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Nanoscale interrogation of α-Synuclein oligomers: Bridging biophysics, interface science, and neurobiology

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ABSTRACT

α-Synuclein (α-syn) is an intrinsically disordered presynaptic protein. In synucleinopathies, it undergoes a structural transition into β-sheet–rich conformers that promote the formation of amyloid fibrils and pathological inclusions. Although fibrillar aggregates have been studied extensively, soluble oligomers, which may have the greatest neurotoxic potential, remain poorly understood because of their transient nature and structural heterogeneity. This review critically examines α-syn oligomeric species, with particular emphasis on advanced biophysical and surface characterization techniques and neurobiological models to elucidate oligomer formation, membrane interactions, and toxic mechanisms. Recent advances in spectroscopy, high-resolution microscopy, and mass spectrometry have significantly expanded the ability to characterize α-syn oligomers and their aggregation pathways. Nanopore-based and single-molecule approaches enable the investigation of transient and structurally heterogeneous oligomeric species at the level of individual particles. Interface science has further clarified direct interactions between oligomers and lipid membranes, providing mechanistic insight into neurotoxicity and the lipid-dependent modulation of α-syn conformation and stability. Neurobiological models have revealed multi-organelle disruption, prion-like propagation, and disease subtype-specific seeding. However, no single approach fully captures oligomer pathogenicity, which emerges from the interplay of structure, interfacial behavior, and cellular vulnerability. However, existing frameworks do not adequately address this complexity. Additional barriers include poor reproducibility and limited sensitivity across approaches. Future work should integrate these technologies with standardized biological protocols, advanced artificial intelligence algorithms, and biocompatible nanomaterials. Such an interdisciplinary approach could enable the development of multiscale platforms for real-time studies of α-syn soluble conformers, with clinical utility in the management of synucleinopathies.

1. Introduction

α-Synuclein (α-syn) is an intrinsically disordered presynaptic protein that plays a significant role in the physiology of the central nervous system, particularly in regulating synaptic vesicle trafficking, neurotransmitter release, and synaptic plasticity [1,2]. As an intrinsically disordered protein, α-syn adopts highly dynamic conformations that facilitate diverse molecular interactions under physiological conditions

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[3]. Under pathological conditions associated with synucleinopathies, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA), α-syn undergoes a profound structural transition toward β-sheet–rich conformers [4]. This conformational shift subsequently promotes the formation of higher-order self-assembled aggregates, leading to the generation of amyloid fibrils and pathological inclusions. Although mature fibrils in Lewy bodies and glial cytoplasmic inclusions were long considered the principal pathological hallmark, accumulating evidence increasingly implicates soluble and metastable oligomers as key contributors to synaptic dysfunction and neurodegeneration [5].

Oligomeric species are structurally and functionally heterogeneous, forming a continuum of conformers. The physicochemical and membrane-binding properties of these oligomers vary widely across disease-relevant conditions. Environmental factors (e.g., ionic strength, pH, lipid composition, and molecular crowding) act as powerful modulators of oligomer conformation, membrane affinity, and stability [6,7]. Consequently, these factors play a significant role in shaping their pathogenic potential. Differences in oligomer size and secondary structure are accompanied by variations in compactness, solvent-exposed surface, and lipid-binding properties of these structures [8,9]. Subtle conformational differences among oligomers can substantially alter their biological activity [10–12]. Mechanistically, oligomer formation may proceed via direct monomer association (primary nucleation) or through secondary nucleation on the surface of preexisting fibrils [13]. From an interface science perspective, oligomers interact with lipid membranes with considerable avidity. This interaction is dictated by the reciprocal interplay among membrane charge, lipid composition, and curvature, leading to the formation of transient, ion-conducting α-syn assemblies that permeabilize membranes, disrupt ionic gradients, and induce calcium dyshomeostasis, thereby mechanistically linking nanoscale interfacial energetics to synaptic dysfunction [14–17].

