Structural Basis of Membrane Disruption and Cellular Toxicity by α‑Synuclein Oligomers

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**One Sentence Summary:** Structural properties and membrane interactions of α-synuclein oligomers unravel a mechanism of neuronal toxicity.

**Abstract:** Oligomeric species populated during the aggregation process of -synuclein have been linked to neuronal impairment in Parkinson’s disease and related neurodegenerative disorders. By using solution and solid-state NMR techniques in conjunction with other structural methods, we have identified the fundamental characteristics that enable toxic -synuclein oligomers to perturb biological membranes and generate cellular toxicity, including a highly lipophilic element that promotes strong membrane interactions and a structured region that inserts into lipid bilayers and disrupts their integrity. We provide support for these conclusions by showing that mutations targeting the region promoting strong membrane interactions by α-synuclein oligomers dramatically suppress their toxicity inneuroblastoma cells and in primary cortical neurons.

The aggregation of α-synuclein (αS) into amyloid fibrils within Lewy bodies has been associated with Parkinson’s disease (PD) and a range of other debilitating neurodegenerative disorders (*1-9*). The primary pathogenic agents in these conditions are thought to be the oligomeric species populated in the self-assembly of αS, particularly through their aberrant interactions with biological membranes (*10-16*). We have investigated here two types of stabilized αS oligomers with significantly different toxicities (*17, 18*), which we designated as *type‑A\** and *type‑B\**, as their Förster resonance energy transfer (FRET) signatures (Fig. 1A) match closely those of the previously identified transient forms of non-toxic (type A) and toxic (type B) αS oligomers (*19*).

The two oligomeric forms of αS have similar sizes and morphologies (Fig. 1B-C), yet exhibit very different abilities to disrupt lipid bilayers. When incubated *in vitro* with small unilamellar vesicles (SUVs), *type‑A\** oligomers induce only a marginal release of encapsulated calcein, comparable to that induced by αS monomers and mature fibrils (Fig. 1D). By contrast, *type-B\** oligomers induce over ten times more calcein release, indicating that these oligomers generate significant disruption of acidic lipid bilayers (Fig. 1D). We observe similar loss of membrane integrity *in vivo* upon incubation of *type‑B\** αS oligomers with human neuroblastoma SH‑SY5Y cells and rat primary cortical neurons (Fig. 1E), and only marginal effects upon incubation with *type-A\** oligomers, monomers and mature fibrils of αS.

The significant disruption of synthetic and cellular membranes by *type-B\** αS oligomers, compared to *type-A\** species, was found to be strongly correlated with their ability to generate cellular toxicity. Thus *type-B\** αS oligomers, but not monomers, *type-A\** oligomers and αS fibrils, were found to reduce significantly the mitochondrial activity in neuronal cells (MTT, Fig. 1F) and to induce very significant increases of intracellular reactive oxygen species (ROS, Fig. S1A). The damage that *type‑B\** αS oligomers induce in this manner reproduces a variety of patho-physiological effects observed in neuronal models of PD obtained by inducing pluripotent stem cell-derived neurons from a patient with triplication of the αS gene (*20-22*).

To probe the structural properties of the two types of αS oligomers, we used solid-state NMR (ssNMR) spectroscopy. Correlations between carbon atoms of residues located in rigid regions of the oligomers were detected using 13C-13C dipolar-assisted rotational resonance (DARR) correlation spectra, measured using magic angle spinning (MAS). 13C-13C-DARR spectra of *type-A\** and *type-B\** oligomers revealed fundamental differences in the nature of the two species (Fig. 2A), with a significant content of β‑sheet identified in the rigid regions of the *type-B\** oligomers (Fig. 2A and S2) and negligible secondary structure content associated with the rigid regions of *type-A\** oligomers. The assignment of the 13C-13C-DARR peaks was performed using an approach (*23-25*) that combines information from solution-state chemical exchange saturation transfer (*23, 26, 27*) (CEST, see Methods and **Fig. S3**) with known assignments of fibrillar (*5*) and monomeric (*25*) states of αS, which reveal that the resonances in the 13C-13C-DARR spectra of the two types ofoligomers belong to specific regions spanning residues 3-36 and 70-88 in *type-A\** and *type-B\** species, respectively (Table S1).

