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Alpha-Synuclein Fibrils Interact with Dopamine Reducing its Cytotoxicity on PC12 Cells

Masoome Khalife[1] · Dina Morshedi[1] · Farhang Aliakbari[1,2] · Amir Tayaranian Marvian[1] · Hossein Mohammad Beigi[1,3] · Sadegh Azimzadeh Jamalkandi[1,4] · Francisco Pan-Montojo[5]

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Abstract Aggregated alpha-synuclein (α-SYN) is the major component of Lewy bodies and Lewy neurites, two of the pathological hallmarks of Parkinson's disease (PD). Aggregation of α-SYN leads to toxic species involved in the degeneration of dopaminergic neurons in the midbrain. Different studies suggest a strong association between the presence of dopamine (DA) and the cell specific degeneration caused by α-SYN aggregates in PD. Despite extensive studies on the effect of DA on α-SYN fibrillation, it remains unclear how the simultaneous presence of DA and α-SYN influences the degeneration of dopaminergic neurons. In this study we show that separate treatments with specific doses of DA or early stage α-SYN aggregates (ESAA) are both cytotoxic to PC12 cells. Surprisingly, simultaneous treatment of cells with DA and ESAA significantly decreased this toxicity. This cytotoxicity was further reduced by the presence of heavier particles of α-SYN aggregates with more fibrillogenic characteristics. Spectrometric analysis revealed that α-SYN fibrils interact with DA even after the sample was dialyzed for 48 h, suggesting a strong interaction. Interestingly, digestion of unprotected N- and C-α-SYN-fibril terminals by proteinase K did not affect this interaction. Our results suggest that fibrillar forms of α-SYN with localized expanded active surfaces may interact with DA and moderate its cytotoxicity. Thus, highlighting the importance of fibrillar proteins in developing clinical approaches for amyloid diseases.

Keywords α-Synuclein · Dopamine · Fibrillation · Fluorescent labeled α-synuclein, Neurodegeneration · Parkinson's disease · Reactive oxygen species (ROS)

Abbreviations

AFM	Atomic Force Microscopy
α-SYN	α-Synuclein
CD	Circular Dichroism
DA	Dopamine
DAQ	Dopamine quinone
ESAA	Early Stages α-SYN Aggregates
FTIR	Fourier Transform infrared
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OD	Optical Density
PD	Parkinson's Disease
PK	Proteinase K
PI	Propidium Iodide
ROS	Reactive Oxygen Species
SNpc	Substantia Nigra pars compacta
ThT	Thioflavin T

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✉ Dina Morshedi
morshedi@nigeb.ac.ir

[1] Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

[2] Department of Medical Biotechnology, Semnan University of Medical Sciences, Semnan, Iran

[3] Department of Chemical Engineering, Tarbiat Modares University, Tehran, Iran

[4] Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

[5] Munich Cluster for Systems Neurology, SyNergy Clinician Scientist Group, Klinikum der Universität München, Munich, Germany

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor symptoms such as tremor, rigidity, bradykinesia and postural instability. Loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and formation of extensive amyloid aggregates are two major pathological hallmarks of the disease [1, 2]. Aggregation of amyloids starting in the pre-synaptic area of neurons leads to intracellular protein inclusions known as Lewy bodies and Lewy neurites, mainly composed of α-synuclein (α-SYN). α-SYN is a natively unfolded protein that is highly prone to constitute amyloid-like fibrils [3]. It has been proposed that oligomeric and protofibrillar forms of α-SYN are toxic to cells through their interaction with intracellular membranes and the disruption of the membrane integrity [4, 5]. Localization of aggregated α-SYN within the inner membrane of mitochondria can decrease mitochondrial activity and promote mitophagy [6]. Furthermore, it was shown that α-SYN aggregates could be transported between cells or neurons and act as seeds, thereby propagating PD pathology [7–9]. According to Braak's staging of the disease, PD-pathology spreads throughout the CNS following a specific pattern that starts at the olfactory bulb and the enteric nervous system (ENS) [10]. According to this staging, all neuronal structures affected are synaptically connected. In a recent study, it was shown that disrupting connecting nerves between the ENS and the CNS stopped the progression of the pathology [11] Thus, supporting the role of α-SYN transmission between neurons in PD pathology progression. Therefore, investigating the extracellular effect of α-SYN aggregates is critical for understanding of the pathogenesis of PD.

Given the involvement of α-SYN in synapse maintenance and storage and release of DA [12, 13], entrapment of α-SYN in the aggregated structures reduces its function and elevates the amount of cytosolic DA [14], which leads to the degeneration of the locus ceruleus and SNpc neurons [15]. Therefore, although L-Dopa is a common and effective treatment for PD, it could be that the long-term treatment of cells with excessive concentration of L-dopa and increased cytosolic concentrations of DA contributes to degeneration of SNpc dopaminergic neurons [16]. DA is an active neurotransmitter, which is prone to spontaneous oxidation or enzymatic degradation within cytoplasm [17, 18]. During its auto-oxidation, dopamine Quinone (DAQ) compounds are generated [19, 20]. These compounds are highly reactive and make molecular adducts subsequently inducing mitochondrial damage, diminishing the activity of the autophagy system, inducing production of reactive oxygen species (ROS), and stimulating the inflammatory response [21, 22]. In addition, DAQs may bind to proteins such as α-SYN and induce the formation of soluble and toxic α-SYN oligomers [23, 24]. Also, oxidized species of DA can make non-covalent interactions with α-SYN and inhibit the formation of α-SYN fibrils [25]. Altogether, these studies point at the importance of further investigating the cytotoxicity and the interactions of DA with other influential factors in PD.

Despite the extensive in vitro and in vivo studies concerning the influence of the DA/α-SYN interaction on the α-SYN fibrillation process, it is still unclear whether any effective interaction between DA and fibrillar and/or prefibrillar α-SYN aggregates exists. In this study, we determined the cytotoxicity of early stages α-SYN aggregates (ESAA) and DA separately and simultaneously on PC12 cells, a pheochromocytoma cell line. Our results indicated that independent extracellular treatment with either ESAA or DA alone induced cell death in a dose dependent manner. Interestingly, co-treatment with DA and ESAA showed an antagonistic behavior on DA toxicity. Our results suggest a scavenging effect of α-SYN fibrillar structures on DA by a direct molecular interaction.

