ M and 35  $\mu$ M were mixed with NPs (50  $\mu$ g.mL<sup>-1</sup>) and fluorescence spectra were collected using a spectrofluorimeter (Perkin-Elmer model LS 50B). An excitation wavelength of 280 nm (slit width = 5 nm) was used and the data were collected over the range width of 290-350 nm (slit width = 5 nm).

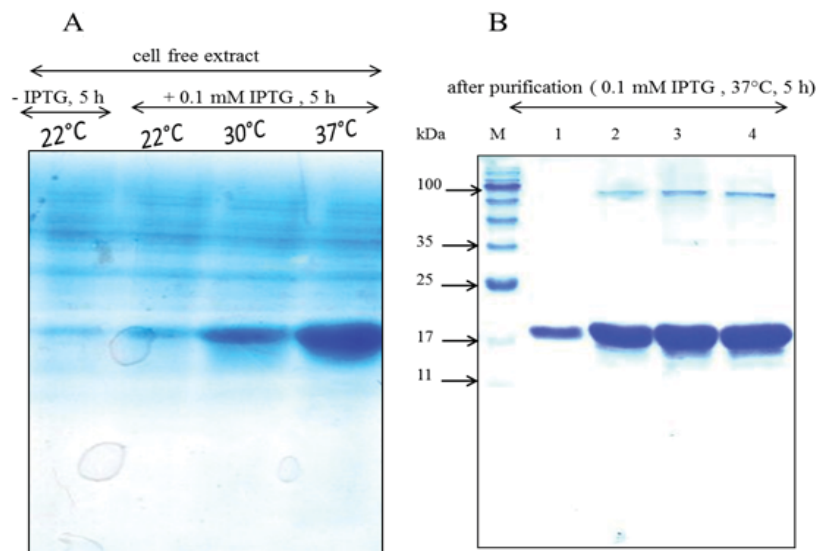
### 3.6. Transmission Electron Microscopy (TEM) Analysis

Aliquotes (10  $\mu$ L) from the  $\alpha$ S fibrillation reaction (with and without TiO<sub>2</sub>-NPs) were placed on formvar/ carbon-coated 300- mesh grids. After 2 minutes, excess fluid was removed, and the grids were negatively stained with 1% uranyl acetate solution for 2 minutes. Finally, excess fluid was removed and the samples were viewed by a Zeiss - EM10C - 80 KV TEM.

## 4. Results

### 4.1. $\alpha$ S Protein Expression and Purification

Transformation of the plasmid pNIC28-Bsa4 –  $\alpha$ S resulted in the high level expression of  $\alpha$ S protein after 5 hours of the induction of the protein expression. The protein was purified by one step metal affinity chromatography and analyzed by gel electrophoresis. While the molecular weight of the protein was estimated to be 16.4 kDa, SDS-PAGE analysis of the purified protein showed a band with a molecular weight of more than 17 kDa (Fig. 1). The



**Figure 1.** SDS-PAGE analysis of A) soluble fractions of the bacterial extracts with and without induction by IPTG at different temperatures and B) the purified recombinant  $\alpha$ S protein using Ni-NTA sepharose column. Four samples with increasing protein concentrations were run in parallel with the molecular mass standards on a 15% polyacrylamide gel and stained with Coomassie Blue R-250. The molecular weight of the marker proteins is shown as M lane in gel B, the first lane from the left.

**Table 1.** The average size and the Zeta potential of the colloidal solutions of the different NPs.

Type of NPs	Average size (d. nm)	Zeta potential (mV)	PdI
TiO <sub>2</sub> -NPs	91.35	-11.9	0.178
SiO <sub>2</sub> - NPs	69.2	-18.3	0.621
SnO <sub>2</sub> - NPs	278	-17.6	0.639

slow mobility of  $\alpha$ S protein can be attributed to the low binding of the SDS to the highly acidic C-terminal sequence of  $\alpha$ S (23).