Highly mobile regions of the two forms of αS oligomers were detected using INEPT measurements in MAS ssNMR (Fig. 2B). The spectra indicate that both types of oligomers are characterized by a significant number of highly mobile residues (45 in *type-A\** and 67 in *type‑B\**, Table S1), whose resonances overlap with those in the 1H‑13C‑HSQC spectra of disordered monomeric αS in solution (Fig. S4). In particular, although both oligomeric species possess a flexible C-terminal region (43 and 40 highly mobile C-terminal residues for *type-A\** and *type-B\**, respectively), the N-terminal region of αS is highly dynamical only in the *type‑B\** oligomers (0 and 26 highly mobile N-terminal residues for *type-A\** and *type-B\**, respectively). Another major difference between the two oligomeric species is that the mobile regions of the *type-A\** αS oligomers also include residues from the NAC region (Fig 2B), a finding consistent with the low FRET efficiency observed for the *type-A\** relative to the *type-B\** oligomers when the fluorophores are attached to residue 90, itself located in the NAC region (Fig. 1A).

The ssNMR characterization of the two types of αS oligomers is supported by Fourier transform infrared (FT-IR) measurements (Fig. S5), showing signals characteristic of both random-coil and β-sheet structure in the case of the *type-B\** αS oligomers, but indicating a predominantly unstructured conformation for the *type-A\** species. Further indication of the association with the core of the oligomers in *type-A\** and *type-B\** species is provided by CEST experiments (Fig. S3), which in the case of *type-A\** oligomers reveal extensive saturation in the 40 N-terminal residues of the protein, indicating a significant degree of association of this region with the core of the oligomers. In the case of *type-B\** oligomers, CEST experiments show a generally lower degree of saturation transfer throughout the sequence, with no saturation associated with either the N- or C- terminal regions of the protein or with residues of three of the four major hydrophobic segments of the αS sequence (residues 36-41, 47-56, 88-95), in contrast to the significant saturation observed for the hydrophobic segment 70-79. Finally, the identification of highly dynamical regions in the two types of αS oligomers is consistent with a dot blot analysis using antibodies targeting the N- and C- terminal regions of the protein (Fig. S6).

We next probed the interaction of the two types of αS oligomers with SUVs composed of DOPE:DOPS:DOPC lipids in a ratio of 5:3:2 (see Methods), which are good mimics of synaptic vesicles for composition and physical properties (*24, 25*). In particular, fluorescence correlation spectroscopy (FCS) in combination with confocal microscopy show that both types of oligomers bind the SUVs with high affinity, with stronger binding being observed for the *type-B\** oligomers relative to the *type-A\** species (Fig. 3A and S7). The high affinity for biological membranes of *type‑B\** αS oligomers is also shown by their strong colocalization with the plasma membrane of primary cortical neurons (Fig. 3B).

We then carried out paramagnetic relaxation enhancement (PRE) experiments using MAS ssNMR, in which small quantities of lipid molecules labeled with a paramagnetic centre (PC) were incorporated into the bilayers (*24, 25*). When the PC was located in the hydrophilic head groups of the lipid molecules (see Methods), selective quenching of a number of 13C‑13C‑DARR resonances was observed in the spectra of both types of αS oligomers (Fig. S8), indicating in both cases strong interactions with the polar head groups of the SUVs. By contrast, when the PC was positioned within the interior of the lipid bilayer, enhanced relaxation of the NMR signals in the 13C‑13C‑DARR spectra was observed only for the *type‑B\** oligomers (Fig. 3C), indicating that they can insert into the hydrophobic interior of the lipid bilayer while the *type-A\** oligomers remain bound exclusively to the membrane surface. In addition, the dynamical regions of the *type-B\** oligomers showed quenching of 1H‑13C‑INEPT resonances only in PRE experiments performed when the paramagnetic center was located in the lipid head groups (Fig. S9).

To characterize the structural nature of the regions of αS that are tightly bound to these lipid bilayers in the two types of oligomers, we measured 13C‑13C‑DARR spectra at -19 °C (Fig. 3D), a condition that enhances the protein signals at the interface with this type of acidic lipid membrane (*24, 25*). In the case of the *type-A\** oligomers, these experiments revealed a set of additional peaks having low intensities and broad linewidths, indicating the absence of a specific and well-defined region of the protein that is tightly bound to the membrane, and suggesting that the interactions involve lysine-rich segments located randomly throughout the first 97 residues of the αS sequence. By contrast for the type-B\* species these experiments revealed a set of additional resonances that match closely those of the N-terminal 25 residues of monomeric αS bound to SUVs, where this region of the protein adopts an amphipathic α-helical conformation (*12, 25, 28*), indicating that the N-terminal region in the toxic oligomers is involved in promoting strong interactions with acidic lipid bilayers.