2 Materials and Methods

2.1 Chemical Reagents

Thioflavin T (ThT), 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Monobromobimane (MBBr), and 2′,7′-Dichlorodihydrofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (United Kingdom). All salts and organic solvents were obtained from Merck (Darmstadt, Germany). Isopropyl-D-thiogalactopyranose (IPTG) was obtained from CinnaGen (Tehran, Iran). The Rat pheochromocytoma cell line (PC12) cells were from Pasteur Institute (Tehran, Iran). Cell culture medium (DMEM high glucose) and antibiotics (penicillin–streptomycin) were from GIBCO BRL (Life Technologies, Paisley, Scotland). Fetal bovine serum (FBS) was obtained from Biosera (East Sussex, U.K). Annexin-V-FLUOS Staining Kit was purchased from Roche Applied Science (Germany).

2.2 Expression and Purification of α-SYN

Recombinant human α-synuclein-containing a pNIC28-Bsa4 plasmid was transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Novagen, Madison, WI, USA). Gene expression was induced using IPTG and proteins were extracted and purified according to Huang et al. [34]. Briefly, the recombinant protein purification was carried out in three steps including osmotic shock, anion-exchange

chromatography and size exclusion chromatography. The purified protein was freeze-dried and stored at −20 °C.

2.3 Site-Directed Mutagenesis of Human α-SYN and Labeling with Monobromobimane

M5C-Syn (a methionine was substituted with a cysteine) and G131C-Syn (a glycine was substituted with a cysteine) mutants of alpha-synuclein were constructed from wild-type pNIC vector contained Kanamycin resistance gene following Quick Change protocol (Stratagene, La Jolla, CA). The property of mutagenesis was demonstrated through sequencing technique using T7 promoter primer. The Mutated forms of α-SYN dissolved in 30 mM Tris buffer pH 7.2. Monobromobimane (MBBr) was dissolved 1 mg/mL in DMSO. Protein/MBBr mixture was made by a ratio of 1:1 (W/W) and incubated at 37 °C for 2 h. Labeled proteins were separated from the unconjugated probe by using size exclusion chromatography (NAP-5). Ultimately, the labeled proteins were freeze-dried and stored at −20 °C to be used in the next steps. In the experiment samples, two kinds of labeled protein, at the α-SYN's N-terminal or at the α-SYN's C-terminal regions, were added by ratio of 1:20 (W/W) to the wild type protein. For Preparation of the α-SYN fibrils, Freeze-dried human recombinant α-SYN (2 mg/mL) was incubated in a shaker incubator (80 rpm) in a buffer including 30 mM Tris (pH: 7.2), 1 mM EDTA and 0.1 mM NaNO3. The agitation was continued to monitor the kinetics of fibrillation.

2.4 ThT Fluorescence Assay

The fibrillation process of α-SYN was monitored by ThT fluorescence intensity. Briefly, 10 μL of every sample was added to 490 μL of 2.5 mM stock solution of ThT in 10 mM sodium phosphate and 150 mM NaCl (pH 7.0). Fluorescence excitation and emission spectra were adjusted at 440 and 450–550 nm, respectively. The excitation and emission slit widths were set as 5 and 10 nm, respectively. All fluorescence experiments were carried out on a Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia) at room temperature.

2.5 Congo Red Absorbance Assay

Congo red was dissolved at 80 μM in a buffer consisting of 150 mM sodium chloride and 5 mM potassium phosphate (pH 7.4). It was filtered using a center-glass N4 filter. A 10 μL of the well-mixed incubated sample was added to 490 μL of the Congo red solution and incubated for 5 min. Absorbance spectra were recorded (400–600 nm) using a PGT80+UV–Visible spectrophotometer (Leicestershire, England).

2.6 Circular Dichroism (CD) Analysis

CD spectra in the far-UV region (190–260 nm) were obtained on an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA), in 0.1-cm circular cuvettes at room temperature. The protein concentration was 0.1 mg/mL.

2.7 Atomic Force Microscopy (AFM)

Samples of the incubated α-SYN (2 mg/mL) were diluted 30 times in filtered deionized water. Then small aliquots (10 μL) were deposited on freshly cleaved mica sheets. Afterwards, samples were dried and washed with deionized water. Then AFM was performed using Veeco with a silicon probe (CP). Imaging was performed under noncontact mode.

2.8 Spectrophotometric Analysis of DA and α-SYN Interaction

The simple absorbance assay was carried out to consider the interaction of α-SYN and DA. First, DA (0.3 mM) dissolved in PBS and optical density (OD) was measured at 280 nm ($OD_{DA\ (Total)}$) using a PGT80+ UV–Visible spectrometer (Leicestershire, England). Next, solutions of incubated α-SYN (2 mg/mL) for 24 h were centrifuged (13,000 rpm, 45 min) and the pellets were suspended in DA solution, then OD_{280} was, once again read. Subsequently, samples were incubated for 15 min at room temperature and were afterwards centrifuged (13,000 rpm, 20 min) where the OD_{280} of supernatant was extracted ($OD_{supernatant}$). To illustrate the content of this test using the following formulas:

1. $OD_{supernatant} = OD_{DA\ non\text{-}interacting}$ (after incubation and centrifugation)
2. $OD_{DA\ interacting} = OD_{DA\ (Total)} - OD_{DA\ non\text{-}interacting\ (sup)}$

2.9 FTIR

Fourier transform infrared (FTIR) was used to analyze the interaction of fibril forms of protein with DA. In order to separate the heavier aggregated species from the small aggregated and monomeric species, the incubated samples were centrifuged (15,000 rpm, 45 min). The collected pellets were washed twice and were air-dried. Powder potassium bromide (KBr) was added to the dried pellets and was thoroughly mixed. After packing the powder as a transparent film, its FTIR spectrum was measured using a Tensor 37 Spectrometer (Bruker OPTIK GmbH, Rodolf Plank, Germany). The detector was DLaTGS and CO spectra of 1700–1600 wavelengths were analyzed. In order

to compare the spectrum of protein with protein-DA, the second-derived data was obtained by OPUS software.

2.10 Cell Culture

PC12 cells were cultured in DMEM (high glucose) enriched with FBS (10 %), penicillin (100 U/mL) and streptomycin (100 μg/mL) with a pH of ~7.4. The cells were incubated in humidified atmosphere with 5 % CO_2 at 37 °C and cultured in 96-well microtiter plates for MTT and ROS tests (200 μL per well). In addition, 6-well microtiter plate (2 mL per well) was used for Annexin V/PI assay.

2.11 MTT Assay

MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used for cell viability evaluation. PC12 cells (with a density of 3×10^4 cell/well) were seeded in 200 μL of growth medium. After adherence of cells to the plates (which took about 24 h), they were treated with different concentrations 10 % (v/v) incubated α-SYN or a mixture of DA and α-SYN. After 24 h of incubation, the supplemented medium was removed (DA may interfere with MTT assay). Thereafter, about 20 μL of MTT stock solution was added to each well and the plates were incubated for 4 h. Crystals of formazan dissolved in DMSO and cell viability was measured at 570 nm by Eliza reader (Expert 96, AsysHitch, Ec Austria).