#### 4.2. Characterization of NPs

The NPs were well suspended in PBS buffer (pH 7.4) by means of sonication. The size and Zeta potential of the nanoparticles have been shown in Table 1.

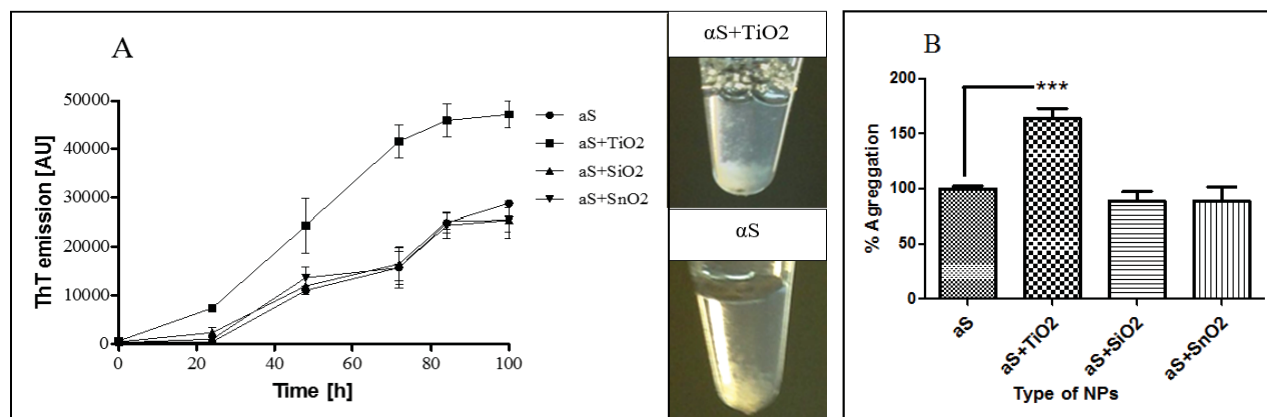
#### 4.3. TiO<sub>2</sub>-NPs Promote $\alpha$ S Fibrillation

Fibrillation of  $\alpha$ S is a two-step process: an initial lag period that reflects the thermodynamic barrier to the formation of a nucleation “seed”, followed by the rapid fibril propagation and aggregation formation stage. The introduction of TiO<sub>2</sub>-NPs in  $\alpha$ S aggregation reaction substantially decreased the lag time (Fig. 2A) and significantly enhanced  $\alpha$ S aggregation as monitored by ThT fluorescence measurements (Fig. 2B). This indicates that the TiO<sub>2</sub>-NPs are able to enhance the nucleus formation. This effect is dependent on the concentration of the TiO<sub>2</sub>-NPs as checked for 5, 25, and 50  $\mu$ g.mL<sup>-1</sup> of the NPs (Fig. 3). However, the kinetics of  $\alpha$ S fibrillation was not altered by SiO<sub>2</sub>, and SnO<sub>2</sub>-NPs (Fig. 2A).

