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Facile "stop codon" method reveals elevated neuronal toxicity by discrete S87p-α-synuclein oligomers

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ABSTRACT

Herein, a new method for preparing phosphorylated proteins at specific sites has been applied to α-synuclein (α-Syn). Three different α-Syn species phosphorylated at Serine 87 (S87p-α-Syn), Serine 129 (S129p-α-Syn) and Serine 87/129 (S87p,129p-α-Syn) were prepared through the 'stop codon' method and verified by LC/MS/MS and immunoblotting. Each type of phosphorylated α-Syn was tested for oligomerization trends and cellular toxicity with dopamine (DA), Cu^{2+} ions and pyridoxal 5′-phosphate. Aggregation trends induced by DA or DA/Cu^{2+} were similar between phosphorylated and non-phosphorylated α-Syn in SDS–PAGE. However, except for the monomer, phosphorylated oligomers showed higher toxicity than the non-phosphorylated α-Syn (Np-α-Syn) oligomers via WST-1 assays when tested on SH-SY5Y human neuroblastoma cells. In particular, S87p-α-Syn and S87p,129p-α-Syn oligomers induced by DA/Cu^{2+}, showed higher toxicity than did S129p-α-Syn. When α-Syn was treated with pyridoxal 5′-phosphate in the presence of DA or Cu^{2+} to determine aggregation effects, high inhibition effects were shown in both non-phosphorylated and phosphorylated versions. α-Syn co-incubated with DA or DA/Cu^{2+} showed less cellular toxicity upon pyridoxal 5′-phosphate treatment, especially in the case of DA-induced Np-α-syn. This study supports that phosphorylated oligomers of α-Syn at residue 87 can contribute to neuronal toxicity and the pyridoxal 5′-phosphate can be used as an inhibitor for α-Syn aggregation.

1. Introduction

Simple protein-based phosphorylation and dephosphorylation events govern a multitude of processes in biology. In addition to being part of healthful normal functions, these events are also linked to various neurodegenerative diseases in ways still not fully elucidated. It has been discovered that Lewy bodies (LBs) are largely fibrils of *phosphorylated* α-synuclein (α-Syn) [1]. The incorporation of phosphates into α-Syn is currently being actively explored in Parkinson's Disease (PD) and Lewy Body (LB) dementia pathology research by chemical and biological researchers [2–4], since it is not abundantly clear how phosphorylation relates to the extent of neurotoxicity, or under what conditions protein phosphorylation (especially at Serine 129), as identified in actual Lewy bodies, leads to oligomerization. Christine et al. reported that Serine 129 phosphorylation induced the toxicity in multiple system atrophy (MSA) [5]. Although the Chen and Feany groups reported that phosphorylation at Serine 129 enhances cellular toxicity, inclusion bodies may protect against cellular toxicity from phosphorylated α-Syn [6]. In addition to phosphorylation of α-Syn, the action of DA and copper ions are also considered important for the progression of neurodegenerative disease. DA can be viewed as a reactive catechol or primary amine; it can be readily aerobically oxidized to quinone, indole and a polymer (neuromelanin) [7]. It has been known that DA can produce oligomers of α-Syn [8]. Additionally, Cu^{2+} can mediate metal-catalyzed oxidation (frequently, Fenton chemistry) has a strong effect on oligomerization, and involves radicals, e.g., ROS [9].

α-Syn monomers or oligomers exist in cerebrospinal fluid and plasma [10,11] and are generated from the action of apoptosis/necrosis [11,12]. Additionally, although many studies report that DA or Cu^{2+} can induce the oligomerization of α-Syn [8,13], there is a paucity of reports dealing with discrete phosphorylated

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oligomers, especially dimers, trimers, tetramers, or multimers, and how they relate with neuronal toxicity. For cellular toxicity of these oligomers, there are limits to studying organisms *in vivo*. Importantly, it is difficult to quantify or regulate the amount of oligomers. Therefore, we designed an *in vitro* experimental strategy which can help quantify the toxicity between α-Syn oligomers, DA and $Cu^{+/2+}$. For the antiaggregation of α-Syn, pyridoxal 5′-phosphate, which has an aldehyde functional group and is controlled naturally in body systems, was considered. Regarding potentially important α-Syn substrate interactions, pyridoxal 5′-phosphate is an activated form of Vitamin B_6 [14] involved in human metabolism. As an aldehyde, we can expect that pyridoxal 5′-phosphate can react with some primary amines of DA or with the 15 lysine residues present in α-Syn and make Schiff bases [15,16] to quench the toxicity of DA.