Studying these assemblies is particularly challenging because of their intrinsic instability, low abundance *in vivo*, and pronounced structural heterogeneity [18]. Even under controlled *in vitro* conditions, small changes in experimental parameters, including agitation rate, incubation time, buffer composition, and ionic milieu, can substantially alter their structural and functional properties [19–22]. Moreover, the transient nature of oligomers means that they are often short-lived intermediates in the aggregation pathway. These can convert into fibrils or dissociate back into monomers on timescales of minutes to hours. This rapid interconversion complicates their isolation and downstream characterization. Traditional ensemble-averaging techniques, such as bulk spectroscopy or conventional electron microscopy, tend to obscure the underlying conformational diversity, which has motivated the adoption of high-resolution single-molecule and nanoscale analytical tools [23–26].

The molecular landscape of α-syn oligomers is further complicated by post-translational modifications (PTMs), which can profoundly influence aggregation kinetics, structural polymorphism, membrane binding, and seeding capacity [27]. In Lewy pathology deposits, more than 90% of α-syn is phosphorylated at serine 129 (pS129). This modification reduces α-syn membrane-binding affinity and modulates aggregation kinetics in a context-dependent manner. Specifically, pS129 can inhibit seeded fibrillization while promoting the accumulation of specific oligomeric species under conditions of oxidative stress or multisite phosphorylation [28–32]. Additional PTMs, such as nitration and glycation, can also remodel α-syn assembly, favoring soluble oligomer formation while inhibiting fibril elongation. Ubiquitination, in contrast, exerts context-dependent effects that can either promote insoluble aggregate formation or facilitate clearance and suppress oligomerization [33]. Moreover, truncation patterns can significantly affect α-syn aggregation. For instance, C-terminal truncations can remove acidic residues, thereby reducing intramolecular shielding of the hydrophobic non-amyloid-β component (NAC) domain and promoting its exposure to solvent. Such modifications accelerate seeding and cross-seeding propensity. Larger truncations generally produce shorter lag times and more heterogeneous, compact fibrils [34]. N-terminal acetylation, in contrast, is associated with enhanced N-terminal α-helicity and membrane interactions. This modification slows down lipid-dependent aggregation and fibril elongation and decreases β-sheet content in mature aggregates [34,35]. O-GlcNAcylation appears to inhibit oligomerization/aggregation and reduce α-syn fibril uptake, thereby mitigating toxicity [36,37]. Notably, PTMs, such as pS129 phosphorylation and C-terminal truncation, can coexist within seeded α-syn aggregates, jointly remodeling fibril organization and influencing protein recruitment to Lewy body-like inclusions [38]. These PTMs modulate not only aggregation kinetics and fibril/oligomer morphology but also seeding potential, thereby modulating prion-like propagation of α-syn pathology. α-Syn oligomers exhibit prion-like behavior, characterized by templated misfolding, or seeding, strain-like heterogeneity, and transcellular spread via uptake/vesicular routes, linking local nucleation events to the network-level propagation of pathology. This phenomenon underpins seed-amplification assays, including real-time quaking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA), which amplify trace α-syn seeds in specimens and thereby support diagnosis [39].

From a clinical perspective, α-syn oligomers are increasingly recognized as promising biomarkers for the early detection, prognosis, and therapeutic monitoring of synucleinopathies [40]. Therapeutically, multiple strategies are under development to mitigate oligomer toxicity or prevent oligomer formation. Small molecules and protein mimetics antagonize α-syn self-assembly [41], molecular chaperones inhibit early oligomerization steps [42], and conformation-specific monoclonal antibodies promote immune-mediated clearance [43]. These strategies have shown promise in preclinical models [41–43]. Doxycycline, for example, has been reported to reduce the β-sheet content in α-syn oligomers, thereby attenuating gliosis. Nevertheless, challenges remain, particularly in achieving sufficient blood–brain barrier (BBB) penetration and maintaining therapeutic concentrations at target brain sites [44].