2.12 Assessing the Production of Intracellular ROS

DCFH-DA probe, a cell-permeable fluorescent reagent, was used to measure the ROS production in the cultured cells. DCFH-DA reacts with ROS where fluorescent 2′,7′-Dichlorodihydrofluorescein (DCF) is generated as a result. Fluorescence intensity is an indicator of cytosolic ROS level. Cells were cultured (density of 6×10^4 cell/well) in 96-well plates and were treated with DA and α-SYN. After 24 h, the cells were incubated with 20 μM DCFH-DA (45 min at dark). Then the cells were collected and washed with phosphate buffer saline (PBS). Fluorescence intensity was measured at 480 nm excitation by Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia).

2.13 Discriminating Apoptosis/Necrosis Using Double Staining Flow Cytometric Assay

In order to further investigate the cytotoxic effect of fibrils and DA, the proportion of apoptosis and necrosis in PC12 cells were measured by cytometric flow method. Cells incubated in 6-well plates for 24 h (density of 5×10^5 cell/well) were treated with three different samples including: 1) ESAA, 2) DA, and 3) ESAA with DA. Then, the treated cells were incubated for more than 24 h. Cells were detached using 0.12 % trypsin and collected through centrifugation (1000 rpm for 5 min). The pellets were washed with PBS and resuspended in 500 μL of binding buffer. Fluorescein isothiocyanate (FITC)-conjugated Annexin, and PI were added to the samples and incubated for 15 min in the dark. Samples were loaded on BD FACS Calibur flow cytometer (Becton–Dickinson, Franklin, Lakes, NJ, USA) for analysis of cell death rate. Flowing software v.2.5 was used to discriminate early or late apoptosis.

3 Results

3.1 Cytotoxicity Assessment of α-SYN Aggregated Species and DA

During the α-SYN fibrillation process, different proto-fibrillar species are formed [26]. In order to determine the presence of high toxic populations of aggregated α-SYN, purified recombinant α-SYN (70 μM) was incubated for 48 h on a shaking device under fibrillation conditions (37° C, pH ~ 7.4). ThT fluorescence intensity, Congo red absorbance and far-UV CD were used to monitor the kinetics of aggregation. These experiments (Sup. 1 a–d) showed that α-SYN undergoes a fibrillation process with a well-known sigmoidal kinetic. We then determined the cytotoxicity of α-SYN aggregates at different fibrillation time-points. For this we stopped the fibrillation process of α-SYN at different incubation times as previously described [27] and evaluated their cellular toxicity by adding the generated α-SYN aggregates from each time-point to the medium of PC12 cells. MTT results (Fig. 1a) show that α-SYN aggregates generated after 7 to 15-h of incubation had a cytotoxic on PC12 cells after 24 h of treatment. Therefore, in this study, we decided to use 7-h incubated α-SYN as ESAA.

Furthermore, we characterized the structural features of generated α-SYN aggregates through AFM imaging. Clearly, AFM images taking from α-SYN aggregates generated after 7- and 24-h of incubation (Fig. 1b, c) show strong morphological and size differences. 24-h incubated samples were mostly composed of fibrous α-SYN aggregates while 7-h incubated samples contained a mixture of different small assembled-species with sphere shapes. Similar to other reports, showing that concentration of oligomeric species is boosted during the lag phase and is diminished during fibrils formation [28], our result suggests that the most cytotoxic aggregated species, α-SYN oligomers, are generated in the ESAA of the fibrillation process.