We next examined the effects that mutations in the N-terminal region of αS exert on the ability of *type‑B\** oligomers to disrupt biological membranes and induce cellular toxicity. We chose the A30P αS variant (αSA30P) associated with an early onset form of PD, which has been shown to reduce significantly, but not completely, the membrane affinity of the N-terminal region (*24*), and also a truncated form of the protein lacking residues 2-9 (αSΔ2‑9), a key region shown to be involved in anchoring the N-terminal region of αS to lipid membranes (*25, 29*).  *In vitro* measurements of calcein release showed intermediate and negligible levels of membrane disruption by *type‑B\** oligomers formed from αSA30P and αSΔ2‑9, respectively, compared to the effects of wild type αS (αSWT) *type‑B\** oligomers (Fig. S10A). Similar trends were observed in the disruption of cellular membranes by these species (Fig. S10B) and in their colocalization with the plasma membrane (Fig. S10D-E). The partial and a total impairment of the binding and disruption of cellular membranes by *type‑B\** oligomers of αSA30P and αSΔ2‑9, respectively, is highly correlated with the levels of cellular toxicity generated upon incubation with these species (MTT and ROS, Figs. S10C and S1B).

In conclusion, we have identified two structural elements that determine the ability of toxic αS oligomers to generate permeability of biological membranes (Fig. 4, Fig. S11), which in primary neurons and astrocytes leads to an increase in basal intracellular Ca2+ and of ROS, with consequent loss of cellular viability (*20, 21*). The first structural element is an exposed highly lipophilic region of the protein in the oligomers that promotes strong interactions with the membrane surface, and the second is a rigid oligomeric core that is rich in β-sheet structure and is able to insert into the lipid bilayer and disrupt the membrane integrity. These conclusions are supported by the introduction of specific mutations to suppress partially (A30P) and completely (Δ2-9) the membrane affinity of the N-terminal sequence of the protein, which show that the ability of the accessible N-terminal region of αS to bind strongly to lipid bilayers is a vital step in enabling oligomers to disrupt both synthetic and cellular membranes and consequently to induce neuronal toxicity.

**References and Notes**

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The data supporting the findings of this study are available within the article and its Supplementary Materials.