Given the complexity of α-syn oligomer biology, multidisciplinary approaches integrating biophysics, interface science, and neurobiology are being developed [45]. High-resolution structural and kinetic analyses provided by atomic force microscopy-infrared spectroscopy (AFM-IR) provide insight into the molecular mechanisms of oligomer formation and transformation [46]. Further, interface science, supported by intact-cell/tissue crosslinking, in-cell fluorescence strategies, such as Förster Resonance Energy Transfer (FRET) or Bimolecular Fluorescence Complementation (BiFC), and lipid-focused analytical workflows, helps elucidate the role of lipid membranes and nanoscale surfaces in modulating oligomer stability, conformational states, and toxicity [18]. Neurobiological models, ranging from primary neuronal cultures and human iPSC-derived systems to transgenic animal systems, anchor these mechanistic findings at physiologically relevant cellular and organismal levels [45]. Advanced single-molecule and nanoscale modalities, such as single-molecule Förster resonance energy transfer (smFRET), atomic force microscopy (AFM), and nanopore sensing, provide the temporal and spatial resolution required to monitor dynamic conformational transitions and heterogeneity in individual oligomers in real time [7,47,48]. When these technologies are coupled with live-cell and *in vivo* imaging platforms, they enable direct observation of oligomer behavior, trafficking, and interactions within living systems [49,50]. Integrating nanoscale and systems-level approaches can refine understanding of the pathogenic roles of α-syn oligomers and position oligomer-focused diagnostics and interventions as key translational priorities.

Although fibrillar aggregates have been extensively studied, a consistent mechanistic and structural understanding of oligomeric assemblies has remained comparatively underdeveloped, mainly due to their heterogeneity and transient nature. Nevertheless, accumulating evidence indicates that specific oligomeric conformers may exhibit pronounced neurotoxic potential. This review integrates current

biophysical and neurobiological evidence with cross-scale experimental approaches. The discussion links the oligomer structure and interfacial behavior to cellular vulnerability and pathology. This perspective establishes the interdisciplinary framework developed throughout the review.

2. Biophysical characterization of α-Syn oligomers

2.1. Conformational flexibility and structural characteristics of monomeric and oligomeric α-syn

α-Syn is a small, acidic protein, comprising 140 amino acid residues and organized into three distinct structural domains (Fig. 1) [51,52]. The first is the amphipathic, lysine-rich N-terminal segment (residues 1–60). This segment comprises seven imperfect 11-residue repeats, each harboring a conserved KTKEGV hexapeptide motif, which facilitates α-helical secondary structure formation upon membrane binding. These amphipathic repeats are implicated in the association of α-syn with phospholipid membranes and in the disruption of lipid bilayer integrity. The second domain is the NAC segment (residues 61–95), which plays a significant role in fibril formation because of its ability to transition from a random coil to a highly aggregative, hydrophobic β-sheet structure [53]. The final domain is a highly acidic and largely unstructured C-terminal segment (residues 96–140). This region mediates interactions with other proteins, ions, polycations and polyamines, and helps protect the protein from aggregation [51]. The charge distribution across the sequence is uneven, ranging from +4 in the N-terminal region through −1 in the NAC region to −12 in the C-terminal region. This asymmetric electrostatic distribution is hypothesized to facilitate intermolecular interactions that lead to pathological aggregation. The NAC domain is considered the key driver of aggregation and forms the core of the amyloid fibril; however, aggregation of the full-length protrein also requires the participation of other domains. The extreme N-terminal residues (1−11) are involved in surface-catalyzed secondary nucleation and the recruitment of monomers into oligomers and fibrils [52,54]. Since both aggregation propensity and domain exposure are strongly influenced by sequence changes, familial PD mutations can be mapped directly onto these structural regions. To date, nine mutations (A53T, A30P, E46K, H50Q, A18T, A29S, G51D, A53E, and A53V) located in the N-terminal region have been identified as being associated with the development of PD [55]. Within the N-terminal region, two segments, namely, P1 (residues 36–42) and P2 (pre-NAC; residues 45–57), have been recognized as regulators of amyloid formation [52]. Six missense mutations (G51D, A53V, A53E, H50Q, E46K, and A53T) in the pre-NAC region are associated with the onset of PD [55]. A recently identified new mutation at position 83 (E83Q) within the NAC region can accelerate α-synuclein fibrillization and increase cellular toxicity [56]. Genetic variants associated with familial PD, such as the A53T mutation, contribute to oligomer heterogeneity, producing species with increased exposure of the hydrophobic NAC domain, elevated β-sheet content, and greater neurotoxic potency than wild-type assemblies [57].