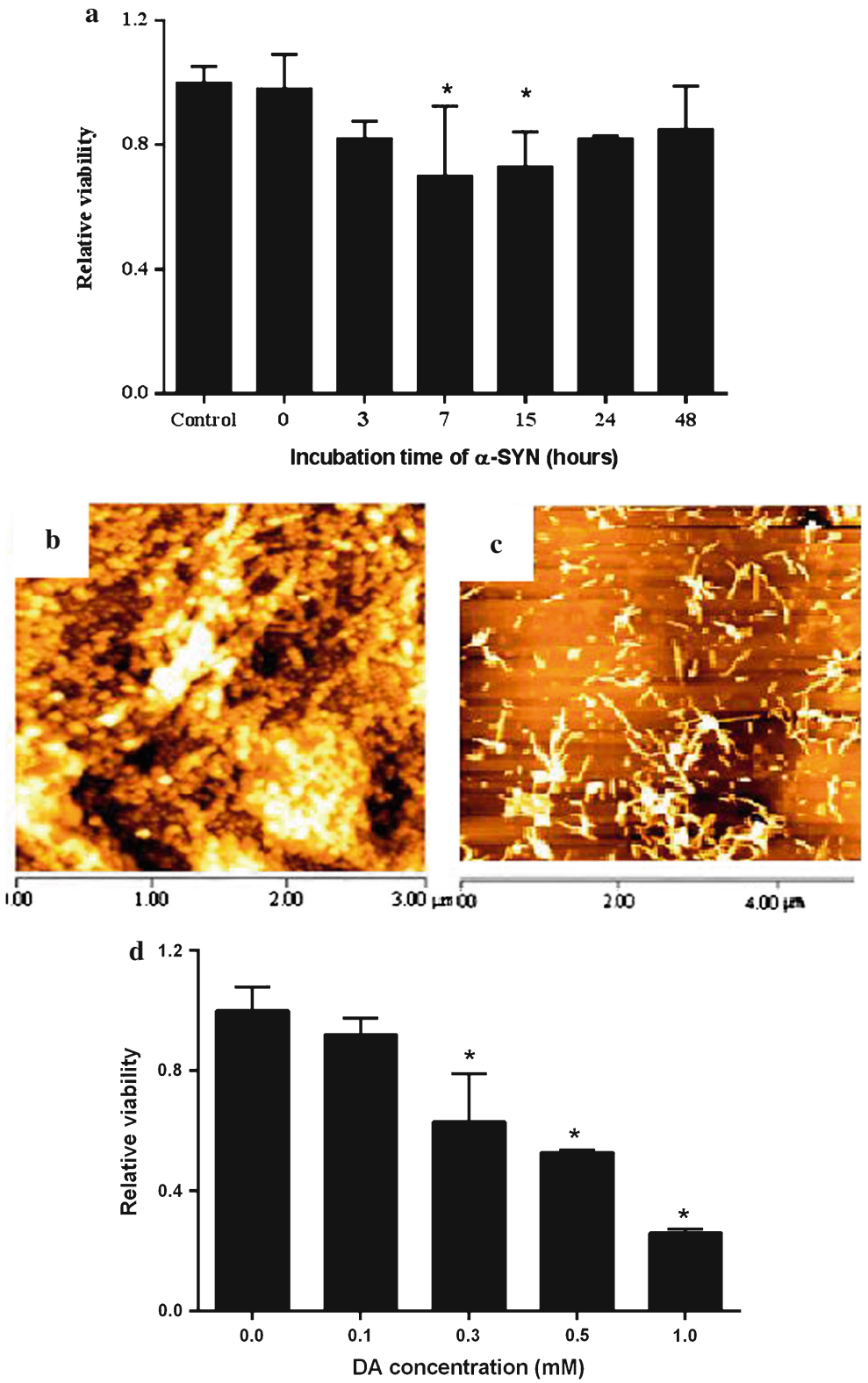

Fig. 1 The effect of aggregated forms of ASN (2 mg/mL) and DA on the viability of the cultured PC12 cells using MTT. **a** ASN incubated under fibrillation condition and sampled at 0, 3, 7, 15, 24 and 48 h during the course. These different aggregated species of ASN (10 %v/v) added to PC12 cells and viability measured after 24-h. **b**, **c** AFM images of ASN incubated for7- and 24-h, respectively. **d** The cytotoxic effect of different concentration of DA (0.1–1 mM) after 24 h. Results are presented as means + SE. n = 3. Significant difference from control, $P < 0.05$; ANOVA followed by Tukey's multiple comparison tests

Among all factors that can trigger degeneration of dopaminergic cells, DA plays an undeniable role [29, 30]. Thus, we examined the effects of different concentrations of DA on PC12 cells to determine the toxic concentrations. Consistent with previous studies, DA significantly causes cellular damage and eventually induces cell death in a dose dependent manner. As it is shown in Fig. 1d, concentrations above 0.3 mM are significantly toxic.

3.2 Simultaneous Treatment with DA and α-SYN Declines Cytotoxic Effects on PC12 Cells

In order to assess the toxic effect of DA in the presence of ESAA, we treated PC12 cells with both factors together at their toxic concentrations. To this end, we exposed the cultured PC12 cells to DA (concentrations of 0.3 and 0.5 mM) in the presence of ESAA for 24 h. Cytotoxicity was then evaluated using MTT, ROS and Annexin V/PI staining assays.

MTT assay showed that cell viability in the presences of ESAA, 0.3 or 0.5 mM DA alone was 70 % ± 1, 50 % ± 6 and 37 % ± 1, respectively (Figs. 1a, d , 2a). Therefore, we expected that co-treatment of the cultured cells with ESAA and DA (0.3 and 0.5 mM) would have a additive effect and increase the death rate when compared to each of the factor alone. Surprisingly, we observed that the treatment with 0.3 and 0.5 mM of DA significantly increased the death rate to 26 ± 2 and 52 ± 4 % in the presence of ESAA, respectively. Given the key role of oxidative stress in PD pathogenesis, we examined the cellular redox levels under each treatment condition by measuring the fluorescence intensity of DCFH-DA. While ESAA and DA treatments dramatically increase ROS levels, co-treatment with ESAA and DA significantly reduced ROS production (Fig. 2b). In order to understand the differences between the approximated rate of death and ROS level with real obtained ones, their proportions have been summarized in Fig. 2c.

To further characterize the type of death induced by these treatments, we stained the cells using Annexin V and Propidium iodide (PI) and analyzed the population using flow cytometry. As shown in Fig. 3a, the patterns of cell death were different between cells treated with DA or ESAA. In the DA-treated cells the ratio of early to late apoptosis was about 45–55 %, while for the ESAA-treated ones this ratio increased and was 83–17 %. Nevertheless, when cultured cells were treated with DA (0.3 mM) and ESAA simultaneously, this ratio was 63–37 %. This result suggests that ESAA and DA treatments induce mostly the early and late cell death, respectively. Interestingly, the population of late apoptosis/necrosis cells in the ESAA–DA treatment was roughly reduced in contrast to the DA treatment.

3.3 The Cross-Neutralization Effect of α-SYN and DA

ESAA was centrifuged to investigate the cytotoxicity of different aggregated forms of α-SYN in the absence and presence of DA. Using High-speed centrifugation, heavier particles (F1) were precipitated and separated from smaller soluble particles (F2). After centrifugation, the F1 and F2 were characterized using AFM imaging and a ThT fluorescence intensity assay. AFM images of the F1 fraction indicate that short fibrils are abundant among the aggregated-forms but no mature long fibrils are formed yet (Fig. 4a). Interestingly, the AFM images from the F2 fraction also show small spherical particles but no fibrils were observed (Fig. 4b). Accordingly, the F2 fraction did not increase the ThT fluorescence intensity while there was a remarkable raise in the fluorescence intensity after adding the F1 fraction (Fig. 4c). After characterizing both fractions, we treated PC12 cells with ESAA F1 or F2 fractions in the presence and absence of DA (0.3 mM). We again used the MTT assay to analyze the viability of the cells. The cell viability after treatment with F1, F2 or total ESAA (without DA) was 92, 78 and 70 %, respectively (data not shown). Considering the concurrent treatment with DA and F1 led to high cell viability rate, a sharp decrease in the rate of the cell death in contrast to the presence of DA alone. Interestingly, the presence of F2 had less effectiveness on the rate of death (Fig. 4d). According to the results, it can be inferred that fibrillar structures might function in escape of cells from DA-induced cell death. To elucidate this assumption, we assessed cell viability and generation of ROS products in the presence of both extended fibrils forms of α-SYN (with incubation for 36-h) and DA (0.3 mM). According to the fibrillation kinetic, fibrillation was ended after 36-h of incubation and samples merely contained mature fibrils. Cell viability results indicate that fibrillar aggregates had no considerable toxic effect on cell viability while significantly increase survival in fibrils and DA co-treatment compared to DA treatment (Fig. 5a). Measurement of ROS indicated that treating the culture cells with DA, strongly promotes ROS generation (Fig. 5b), however exposing cells to both DA and fibrils diminished ROS generation.