**Supplementary Materials**

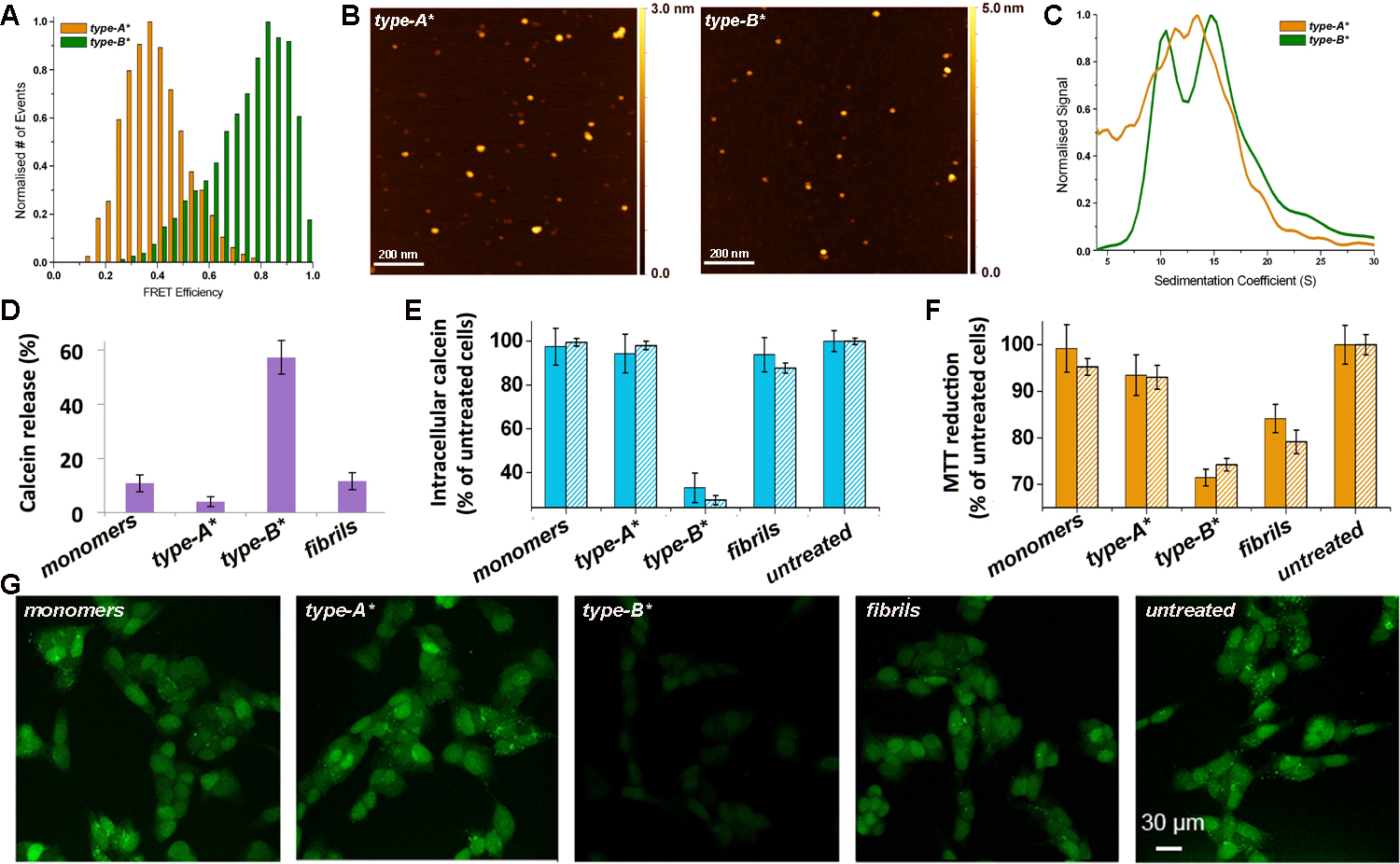
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Materials and Methods