#### 4.4. The Interaction of TiO<sub>2</sub>-NPs and $\alpha$ S Fibrillation Products

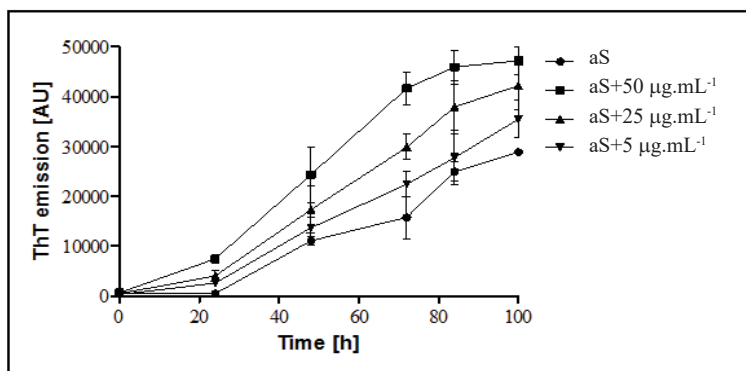
To analyze the conformational changes exerted by  $\alpha$ S adsorption on the surface of TiO<sub>2</sub>-NPs, tyrosine fluorescence emission spectra of the protein in the presence of different NPs were acquired (there isn't a tryptophan residue in  $\alpha$ S). It has been suggested that NPs can interact with protein monomers, eventually leading to a locally high concentration of monomers on the surface of the NPs, and in turn facilitate the nucleation and the fibrillation process (5). In this study, tyrosine fluorescence emission spectra of  $\alpha$ S were acquired in the presence of different NPs. Interestingly, all NPs showed the quenching effect on the intrinsic fluorescence of  $\alpha$ S, and all the examined NPs have induced structural change to some extent in  $\alpha$ S, however, the effect of TiO<sub>2</sub>-NPs was more prominent than the other NPs (Fig. 4).

The higher quenching effect of TiO<sub>2</sub>-NPs may implicate the better adsorption of  $\alpha$ S protein on the surface of TiO<sub>2</sub>-NPs. It could be suggested that TiO<sub>2</sub>-NPs can adsorb  $\alpha$ S onto their surfaces and provide a locally high concentration of  $\alpha$ S monomers, which may explain the promoting effect of TiO<sub>2</sub>-NPs on  $\alpha$ S fibrillation.

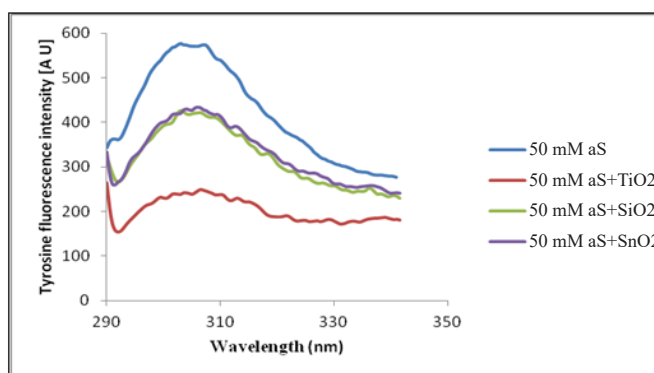


**Figure 2.** **A)** Kinetics of  $\alpha$ S fibrillation in the presence of different NPs. ThT fluorescence was plotted as a function of time for 35  $\mu$ M  $\alpha$ S at 37 °C in PBS (pH 7.4) without or with 50  $\mu$ g.mL<sup>-1</sup> of NPs. The NPs include TiO<sub>2</sub>, SiO<sub>2</sub>, and SnO<sub>2</sub>. The values are means  $\pm$  SD, n = 3. **B)** The percentage of  $\alpha$ S aggregation in the presence of different NPs (50  $\mu$ g.mL<sup>-1</sup>) compared to the control ( $\alpha$ S) after 100 hours of incubation under an aggregation inducing conditions. Each experiment was conducted three times. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001, one-way ANOVA and LSD's post-hoc test.





**Figure 3.** Kinetics of the  $\alpha$ S fibrillation in the presence of different concentration of TiO<sub>2</sub>-NPs . ThT fluorescence was plotted as a function of time for 35  $\mu$ M  $\alpha$ S at 37 °C in PBS (pH 7.4). The values represent the means obtained for 3 separate experiments (n = 3) and  $\pm$  SD.