For this study, the first thing to consider is the synthesis of site-specific phosphorylation of α-Syn. New methods of specific incorporation of phosphorylation units, without reliance on phosphomimics would help further understanding in the field of neurodegenerative disease research. Usually, Casein Kinase 1 was used with wild-type or mutant α-Syn to generate a phosphorylated version [17,18]. Also, Tyr phosphorylation has been implemented involving a semi-synthetic strategy; and a total synthetic strategy has recently come out too strategy [19]; these approaches allow for site selectivity. While the production of discrete phosphorylation in full length proteins has significant technical limitations, Park et al. recently reported genetic incorporation of phosphoserine into proteins at desired positions using an engineered bacterial expression system [20]. Thus, herein we have tried to synthesize α-Syn with site-specific phosphorylation and measure oligomerization trends and the neuronal toxicities using phosphorylated or non-phosphorylated α-Syn oligomers induced by DA, or DA/Cu^{2+}. We further studied the oligomerization inhibition effect by pyridoxal 5′-phosphate through *in vitro* protein level and cellular level experiments.

2. Materials and methods

2.1. Synthesis of phosphorylated α-Syn

Three types of phosphorylated α-Syn were synthesized by incorporating phosphoserine (sep) into protein site-specifically using the orthogonal SepRS-EF-Sep-$tRNA^{Sep}$ system. Engineered phosphoseryl-tRNA synthetase (SepRS) aminoacylates $tRNA^{Sep}$ carrying a stop anticodon (CUA) with phosphoserine. EF-Sep, engineered EF-Tu, specifically recognizes Sep-$tRNA^{Sep}$ and delivers it to the ribosome (ESI, Fig. S8).

2.1.1. Construction of plasmids

pCDFDuet-α-syn-wt (wild type) was created by adding a codon-optimized α-Syn gene into the streptomycin resistant vector pCDFDuet-1(Novagen) using BamHI and AscI sites. A TEV (Tobacco Etch Virus) protease recognition site was inserted between His6-tag and α-Syn wild-type gene. The resultant plasmid was constructed to express the recombinant protein His6-TEV protease cleavage sequence (ENLYFQG)-α-syn-wt. The vectors pCDFDuet-α-Syn87/129TAG were constructed by cloning the cleavable His6-tagged α-Syn mutants into pCDFDuet-1 using NcoI and AscI. The Ser87 or Ser129 residue was replaced with an amber stop codon (TAG) by PCR mutagenesis. The TEV protease recognition site was added downstream of the α-Syn gene; the His6 tag was inserted consecutively, next to TEV protease cleavage site with PCR. The resultant plasmid was created to express α-Syn-Sep87/129-TEV protease cleavage sequence (ENLYFQG)-His6. Construction of the plasmids pKD–SepRS–EFTu and pSepT was described in the previous report [20]. Engineered SepRS and EF-Tu (EF-Sep) that were evolved in the recent report [21] were used in this paper.

Note: Additional information about *protein expression and purification*, *Oligomer purification*, *Changing buffer using centrifugation method, SDS–PAGE and immunoblotting, Neurotoxicity experiments and the WST-1 assay, Aggregation of phosphorylated α-Syn by DA or DA/Cu^{2+} in SH-SY5Y cells, Intensity calculations from SDS–PAGE data, and Transmission electron microscopy* (*TEM*) can be found in the Supporting Information.

3. Results

3.1. Synthesis of phosphorylated α-Syn; S87p-, S129p-, and S87p,129p-α-Syn

The α-Syn variants were expressed and purified under the same conditions as those described for non-phosphorylated systems (ESI, Fig. S1 and S2). Full length α-Syn variants were obtained only in the presence of EF-Sep, signifying the importance of the engineered elongation factor for phosphoserine incorporation, as previously reported [20]. Phosphoserine incorporation was further verified by LC–MS/MS analysis or immunoblotting (Fig. 1). When S87p-α-Syn was digested with trypsin, and each fragment was subjected to collision-induced dissociation (CID), the neutral loss of phosphoric acid (~–98 Da, H_3PO_4) was observed at position 87 [22], confirming the expected phosphoserine incorporation.

3.2. Oligomerization of α-Syn phosphorylated with in vitro DA and Cu^{2+} incubation

Phosphorylated α-Syn and Np-α-Syn were incubated with DA or DA/Cu^{2+} for 14–20 h, at 37 °C. For DA-induced oligomerization, 4- and 10-fold increases of oligomer formation were shown at the ratio of 1:1 and 1:10 (α-Syn:DA), respectively. Some DA-dependent oligomerization increases were found in both cases of Np-α-Syn (Fig. 2A and B) and phosphorylated α-Syn (Fig. 2C and D). However, no definite differences exist between non-phosphorylated and phosphorylated oligomerization.