Both the physiological functions of α-syn and its involvement in the pathogenesis and progression of PD are intricately dependent on its structure. Monomeric α-syn is an intrinsically disordered protein that lacks a stable tertiary structure [58]. Endogenous α-syn has been reported to exist predominantly as a folded tetrameric species with a molecular weight of approximately 58 kDa. An imbalance in the tetramer-to-monomer ratio can shift the equilibrium toward aggregation-prone species [59]. Although physiological α-syn predominantly adopts a random coil conformation, it assumes a partially α-helical structure upon membrane binding [58]. The high structural flexibility of the monomeric form is believed to render it susceptible to post-translational modifications, misfolding, and aggregation [60,61]. Under pathological conditions, α-syn adopts fibrillar conformations enriched in β-sheet secondary structures characteristic of amyloids [58]. This finding highlights the impact of protein conformation on its

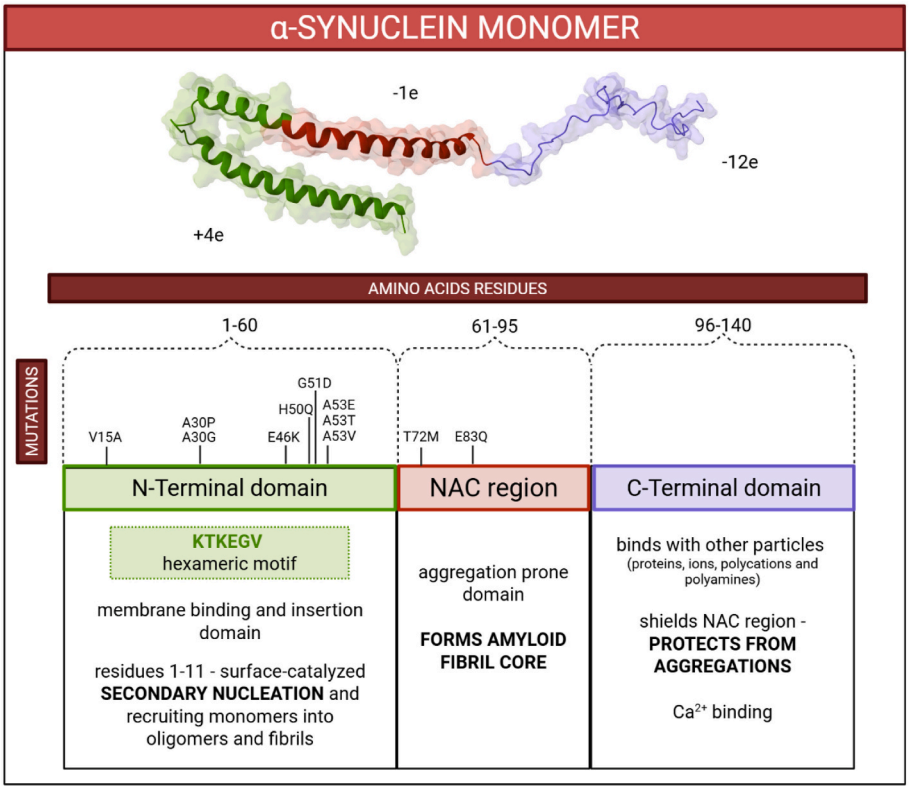


Fig. 1. α-Syn monomer structure and schematic representation of an N-terminal amphipathic repeat domain, a central non-amyloid-β component (NAC) region, and a C-terminal acidic domain.