**Figure 4.** Tyrosine fluorescence signal of  $\alpha$ S in the presence of the different applied NPs.

#### 4.5. TEM Analysis of $\alpha$ S Fibril Formation in the Presence of TiO<sub>2</sub>-NPs

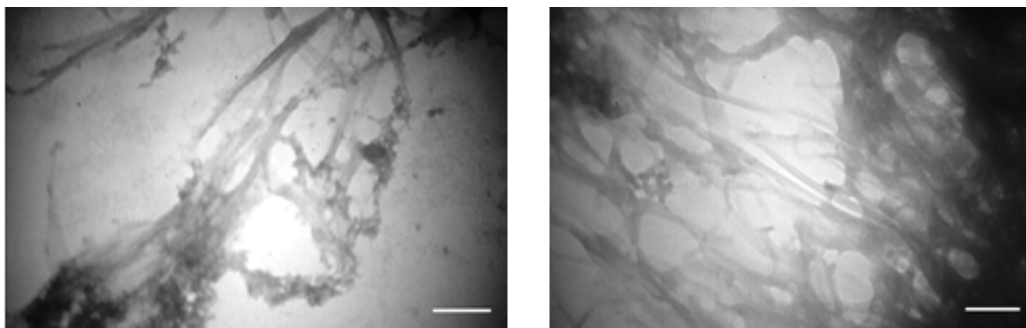
TEM analysis was performed on the samples of  $\alpha$ S incubated under the amyloid-inducing conditions (alone or in the presence of the TiO<sub>2</sub>-NPs), to confirm the formation of  $\alpha$ S amyloid fibrils and to explore the ultrastructure of the aggregates. Samples were taken from the aggregation reactions after 100 hours. Consistent with the results of ThT experiments, TEM analysis has also confirmed the formation of amyloid fibrils in both the  $\alpha$ S and  $\alpha$ S supplemented with TiO<sub>2</sub> nanoparticles samples (Fig. 5). A higher amount of amyloid fibrils formation could be seen in the presence of TiO<sub>2</sub> nanoparticles as shown in Figure 5.

### 5. Discussion

In brief, this study has investigated the effect of TiO<sub>2</sub>, SiO<sub>2</sub>, and SnO<sub>2</sub>-NPs on  $\alpha$ S protein aggregation. Here we have shown that TiO<sub>2</sub>-NPs might be one of the environmental factors involved in PD. We found that TiO<sub>2</sub>-NPs are able to promote  $\alpha$ S assembly into amyloid fibrils *in vitro* by presumably accelerating the nucleation process. This feature could result from a locally high  $\alpha$ S

monomer concentration owing to the adsorption on the TiO<sub>2</sub>-NPs surface. These results unveil the potential neurotoxicity of TiO<sub>2</sub>-NPs exposure, which may cause PD.

Based on previous reports regarding neurotoxicity of the ferrous oxide and silicon dioxide (24, 25), recently Jie Wu *et al.* (21) have investigated the effects of TiO<sub>2</sub>-NPs on  $\alpha$ S aggregation and explored the mechanism of NPs action by establishing a co-culture model of TiO<sub>2</sub>-NPs and dopaminergic neurons. It has been shown that TiO<sub>2</sub>-NPs could induce PD-related  $\alpha$ S protein aggregation through oxidative stress, thus causing damage to the dopaminergic neurons and subsequently PD. Jie Wu *et al.* (25) have also demonstrated that SiO<sub>2</sub>-NPs could possibly have a negative impact on the striatum and dopaminergic neurons as well as a potential risk for neurodegenerative diseases, which is in contrast to the results of the present study in which an effect on  $\alpha$ S assembly into amyloid fibrils was not observed in the presence of SiO<sub>2</sub>-NPs. The acceleration of  $\alpha$ S fibrillation in the presence of  $\alpha$ S-conjugated CdSe/ZnS quantum dots has been reported by the Roberti *et al.* (26). In addition, Yang *et al.* (27, 28) have



**Figure 5.** TEM micrographs of the samples taken from the  $\alpha$ S fibrillation reaction.  $\alpha$ S protein was incubated without (left) TiO<sub>2</sub>-NPs or with 50  $\mu\text{g.mL}^{-1}$  of TiO<sub>2</sub>-NPs under amyloid inducing conditions for 100 h (scale bar represents 120 nm for both micrographs).

studied the interaction of the  $\alpha$ S with both negative and positively charged gold NPs and have proven a large conformational change of  $\alpha$ S upon interaction with the gold nanoparticles.