For DA/Cu^{2+}-induced oligomerization, marginal increases (×0.9–1.2) were found for a 1:1:10 stoichiometry (α-Syn:DA:Cu^{2+}), relative to control values. When copper ions are present, oligomerization did not correlate with the concentration of Cu^{2+}, although some other variations were shown between proteins. Thus, DA or DA/Cu^{2+} appear to increase oligomerization of α-Syn, but do not induce a different oligomerization trend of phosphorylated protein, relative to the non-phosphorylated α-Syn *in vitro*.

3.3. Aggregation of phosphorylated α-Syn by DA or DA/Cu^{2+} in SH-SY5Y cells

We performed SDS–PAGE and immunoblotting to assess the extent of aggregation of phosphorylated α-Syn by DA or DA/Cu^{2+} in SH-SY5Y cells. Cu^{2+}, DA or DA/Cu^{2+} can induce aggregation of α-Syn phosphorylated at Serine 87 or α-Syn phosphorylated at Serine 129 in SH-SY5Y cells (ESI, Fig. S7). There were no significant differences found between results for Cu^{2+}, DA, and DA/Cu^{2+}. Aggregation trends between S87p-α-Syn and S129p-α-Syn were similar. Thus, at the level of dopaminergic cell assays, DA or Cu^{2+} appears to induce the aggregation of α-Syn similarly to that seen between S87p-α-Syn and S129p-α-Syn.

3.4. Cellular toxicity assays

We attempted to purify all α-Syn oligomers induced by DA or DA/Cu^{2+} through gel electrophoresis as described in the Experi-