3.4 Determination of the Interaction Between DA and Fibrillar α-SYN

To further elucidate the existence of an interaction between DA and fibrillar aggregates, we performed spectrophotometry and FTIR analysis. For spectrophotometric analysis we dissolved DA (0.3 mM) in PBS buffer and OD was measured at 280 nm (the absorbance wavelength of DA) (A in Fig. 6a). Next, monomeric or fibrillar α-SYN (2 mg/

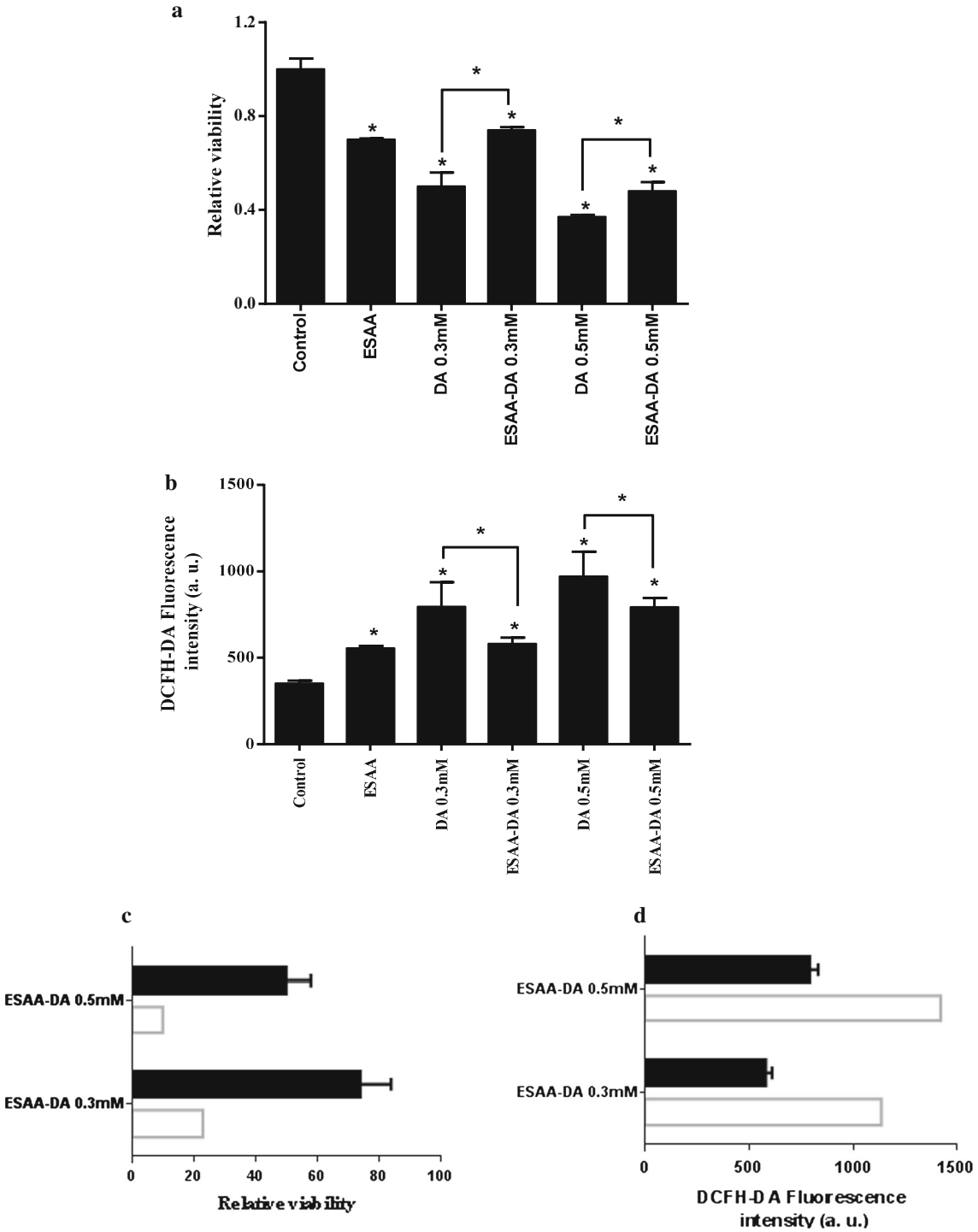

Fig. 2 The effect of the simultaneous treatment with ESAA and DA on cell viability and ROS production. Cells treated with DA, ESAA alone, and both concomitantly for 24-h; the concentrations of dopamine was 0.3 and 0.5 mM and the concentration of protein (2 mg/mL) was 10 % v/v. **a** Viability of cells measured by MTT and **b** ROS production evaluated by intensity of DCF fluorescence intensity. **c** The predicted viability (*black part*) or ROS production (*total column*) and the observed viability (*total column*) and ROS production (*black part*) of the treated cells with ESAA (10 % v/v) along with DA (0.3 mM or 0.5 mM). Results are presented as means + SE. n = 3. All treatment are significantly different from control and DA-treated groups, $P < 0.05$; ANOVA followed by Tukey's multiple comparison test

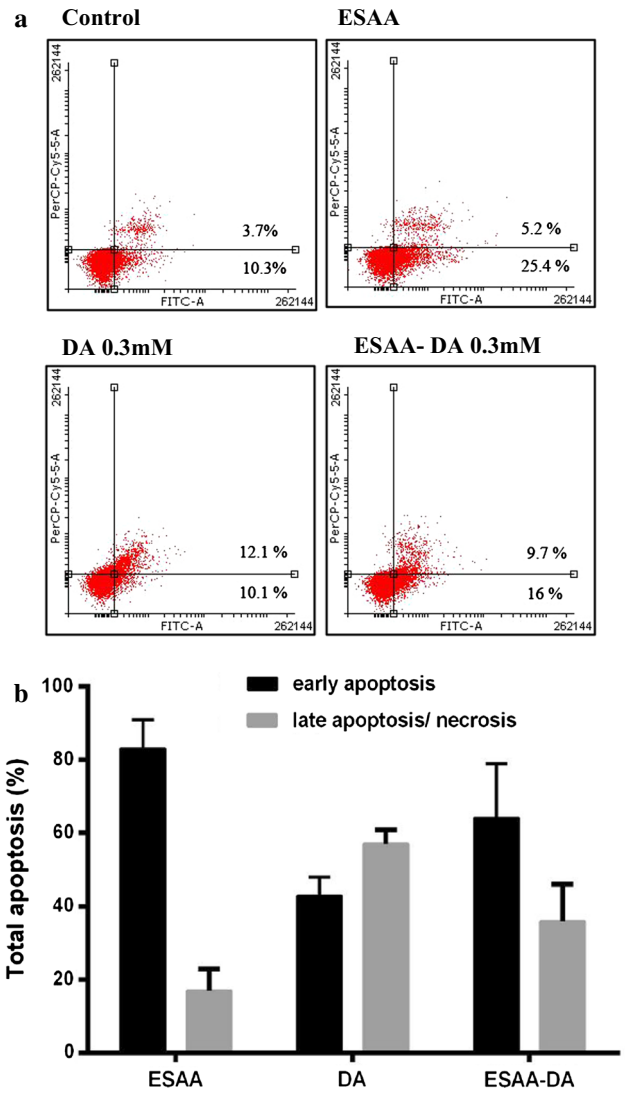

Fig. 3 Cell death assessment using Annexin V/PI staining. The cells were treated with ESAA (10 %v/v) and 0.3 mM DA separately and simultaneously and then the rate of apoptosis was measured using flowcytometry. **a** The pattern of flowcytometry for the cell death. **b** The ratio of early apoptosis to late apoptosis/necrosis in the different treated cells obtained from the average analysis of three experiments of the flowcytometry. The concentration of the protein was (2 mg/mL)