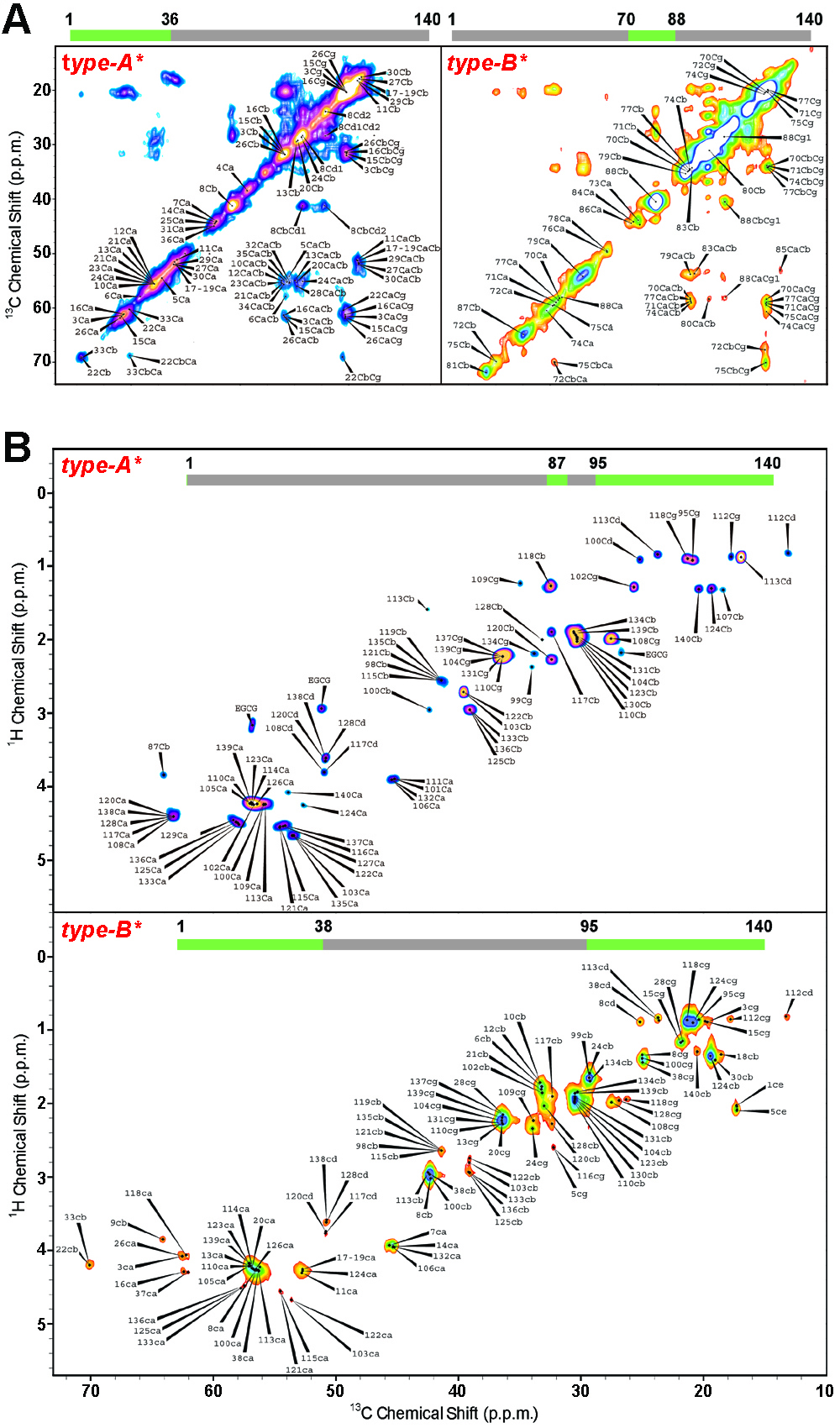
Figs. S1 to S11

Tables S1 to S2

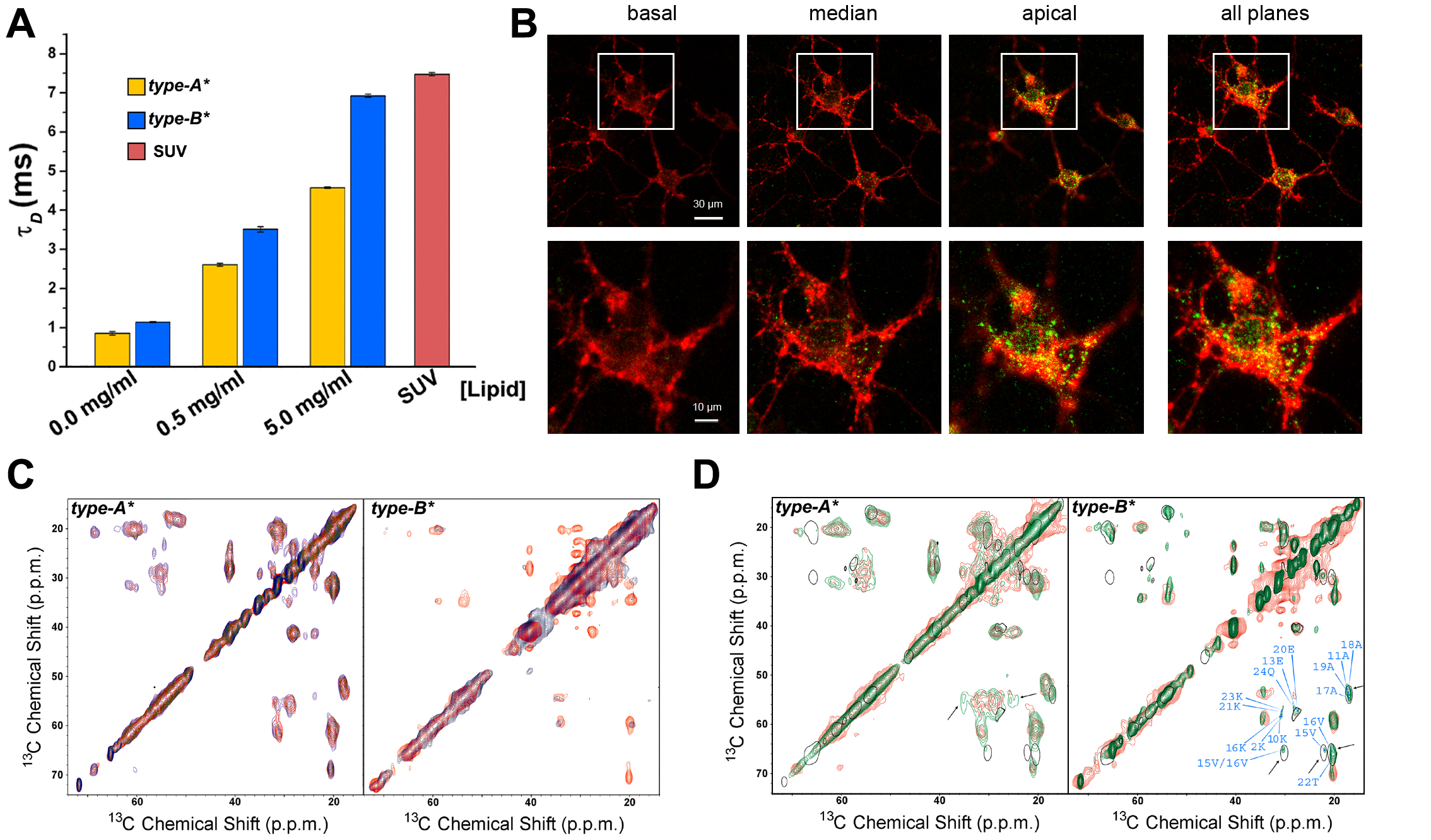
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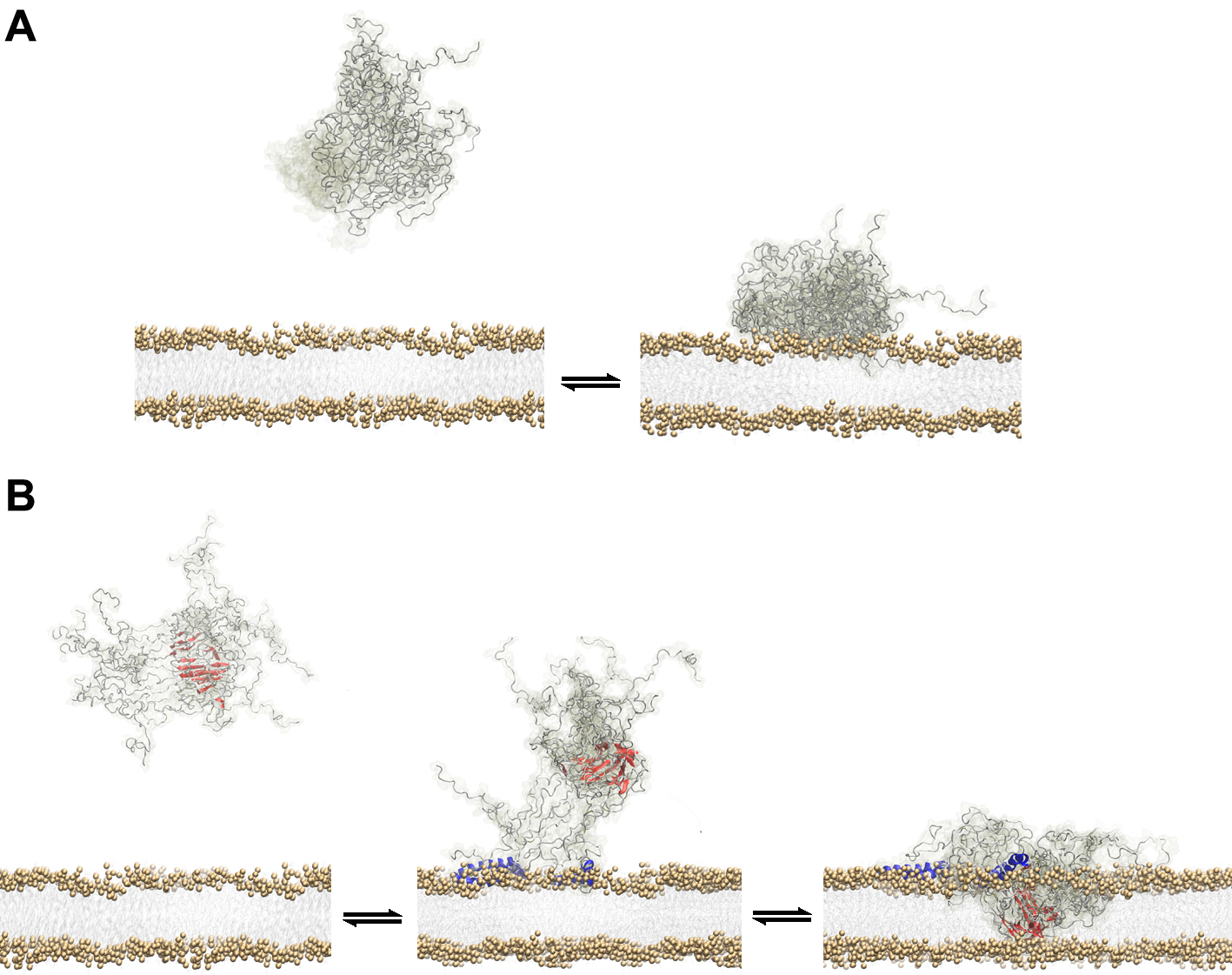
**Figure 1. Properties of the two types of αS oligomers.** (**A**) Intermolecular FRET efficiencies from measurements of *type-A\** (orange) and *type‑B\** (green) αS oligomers. (**B**) Images of the *type-A\** and *type-B\** samples of αS oligomers probed by AFM (scale bar 200 nm). (**C**) Sedimentation coefficients of the *type-A\** (orange) and the *type-B\** (green) oligomers measured using AU. (**D**) *In vitro* calcein release assay from POPS SUVs (see Methods). (**E-F**) Intracellular calcein-induced fluorescence (**E**) and mitochondrial activity monitored by the reduction of MTT (**F**) measured on human neuroblastoma SH-SY5Y cells (filled bars) and rat primary cortical neurons (striped bars) upon incubation with the various αS species. (**G**) Representative confocal scanning microscopy images of SH-SY5Y cells (scale bar 30 μm). P values for the data shown in panels D-F are reported in Table S2.