physiological properties and toxicity. Before the conversion of monomers into highly ordered fibrils, α-syn can exist in other misfolded and toxic forms, such as amorphous aggregates, protofibrils, and oligomers. Intermediate oligomeric species are considered among the most neurotoxic entities in synucleinopathies. These species are soluble α-syn forms that have not yet acquired the structural and biophysical features typical of fibrils. α-Syn oligomers are transient, early-stage aggregates composed of multiple polypeptide chains and are formed via non-covalent protein-protein interactions. These protofibrillar forms can range from dimers and trimers to spherical, chain-like, or ring-shaped structures. α-Syn oligomers display pronounced size heterogeneity, comprising assemblies of 2–150 monomers with diameters ranging from 0.8 to 90 nm [62]. Due to their transient and metastable nature, the precise biological role of these forms remains elusive [63]. Their molecular composition is highly heterogeneous, exhibiting variability in molecular weight, hydrophobicity, and β-sheet content [64]. The proportion of β-sheet content can range from less than 20% to more than 60%, depending on several environmental factors, including pH, ionic strength, lipid composition, and the presence of specific small molecules [6,7]. The organization of β-sheet structures in α-syn oligomers differs during protein misfolding, directly affecting their biological properties and toxicity. Long-lived, protease-resistant oligomers with an antiparallel β-sheet arrangement tend to accumulate in cells and exhibit higher toxicity. In contrast, more fibrillar forms with a parallel β-sheet structure promote elongation and pathological spreading [65]. The antiparallel structure also promotes the formation of pore-like β-barrel complexes in membranes, leading to their destabilization and increased permeability [66]. The transition of oligomers from an antiparallel to a parallel arrangement is a crucial step in fibril formation. Anti-parallel forms exhibit stronger reactive oxygen species (ROS) generation and greater membrane damage, thereby increasing cellular toxicity [46]. The presence of dopamine in enteroendocrine intestinal cells can enhance nitrite-induced α-syn aggregation in enteric neurons [67]. Experimental conditions have been shown to strongly influence oligomer structure. For instance, small alterations in agitation rate, buffer composition, or ionic strength can shift the average oligomer diameters by more than 30% and β-sheet content by over 15% [19–22]. These alterations can lead to divergent biological activities in neuronal culture assays. Single-molecule and biophysical analyses indicate that differences in oligomer size and secondary structure are accompanied by variations in compactness, solvent-exposed surface area, and lipid-binding properties [8,9]. Such variability is not merely a structural curiosity; subtle conformational differences have been linked to marked differences in biological activity. For example, changes in β-sheet content or hydrophobic surface exposure can translate into two- to threefold differences in the ability of oligomers to permeabilize lipid bilayers, depolarize mitochondria, or interfere with synaptic vesicle fusion [10–12]. Modulatory small molecules, such as dopamine and norepinephrine, can stabilize distinct oligomeric conformers by preventing the formation of free radicals on the amyloid-β peptide [68].

α-Syn oligomers display considerable structural diversity, adopting either β-sheet-rich conformations or largely disordered states. Nevertheless, biophysical studies have shown that a distinguishing feature of these oligomers is a helical, hollow cylindrical morphology. Two principal classes of α-syn oligomers have been identified based on structural characteristics, namely, Type A and Type B, and functional properties, namely, on-pathway and off-pathway species (Fig. 2). Type A and Type B oligomers differ in terms of β-sheet content and toxicity. Type A oligomers are predominantly unstructured with no detectable cytotoxicity. These oligomers are primarily associated with membrane surfaces. By contrast, Type B oligomers are rich in β-sheet structures and contain a stable core capable of disrupting neuronal membrane integrity, consistent with their pronounced neurotoxic potential [5]. Further, compared with off-pathway oligomers, on-pathway oligomers serve as precursors in the formation of toxic fibrils [69,70]. Interestingly, off-pathway oligomers may actively inhibit secondary nucleation of α-syn, a critical step in amyloid formation [71]. These classification systems, namely, Type A/Type B and on-/off-pathway categories, are significantly correlated. Type A oligomers, which have low β-sheet content and their ability to dissociate into monomers, are commonly considered off-pathway species. In contrast, type B oligomers, which are rich in β-sheets and capable of inducing fibril formation, typically correspond to on-pathway species. However, each class includes a heterogeneous range of structures, and belonging to one class does not necessarily determine functionality in the other classification system.