mL) was added to the DA solution, OD was then measured at the same wavelength (B in Fig. 6a). Samples were then incubated at 37 °C for 15 min, then monomeric or fibrillar α-SYN were removed from the supernatant by precipitation and the OD_{280} of the supernatant was measured again (C in Fig. 6a). Our results suggest that by adding α-SYN fibrils to the DA solution and subsequent extracting them from the solution, the level of DA decreased in the solution, a phenomenon that was not observed when using the monomeric form of the protein (Fig. 6a). Thus, suggesting that DA extensively bound to α-SYN fibrils but not its monomeric form. We speculate that this is probably due to the dense and extended structure of α-SYN fibrils, that lead to a quick interaction between DA and fibrils. Although it has been shown that the interaction between DA and α-SYN can occur in the monomeric forms [31], It seems that this interaction is not as efficient in removing DA as that of α-SYN fibrils, either because it needs more time to interact or because the number of DA molecules captured by each monomer is much less than the number of molecules capture by each fibril.

On the other hand, in the FTIR study (at 1600–1700 wavenumbers belongs to amid I) there were some differences between the fibrils and DA-fibrils spectra. Amide I is important region for research about secondary structure of protein that provides appropriate data concerning about interaction of small molecules with proteins [32]. Figure 6b indicates the second derivative of FTIR absorbance related to the amide I region in fibrillar α-SYN associated with strong peaks at 1624 and 1692 wavenumbers as characteristics of fibrils structures. On the other hand, simultaneous incubation of DA with fibrils have been changed the spectral pattern thereby the peak in the area of 1624 shifted to 1634 and raised sharply (Fig. 6c). Results indicate that there is a strong alteration on 1624 wavenumber for fibrils, which might be caused by an interaction between DA and fibrils. The investigation of the FTIR spectrum of DA demonstrated that DA has a strong peak at 1634 wavenumber. We also analyzed far-UV CD spectrum of the fibrils. Far-UV CD spectra did not indicate any considerable change in the secondary structure of fibrils and contained a deep negative peak at 218 nm for fibrils in the absence and presence of DA (data not shown). The change in FTIR spectrum of protein is likely related to the co-presence of DA.

To find the strength of this interaction, after incubation of the fibrils with DA, the sample again was centrifuged and the pellet was suspended and dialyzed against tris buffer for more than 48 h. The pellet of fibrils with DA and without DA after dialyzed is presented in Fig. 7a. It is clear that the color of the pellet became black in the presence of DA and it can be pointed of a strong interaction between DA and fibrils.

To further clarification the status of interaction between DA and fibrils, N-terminal and C-terminal fluorescence labeled fibrils were incubated with PK. The SDS-PAGE images showed that after centrifuging the treated fibrils with PK, the fluorescence regions presented at N-terminal and C-terminal were removed from the pellet. Figure 7b indicate the images of a SDS-PAGE which was visualized with UV-light and it was then stained with Coomassie blue. As indicated in the SDS-PAGE images after treatment with PK, the fluorescence band disappeared and the non-fluorescence band appeared with different size that was colored

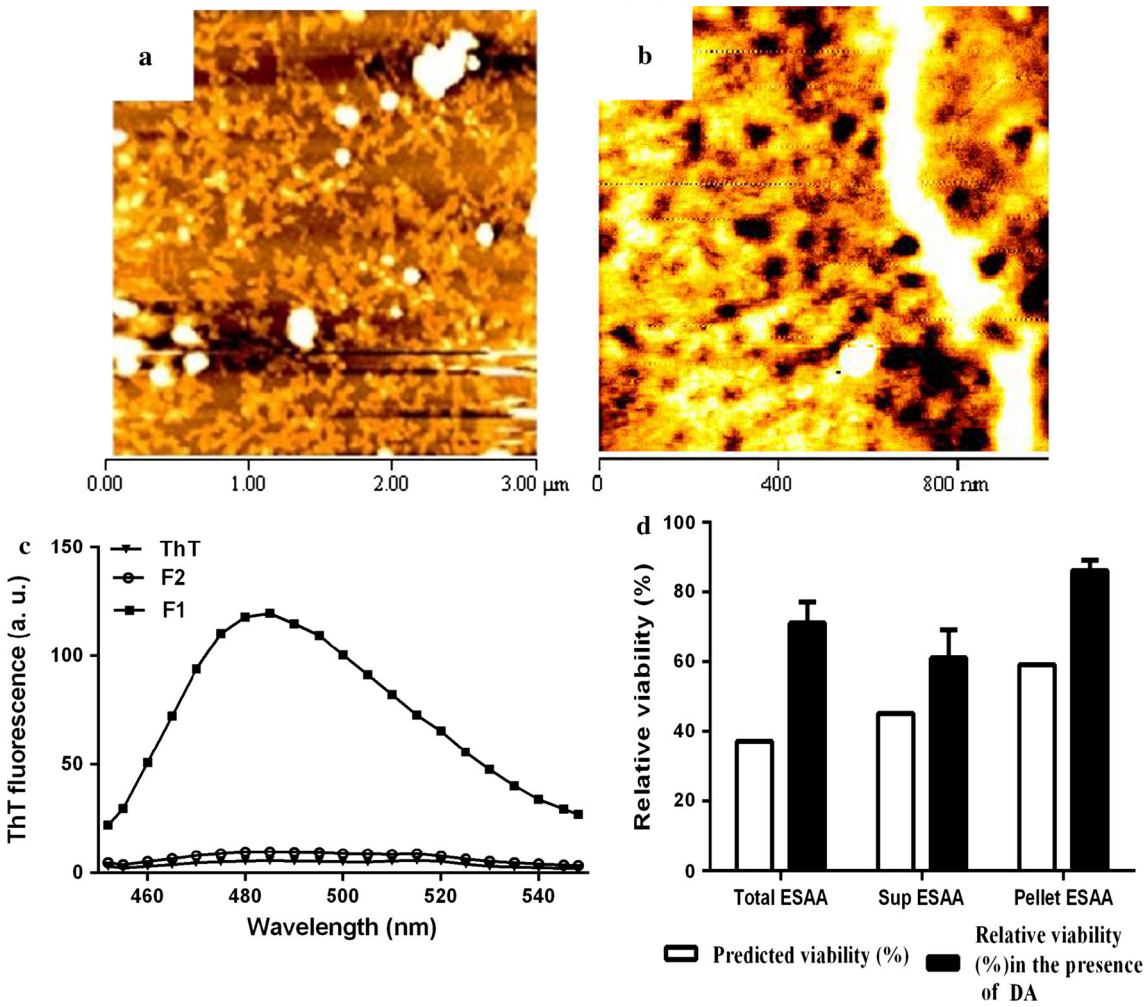

Fig. 4 Characterization of different fractions of ESAA after centrifugation. The AFM images of the pellet (**a**) and supernatant (**b**) of ESAA after centrifugation. **c** ThT fluorescence intensity of F1 (pellet) and F2 (supernatant). **d** The predicted viability (*black column*) and the observed viability (*white column*) of the treated cells with different fractions of ESAA (10 % v/v) along with DA (0.3 mM)