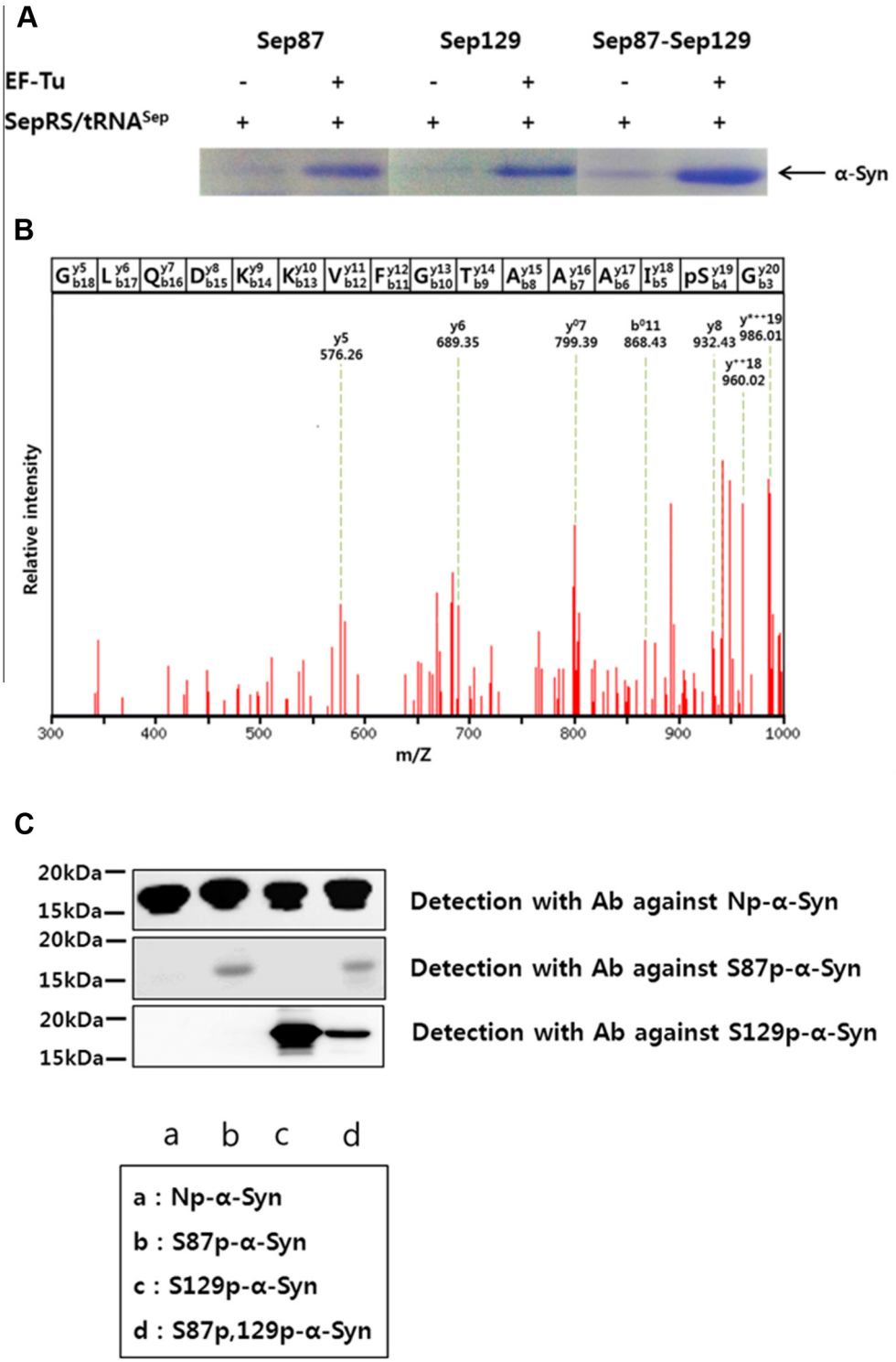


Fig. 1. Expression and analysis of α-Syn variants with phosphoserine. (A) SDS–PAGE analysis of purified α-Syn variants; S87p-α-Syn expression without (lane 1) or with (lane 2) EF-Sep; S129p-α-Syn expression without (lane 3) or with (lane 4) EF-Sep; and S87p,129p-α-Syn expression without EF-Sep (lane 5) and with EF-Sep (lane 6). (B) LC–MS/MS analysis of the purified S87p-α-Syn variant. (C) Verification of α-Syn of phosphorylation with immunoblotting by antibodies against Np-α-Syn, S87p-α-Syn, S129p-α-Syn and S87p,129p-α-Syn. The antibody (Ab) against Np-α-Syn showed broad detection ability regardless of extent of phosphorylation.

mental section. Immunoblotting results of purified oligomers are shown in the ESI section (Fig. S4) and were verified with TEM in ESI, Fig. S5. Neuronal cellular toxicities of discrete oligomers were then tested via WST-1 toxicity.

Whereas *oligomerization trends* were the same between phosphorylated and non-phosphorylated proteins (Fig. 2), *toxicity* differences were found to be large (Fig. 3). Phosphorylated proteins induced more toxicity at dimer and trimer levels ($p < 0.05$). It is especially interesting that S129p-α-Syn showed less toxicity than S87p-α-Syn regarding the DA/Cu^{2+}-induced tetramer and multimers, and DA induced tetramers ($p < 0.01$). However, S129p-α-Syn showed more variation than S87p-α-Syn did. Interestingly,