2.2. *Mechanisms of α-syn oligomerization and fibrillization*

α-Syn monomers assume distinct conformational states depending on their context, including whether they are in solution, associated with membranes, or incorporated into dimers, oligomers, or higher-order aggregates. The highly dynamic conformation of α-syn is influenced by environmental factors, including pH, temperature, and the degree of molecular complexation. These structural changes modulate distinct aggregation pathways. Therefore, detailed atomic-level characterization of the monomer is crucial for understanding the diverse biological functions and aggregation pathways of its conformers. For instance, dimer formation represents the initial step toward the assembly of α-syn oligomers. Dimers can adopt an antiparallel configuration that facilitates subsequent aggregation. Propagation-prone α-syn dimers can polymerize by recruiting additional free monomers, thereby leading to the formation of ring-like oligomers. Molecular dynamics (MD) simulations have revealed the formation of multiple possible dimer structures from membrane-binding and β-sheet conformations of α-helical monomers [72]. The central hydrophobic region has been identified as crucial for dimerization, underscoring the significance of hydrophobic interactions in dimer-dimer binding affinity, which likely represents an early step in α-syn oligomerization. While α-helical dimers exhibit greater stability, more compact β-structured dimers can promote stronger inter-monomer interactions. These interactions drive oligomerization toward fibrillar assemblies [72]. In contrast to the elongated conformations of monomers, dimers adopt a less dynamic “dumbbell”-like conformation that evolves over time, as confirmed by AFM and theoretical discrete molecular dynamics (DMD) [17]. Monomers within dimers exhibit extensive interfacial interaction surfaces that are stabilized by contacts within the central NAC region and form β-sheet-rich segments in the hydrophobic dimer core [73]. α-Syn amyloid fibril formation is a complex process involving nucleation-dependent polymerization of α-syn in a series of interconnected stages. The three main stages are the lag phase, elongation phase, and stationary phase (see Fig. 3). During the lag phase, the native α-syn monomer undergoes a conformational change, misfolds, and forms small, aggregation-competent “seeds” or nuclei. These nuclei are the smallest kinetically stable aggregates, capable of promoting further growth by adding monomers [61]. The elongation phase is characterized by the transformation of α-synuclein nuclei into oligomers, then protofibrils, and finally fibrils. During the stationary phase, insoluble, amyloid-like fibrils form. At this stage, monomers involved in secondary nucleation or toxic oligomeric forms may be released. Based on distinct oligomer growth patterns, two primary theories describe aggregation mechanisms: (a) the nucleation-polymerization model and (b) the nucleation-conversion-polymerization model. The first model describes the transition of α-syn monomers from an intrinsically disordered state to β-sheet-rich conformations. By contrast, the second model proposes the formation of disordered oligomers via hydrophobic collapse. Both models emphasize the necessity of the primary nucleation step, followed by gradual oligomer growth into protofibrils and mature amyloid fibrils [74]. Initial oligomer formation is a kinetically hindered slow process [75]. During primary nucleation, monomers self-assemble from their native state to form soluble oligomeric nuclei in solution and at lipid interfaces [61,75,76]. Cremades et al. [77] described the formation of two major oligomer populations during *in vitro* α-syn fibrillization: initially