only with Coomassie blue. The pellet of the treated fibrils with PK was incubated with DA. Its FTIR spectrum also indicated that the interaction was still happened between fibrils and DA even without unprotected N-terminal and C-terminal regions (result has been presented in Fig. 7c).

4 Discussion

It has been suggested that the high level of DA in dopaminergic neurons makes them susceptible to neurodegeneration [33]. Previous studies suggest that DA cytotoxicity might be mediated by ROS generation [18, 34]. Accumulation of ROS plays a crucial role in the induction of apoptosis by releasing cytochrome c from mitochondria [35, 36]. On the other hand, α-SYN oligomerization is an important factor involved in pathogenesis of PD. The oligomers, the most toxic intermediates of α-SYN, may diffuse to membrane and disrupt normal function of cell membrane, which can lead to the dysregulation of intracellular calcium homeostasis [37–39]. Recent investigations showed that metabolites derived from DA oxidation could interact with α-SYN and induce its aggregation through SDS-resistant oligomers [23, 40, 41]. Although there are many studies investigating the interaction of DA with α-SYN, which seems to stop the fibrillation process [40, 41], no studies have been made on aggregated α-SYN and DA. In the present study, we examined the simultaneous effect of ESAA, mainly composed of oligomer α-SYN species, and toxic concentrations of DA on cultured PC12 cells. Our cytotoxicity assays show that simultaneous treatment with ESAA and DA increased the viability of cells in comparison with each treatment alone. Remarkably, all co-treatment experiments all increases in viability were correlated with a decrease in ROS production. Given that both ESAA, as a toxic

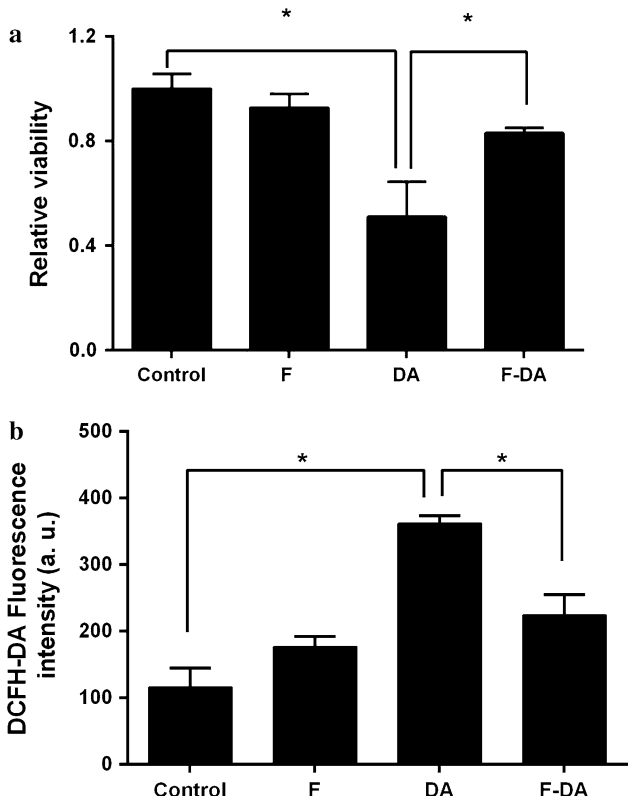

Fig. 5 The effect of simultaneous treatment of fibrillar form ASN and DA on cell viability and ROS production. Cells treated with 0.3 mM DA and ASN fibrils (36 h incubated) separately (F) and together (F–DA) for 24 h. **a** Cells viability assessed by MTT and **b** ROS production measured via DCF fluorescence intensity. Results are presented as means + SE. n = 3. DA treated cells are significantly different from control and F-DA treatment, $P < 0.05$; ANOVA followed by Tukey's multiple comparison test

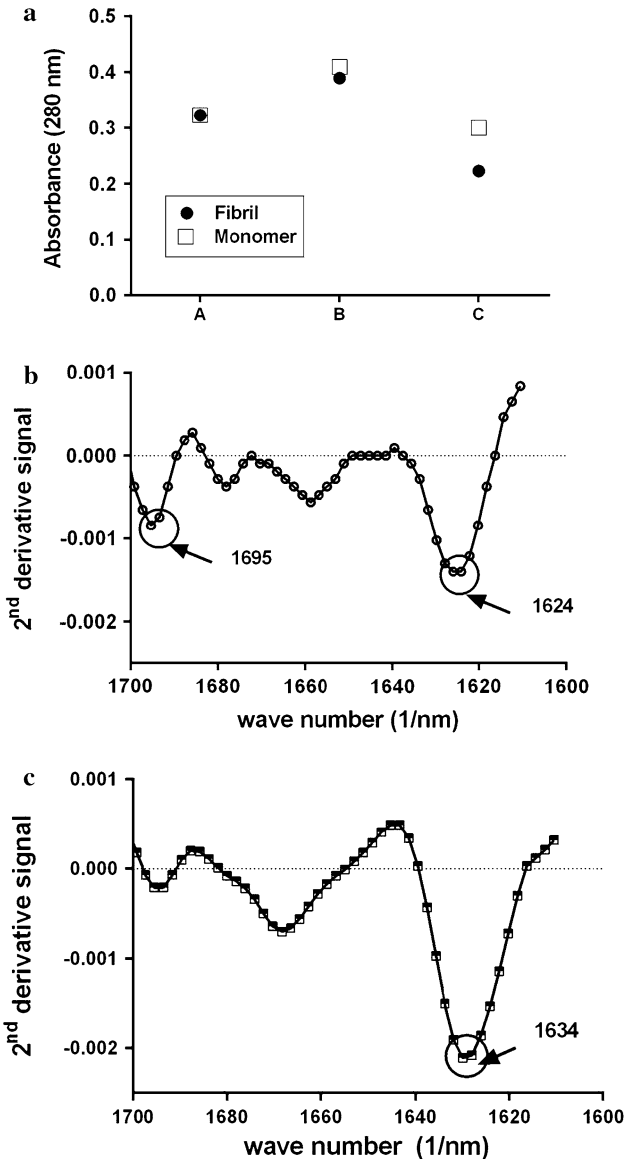

Fig. 6 Interactions of ASN with dopamine. **a** The monomeric and fibrillar species of ASN with 0.3 mM dopamine were incubated for 15 min and the absorbance were measured at 280 nm before adding ASN (A), after adding ASN (B) and after incubation and elimination of proteins from the solutions (C). FTIR spectra of ASN fibrils alone (**b**) and the fibrils incubated with 0.3 mM DA (**c**) at the range of 1600–1700 wavenumber