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**Figure 2.** **MAS ssNMR spectra of αS oligomers**.(**A**) 13C-13C DARR correlation spectra (aliphatic regions) of *type-A\** and *type-B\** αS oligomers (left and right panels, respectively). (**B**)1H-1**3**C correlations *via* INEPT transfer. The labels ca, cb, cg, cd and ce indicate Cα, Cβ, Cγ, Cδ and Cε atoms, respectively. The regions of the protein sequence detected in the spectra are highlighted in green in the bars at the top of the spectra.



**Figure 3. Interactions of αS oligomers with lipid bilayers**. (**A**) Diffusion times, ***τD***, from FCS experiments of *type-A\** and *type-B\** αS oligomers in the presence of variable quantities of small unilamellar vesicles of DOPE:DOPS:DOPC lipids (SUV, see Methods and Fig. S8). (**B**) Representative confocal scanning microscope images of basal, median, apical planes and their combination (fourth column) of *type-B\** αS oligomers incubated with primary cortical neurons for 15 min. Red and green fluorescence indicates the plasma membrane and the αS oligomers, respectively. (**C**) PRE effects measured using MAS ssNMR for *type-A\** (left) and *type-B\** (right) αS oligomers using SUVs with a paramagnetic centre at the position of carbon 16 of the lipid chain. 13C-13C-DARR spectra measured in the presence and absence of the paramagnetic labeled lipids are shown in blue and red, respectively. (**D**) 13C-13C-DARR spectra of isolated *type-A\** (left) and *type-B\** (right) αS oligomers (red) compared with spectra measured at -19 °C in the presence of DOPE:DOPS:DOPC SUVs (green) and of monomeric αS bound to the same type of vesicles (*24*) (black contour lines). The arrows identify the signals of residues in the rαS oligomers arising from the membrane-bound protein regions. In the case of *type‑B\** αS oligomers these regions were assigned from a previous study of the monomeric state of αS bound to SUVs (*24*).



**Figure 4. Structural basis of membrane binding of αS oligomers.** Schematic summary of the findings of this investigation (further details in Fig. S11). (**A**) The non-toxic *type-A\** αS oligomers, represented in disordered conformations using grey ribbons, bind to the surface of acidic lipid bilayers via various lysine rich segments randomly exposed on their surfaces. (**B**) The toxic *type-B\** αS oligomers feature both structured (red) and disordered (grey) regions, and bind to the surfaces of the lipid bilayers *via* the exposed N-terminal regions of αS molecules in the oligomer, which fold into amphipathic α-helices (blue) upon membrane binding, with the rigid core of the oligomers inserting into the bilayer.