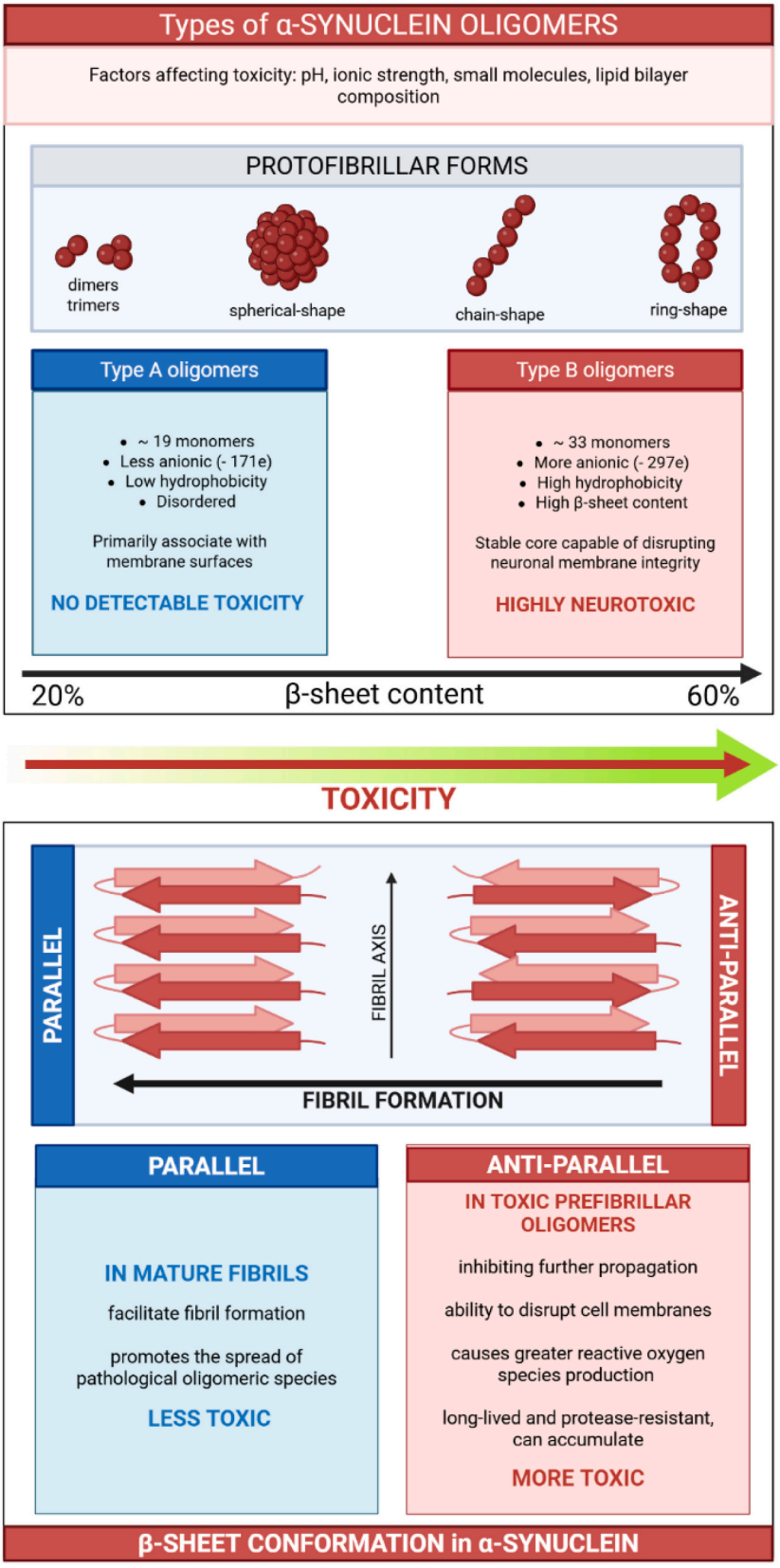


Fig. 2. Dependence of α-syn oligomer toxicity on oligomer type, morphology, β-sheet content, and β-sheet topology (parallel or antiparallel).