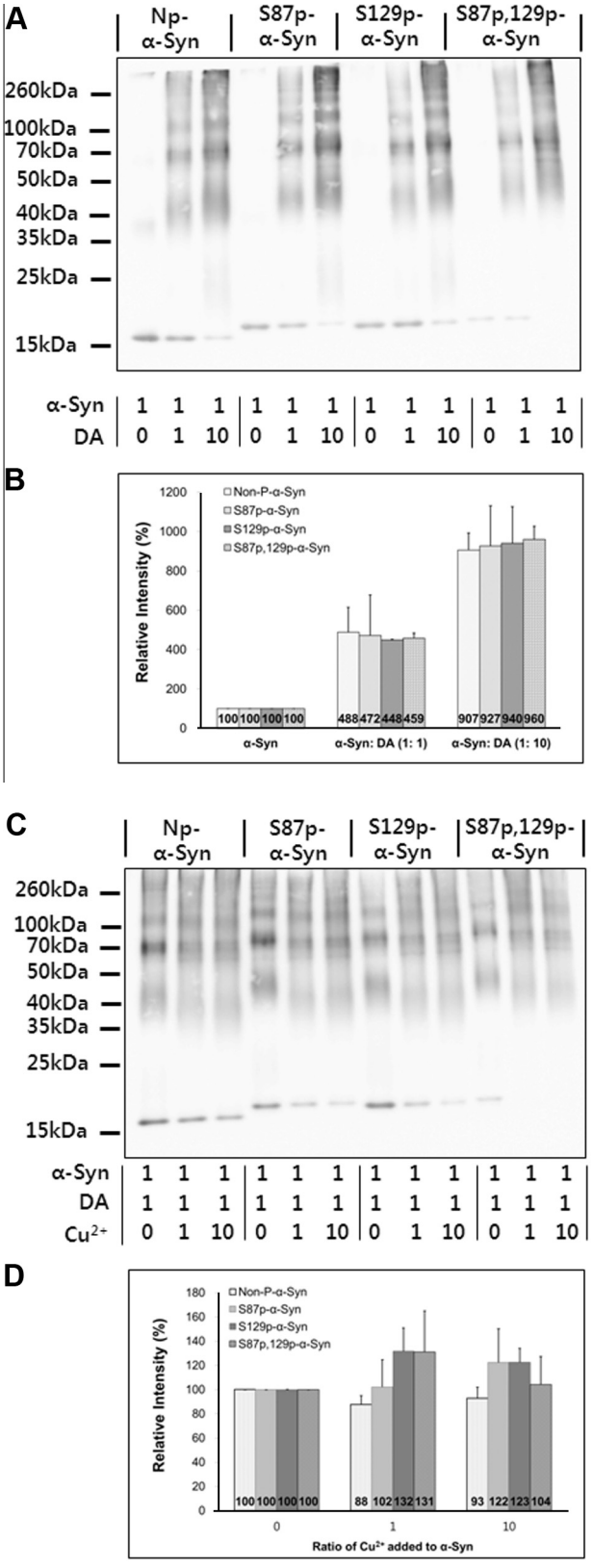


Fig. 2. (A and B) Oligomerization trends of phosphorylated α-Syn in the presence of DA. (C and D) Oligomerization of different types of α-Syn in the presence of DA and Cu^{2+}, Control reactions involved no added copper; α-Syn and DA were present only. Error bar = standard deviation. Conditions: 10 mM sodium phosphate buffer (pH 7.4), 37 °C, 14–20 h incubation. Experiments repeated 3 times.

S87p-α-Syn and S87p,129p-α-Syn showed similar cellular toxicities, suggesting that residue 87 for phosphorylation may be more important than that for 129 in cellular toxicity. DA/Cu^{2+}-induced oligomers of non-phosphorylated systems (except monomer) showed slightly higher toxicities than those formed via only DA oligomerization induction (mean value, $p < 0.01$). This corresponds with results from the Brown group [9] which reports that the incubation of α-Syn with copper ions gives cellular toxicity.

3.5. Effect of pyridoxal 5′-phosphate on α-Syn oligomerization induced by DA and $Cu^{+/2+}$

The inhibition effects on oligomerization with pyridoxal 5′-phosphate was investigated using protein level experiments; pyridoxal 5′-phosphate is an activated form of Vitamin B_6 [14]. As with S87p- or S129p-α-Syn, it is also a mono-phosphorylated species. Inhibition effects of pyridoxal 5′-phosphate have also been previously reported: Remani et al. performed pyridoxal 5′-phosphate blocking experiments of the citrate transport protein lysine residues in yeast mitochondria [23].

When pyridoxal 5′-phosphate was incubated with Np-, S87p-, S129p-, and S87p,129p-α-Syn under various combinations of DA and Cu^{2+} *in vitro*, clear oligomerization inhibition was demonstrated (Fig. 4A–C). In particular, DA-induced oligomerization was thoroughly inhibited by pyridoxal 5′-phosphate, which showed initial levels (same as that for the untreated sample). Pyridoxal 5′-phosphate may act to scavenge DA away from its protein cross-linking oligomerizing capacity. There is *little* difference in inhibition by pyridoxal 5′-phosphate between phosphorylated α-Syn and non-phosphorylated versions (Fig. 4A–C). However, differences between oligomers induced by DA and DA/Cu^{2+} do exist. Oligomers induced by DA/Cu^{2+} exposure showed more resistance to treatment of pyridoxal 5′-phosphate.

Further cellular assays were performed to determine if pyridoxal 5′-phosphate can inhibit the toxicity of oligomers. Each type of α-Syn was incubated with DA, pyridoxal 5′-phosphate or DA/Cu^{2+} at first. Centrifugal filtering was performed after incubation of α-Syn, DA or DA/Cu^{2+} to help in the removal of unreacted species. Monomers, dimers, trimers, tetramers and multimers were not separated. Instead, oligomers for each type of α-Syn were treated into SH-SY5Y cells for cellular toxicity test. Significant decrease of cellular toxicity was shown only for Np-α-Syn-derived aggregates which formed from incubation with DA (arrow, $p < 0.05$, Student's *t*-test). No significant differences existed for the other cases (Fig. 4D).

4. Discussion

Through stop codon methods, three types of α-Syn were synthesized and verified with LC–MS–MS and immunoblotting. Four types of α-Syn proteins, Np-, S87p-, S129p-, S87p,129p-α-Syn, were synthesized and incubated with DA or DA/Cu^{2+} to induce each type of oligomers. Higher oligomerization could be observed in a dose-dependent manner of DA. Almost 9 times increase of overall aggregation could be observed at the ratio of 1:10 (Syn:DA). When Cu^{2+} was added into the solution of α-Syn and DA (ratio, 1:1), there were no significant increases, which means copper did not effect on aggregation in the presence of DA (ESI, Fig. S6). Similar results were observed between phosphorylated and nonphosphorylated α-Syn, where the oligomerization of α-Syn by DA and copper ions is not dependent on phosphorylation.