complex of α-SYN aggregates, and the doses of DA used were toxic, we expected to have a considerable additional decrease in cell viability. In this respect it is interesting to note that Pham and colleagues observed that the formation of oligomer-DA adducts decreases α-SYN toxicity. The authors suggest that this interaction prevents the interaction of oligomers with the membrane to form pore-like structures [42]. In our study, fractionation of ESAA revealed that the presence of particles with more fibrillogenic characteristics (F1) in a heterogeneous mixture of α-SYN species noticeably reduced the toxicity. The spectrophotometric and FTIR results confirmed the existence of an interaction between α-SYN fibrillar structures and DA. We have previously shown that fibrils strongly tend to absorb small molecules such as Azo dyes. These interactions are usually formed even with macroscopic coagulants, which are visible to the naked eye [43]. In this study, it also seems that a similar interaction may be present between fibrillar aggregates of α-SYN and DA. The interaction between the fibrils and DA should be strong because after 48 h of dialysis, the color of fibrils was black which indicated of the presence of DA. As previously shown, DA is transported from the extracellular space into cells through the dopamine transporter (DAT), a membrane protein present on the membranes of dopaminergic neurons that increases intracellular level of DA. DAT has a central role in DA turnover. It was shown that in DAT knockout mice, stored DA levels were attenuated when compared with the controls [44]. On the other hand, L-dopa treatment in a transgenic mouse model overexpressing DAT, increase

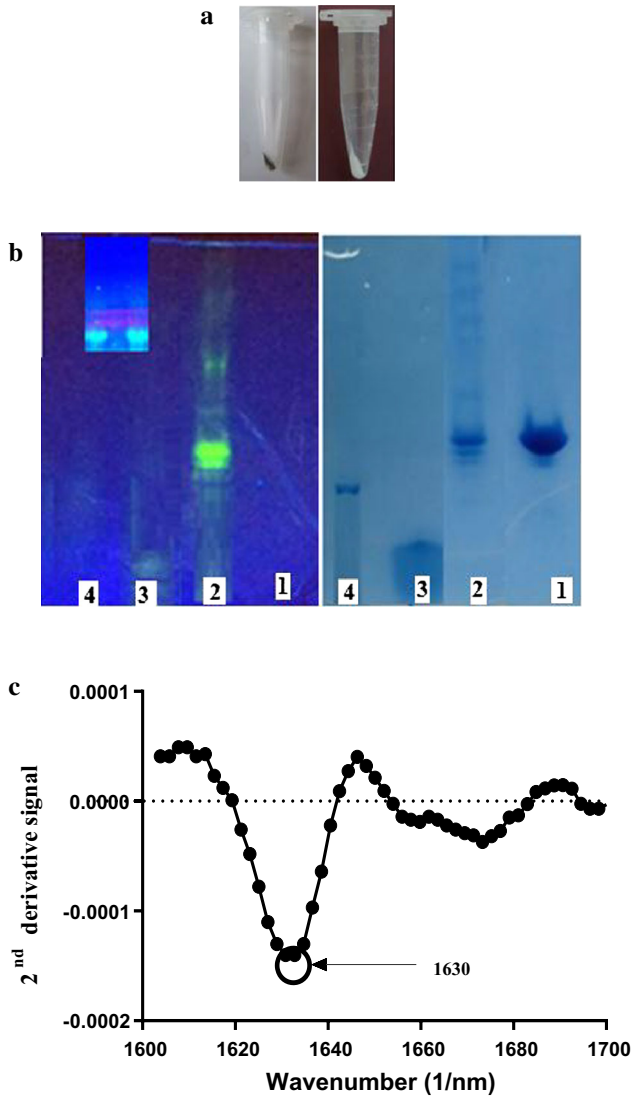

Fig. 7 Investigation of more details about fibril–DA interaction. **a** The pellets of α-SYN fibrils which had been treated with DA (*left*) or without DA (*right*) and then were dialyzed for 48 h at ambient temperature. **b** The SDS-PAGE of α-SYN fibrils which had been treated with PK, *1* the fibrils of non-labeled α-SYN, *2* the fibrils of mixed labeled α-SYN (with both the N-terminal and C-terminal labeled α-SYNs), *3* the supernatant of *2* after treated with PK, *4* the pellet of *2* after treated with PK *left*: visualized with UV light, *right*: stained with Coomassie blue. The *inset image* shows the SDS-PAGE of the N-terminal (*left*) and C-terminal labeled (*right*) monomeric form and also non-labeled α-SYN (middle). **c** The FTIR spectrum of the pellet *4* after treating with PK

cytosolic DA level resulting in the development of neuronal degeneration [45]. Therefore, according to both in vitro and in vivo studies, chronic exposure to DA or L-dopa may lead to a selective loss of dopaminergic cells [46–48]. In our experiments, cells were treated with DA and aggregated α-SYN, our results suggest that interaction between DA and fibrillar α-SYN in the extracellular space may sequester DA and prevent its entrance to the cells. As shown in previous studies, α-SYN has 140 residues where the residues 34–100 are involved in fibrillation. Some reports claimed that the major areas interacting with DA and its derivatives are located outside the fibril structure, in both the N-terminal and C-terminal regions [49, 50]. However, our experiments on labeled N-terminal and C-terminal parts of α-SYN revealed that even after digesting the fibrils with PK, a process that removes these regions, the interaction between fibrils and DA was still present. Thus, suggesting that this interaction is independent of these areas.

Our finding concludes that the co-treatment with DA and ESAA have a paradoxic antagonistic effect on cell survival and that this effect depends on the α-SYN fibrillar structures present in the ESAA mixture. These findings are remarkable and reveal an unknown protective effect of extracellular α-SYN fibrils on DA toxicity. Opening new questions that need further investigations to clarify the exact mechanisms by which fibrils contribute to reduce DA toxicity.

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Compliance with Ethical Standards

Conflict of interest The authors declare no potential conflict of interest.

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