Linse *et al.* (29) have found that all TiO<sub>2</sub>, SiO<sub>2</sub>, ZrO<sub>2</sub>, CeO<sub>2</sub>, C60, and C70 NPs can promote  $\beta$ 2-Microglobulin fibrillation *in vitro*. They observed that the duration of the lag phase depends on the amount and the nature of the particle surface. Wei-hui Wu *et al.* (30) have shown that all six kinds of the above-mentioned NPs can bind to A $\beta$ 42, but only TiO<sub>2</sub> can strongly be adsorbed to the A $\beta$  protein and influence the fibrillation process. The difference between the Linse's results with that of Wei-hui Wu may be correlated to the differences that the two proteins might have in their fibril formation capacity. A $\beta$ 42 has a strong tendency to form a nuclear seed (31) while  $\beta$ 2-Microglobulin is relatively more resistant to fibril formation, and therefore any weak adsorption of the proteins on the surface of the nanoparticles may be important for shortening the lag time.

Colvin *et al.* (32) have proposed that NPs may act much like conventional catalysts in promoting fibrillation. NPs would reduce the energetic barriers for fibril formation by enhancing the population of prefibril aggregates. Although the detailed molecular mechanism of the nucleation on the NPs surface remains elusive, but it could involve by mean of establishing a higher concentration of monomers which in turn increase the probability of the nucleation events. Alternatively, the interaction with particle surfaces could actively promote a conformational change and consequently the aggregation of the protein (33). Nonetheless, the dependence of the lag phase length on the accessible surface area and its characteristics (relative hydrophobicity) suggest that the nucleation mechanism involves association of the protein with the particle surface. This effect has been studied by using

surface plasmon resonance (SPR) (29).

Herein, it was demonstrated that TiO<sub>2</sub>-NPs interact more strongly with  $\alpha$ S than SiO<sub>2</sub> and SnO<sub>2</sub>-NPs, (Fig. 4). This interaction caused a significant change in the conformation of  $\alpha$ S, which led to the exposure of the hydrophobic regions of the protein to the solvent and hence presumably increased the occurrence of the nucleation events.

Overall, it seems that the control of amyloid formation via NPs can be explained based on two factors: first, the surface chemistry of NPs, and the second, the protein's intrinsic stability. These factors determine the interaction between NPs and the proteins or peptides, and hence, they may act either as a platform for nucleation or as destabilizing agents, causing acceleration of amyloid formation (14).

Studies have shown that many NPs are small enough to access all parts of the body including the brain (34). The invasion of the nanomaterial into the brain, especially the NPs with a strong ability to promote  $\alpha$ S fibrillation, may be an important environmental factor in the pathogenesis of PD. These findings also suggest other more positive future directions in which NPs could be used to form new protein assemblies or to enhance and control the rate of protein or peptide self-assembly into amyloid-like materials with novel biotechnological properties and applications.

## 6. Conclusion

The pathogenesis of PD is associated with  $\alpha$ S fibrillation. There is limited data on the interaction of  $\alpha$ S with NPs and their effects on its aggregation kinetics. In this study, we surveyed the effect of three different NPs on  $\alpha$ S fibrillation and found that TiO<sub>2</sub>-NPs can promote  $\alpha$ S fibrillation by shortening the nucleation process, which is the key rate determining step of  $\alpha$ S fibrillation. Hereby, we suggest that the interaction between  $\alpha$ S and TiO<sub>2</sub>-NPs may contribute to the PD etiology.

Nanotechnology offers great promises in many industrial applications. It is necessary to control and prevent the unwitting use of potentially hazardous nanomaterials. To keep PD away from human beings, the potential of NPs and nanomaterials hazards should be seriously taken into account and be eliminated.

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