For purification, oligomer solution which was induced by DA or DA/Cu^{2+} with α-Syn for 16 h at 37 °C, were centrifugal-filtered (Cutoff: 3 kDa) to remove unreacted species. We could acquire five types of oligomers: monomer, dimer, trimer, tetramer and multimers (above tetramers). Each purified oligomer sample was treated on neuronal SH-SY5Y cells for cellular toxicity with

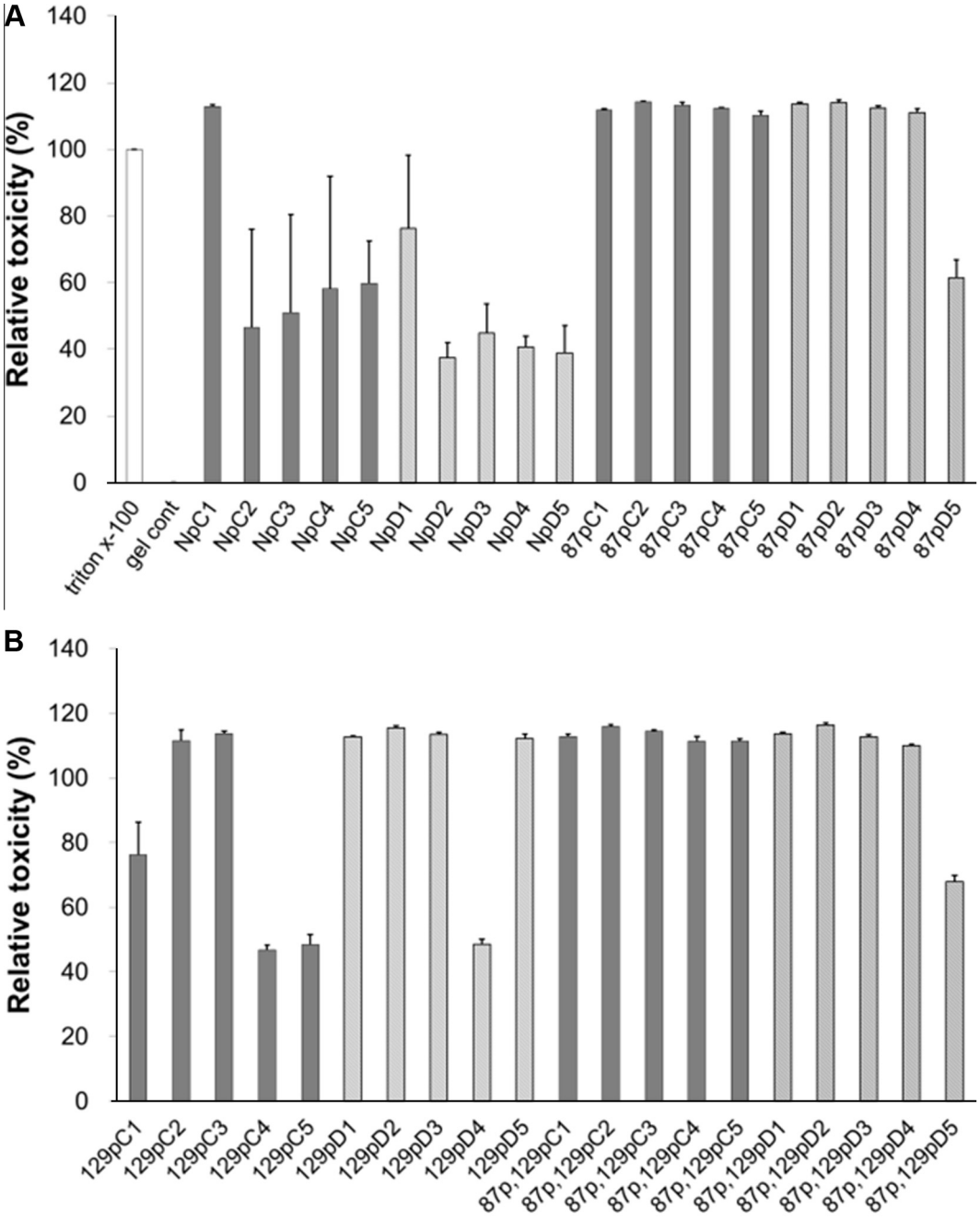
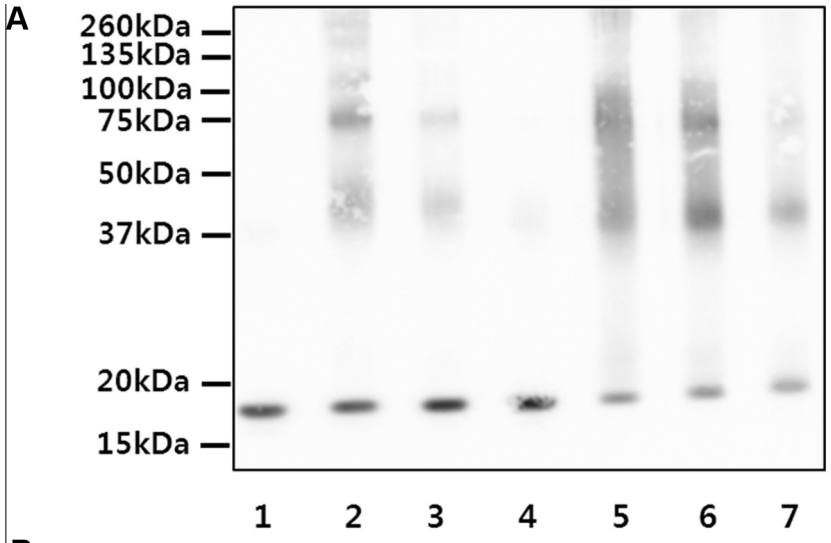
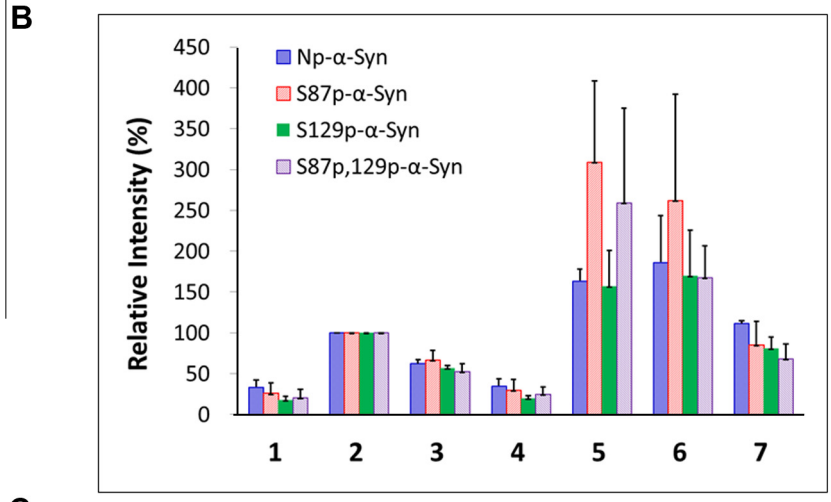


Fig. 3. (A) Cellular toxicity of Np-α-Syn, S87p-α-Syn and (B) S129p-α-Syn, S87p,129p-α-Syn on SH-SY5Y dopaminergic neuronal cells via WST-1 assay. (Student's *t*-test analysis in ESI, Fig. S3). "Np" = Np-α-Syn, "87" = S87p-α-Syn, "129" = S129p-α-Syn. "87p,129p" = S87p,129p-α-Syn, "1" = monomer, "2" = dimer, "3" = trimer, "4" = tetramer, "5" = multimers beyond that of the tetramer. "D" = DA-induced, "C" = DA/Cu^{2+}-induced. 'Triton X-100' was positive control (100% cell deathGel cont. was set as the negative control (0% cell death); only buffer without α-Syn which was from crushed gel, followed the same procedure to check the effect of crushed gel during oligomer purification process. Final concentration of each oligomer was 14.5 μg/L. All measurements were performed in triplicate. Error bars indicate standard deviation. Incubation of each oligomer with SH-SY5Y cells was performed over 24 h; 1 h incubation for each sample was undertaken with WST reagent, 37 °C.

via WST-1 assay. Surprisingly, every monomer showed most severe toxicity. We discussed this peculiarity because oligomers were treated outside of the cell. The monomer type is the most useful for penetration of cellular membrane. Some studies reported α-Syn migration occurs between neuronal cells [24–26], which can induce the toxic effect on the progression of PD. We discussed that good permeability of small size of monomer can induce the cellular toxicity.

Interestingly, the same trend can be observed between S87p-α-Syn and S87p,129p-α-Syn which showed severe toxicities, especially, in the DA/Cu^{2+} induced cases. Relatively, S129p-α-Syn showed less toxicities than S87p-α-Syn or S87p,129p-α-Syn, especially, at the monomer, tetramer and multimer levels (DA/Cu^{2+}) and tetramer level (DA). Oligomers of S129p-α-Syn showed less toxicity than S87p-α-Syn and S87p,129p-α-Syn, which means a relative protective effect of S129p-α-Syn relative to the α-Syn proteins phosphorylated at the Ser87. Another important point relates to the importance of site Ser87. Phosphorylation at 87 can induce severe toxicity effect on neuronal cells. Although cellular studies are need for more elucidation, we could know the phosphorylation





C

Lane	1	2	3	4	5	6	7
α-Syn	1	1	1	1	1	1	1
DA	0	1	1	1	1	1	1
Cu^{2+}	0	0	0	0	10	10	10
Pyridoxal 5´-phosphate	0	0	1	10	0	1	10

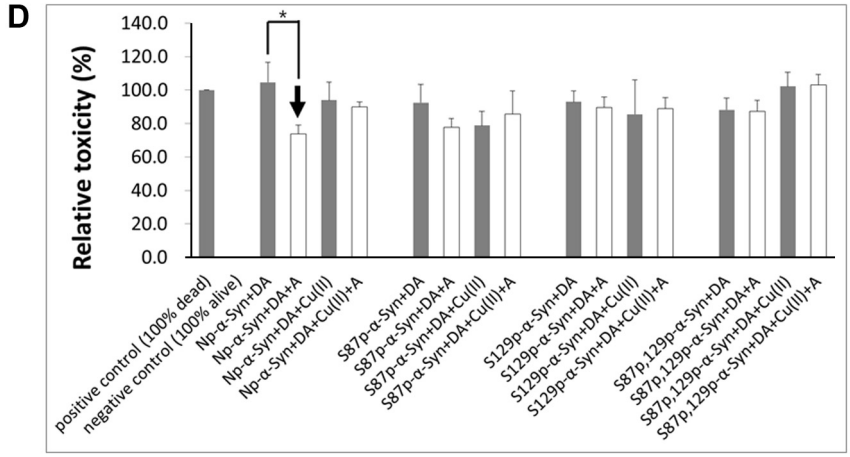


Fig. 4. SDS–PAGE result of S87p,S129p-α-Syn (A), with pyridoxal 5′-phosphate in the presence of DA and Cu^{2+}, calculated intensities (B) of the four types of α-Syn from the SDS–PAGE data, and reaction ratio (C). α-Syn (1 equiv., 7 μM). Error bar means standard deviation. Repetition 3 times. Conditions: 10 mM sodium phosphate buffer (pH 7.4), 37 °C, 14–20 h incubation. (D) Pyridoxal 5′-phosphate effect on α-Syn oligomer toxicity in SH-SY5Y cells via WST-1 assay. DMEM culture media supplemented with 20 mM glucose, 38 mM sodium bicarbonate and treated with 10% fetal bovine serum, 5% CO_2 incubation, 37 °C, 16 h. A: Pyridoxal 5́-phosphate (Additional data in ESI, Fig. S9).

at Ser 87 is an important site for cellular toxicity compared to that for Ser129.

DA is an important molecule in the context of PD research with its two reactive groups, primary amine and catechol. Although Rees et al. and other groups reported that catechol is important for reactivity [27], we focused here on its primary amine. For quenching of primary amine, it is well known that the aldehyde gives Schiff bases [15] (ESI, Fig. S10). Pyridoxal 5′-phosphate has an aldehyde group and is a natural product from human body because it is an active form of vitamin B_6 and thus it is non-toxic. When pyridoxal 5′-phosphate was treated with four types of α-Syn, inhibition effect of pyridoxal 5′-phosphate can be observed. At the ratio of 1:1:10 (α-Syn:DA:pyridoxal 5′-phosphate), aggregation was inhibited thoroughly which suggests pyridoxal 5′-phosphate can interact with DA to block aggregation mechanism. Pyridoxal 5′-phosphate more efficiently inhibits the oligomerization by DA over DA/Cu^{2+}, suggesting oligomerization by DA occurs along a different pathway to that from DA/Cu^{2+}.

Inhibition effects with pyridoxal 5′-phosphate were investigated through similar methods in Fig. 2. Each type of α-Syn was incubated with DA, pyridoxal 5′-phosphate or DA/Cu^{2+} at first. After centrifugal filtrations (Cutoff size: 3000 Da) for removal of unreacted species, e.g., DA, pyridoxal 5′-phosphate or Cu^{2+}, purified oligomers were dosed into SH-SY5Y dopaminergic neuronal cells for cellular toxicity determinations. Some decreases in toxicity occurred from mean values. A significant decrease of toxicity was shown only at the case of Np-α-Syn incubated with DA (arrow, $p < 0.05$ checked with student's t-test), whereas there were not significant differences in the other cases. From this result, we could know that pyridoxal 5′-phosphate can appear to decrease toxicity by oligomers induced by DA from interaction between DA and pyridoxal 5′-phosphate during incubation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.099.

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