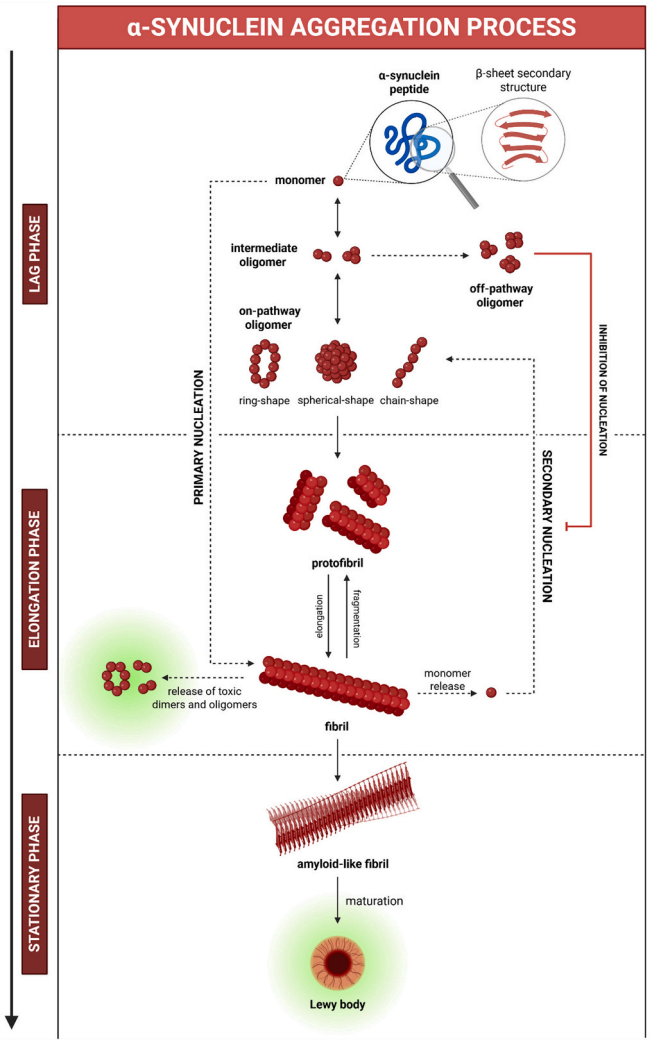


Fig. 3. The aggregation mechanism of the α-syn monomer into oligomeric, prefibrillar, and fibrillar forms, divided into three main phases (log phase, elongation phase, and stabilization phase). This process involves α-syn oligomer formation, along with pathways and oligomer types.

disordered species and later, more compact and stable β-sheet-enriched oligomers. This fibrillization process follows the nucleation-conversion-polymerization model. In this model, monomers first form loose oligomers at hydrophobic interfaces and then convert into β-sheet-rich structures. Further, these oligomers elongate by recruiting additional monomers to form fibrils [65,77,78]. The elongation phase results in linear aggregate growth through monomer addition at fibril ends. The final stage involves secondary processes, including fibril fragmentation and secondary nucleation [76], and a pathway particularly favored under near-physiological ionic strength and temperature [13]. Protofibrils and elongation-formed fibrils can harbor additional nucleation sites, facilitating secondary nucleation and promoting autocatalytic amyloid amplification. Xu et al. [54] demonstrated that under physiological pH and membrane-free conditions, secondary nucleation is the dominant mechanism driving both oligomer and fibril formation in α-syn.

2.3. *Experimental approaches for probing oligomer formation*

Experimental approaches for probing α-syn oligomer formation include a broad range of bulk, microscopic, nanoscale, and interface-sensitive techniques that provide complementary yet method-dependent insights into aggregation pathways and oligomer heterogeneity. Recent advances have improved the ability to characterize transient and structurally diverse oligomeric species with increasing spatial and temporal resolution. Together, these approaches contribute to a more comprehensive understanding of oligomer formation, membrane interactions, and mechanisms associated with toxicity and disease progression. The following sections discuss these experimental approaches in detail, together with their advantages, limitations, and applicability to the study of α-syn oligomers. A comparative overview of solution-based, microscopic, and interface-sensitive techniques, together with their principal applications, strengths, and limitations, is summarized in Table 1.

2.3.1. *Spectroscopic insights into α-syn oligomerization*

The formation of α-syn oligomers plays a key role in the pathogenesis of neurodegenerative diseases, including PD and other synucleinopathies. Understanding the structure, dynamics, and aggregation pathways of this protein requires precise and complementary analytical techniques. Different spectroscopic techniques, such as circular dichroism (CD), Fourier-transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR), are most commonly used for structural characterization and for monitoring α-syn fibrillization.

FTIR and CD are complementary methods that are widely used to assess secondary structure, monitor conformational changes, and non-invasively track protein aggregation under various environmental conditions. FTIR studies of amyloids focus on the amide I band, where signals arise mainly from carbonyl group vibrations that are sensitive to the local electronic environment and chain conformation [79]. Therefore, because β-sheet-containing structures can be readily differentiated, FTIR is used for structural analysis of α-syn monomers, oligomers, and fibrils. CD is one of the most commonly used techniques for structural characterization of α-syn during aggregation, for monitoring the kinetics of α-syn fibril formation over time, and for monitoring membrane-induced conformational changes [79–81]. Collectively, these methods consistently indicate that α-syn does not undergo a simple two-state transition from random coil to β-sheet. Instead, multiple studies support the presence of intermediate conformations, often characterized by transient α-helical content [81,82]. For example, CD spectroscopy frequently reveals a progression from disordered structures to α-helical intermediates, followed by β-sheet-rich fibrillar assemblies [82]. This pattern is supported by FTIR analyses, which similarly detect early random coil signatures that evolve into helix-containing intermediates and ultimately β-sheet-dominated structures. Importantly, the absence of a single isodichroic point in CD spectra further suggests a complex, multistep aggregation pathway rather than a direct structural conversion (Fig. 4 A,B) [82]. Despite this general agreement, the extent and stability of intermediate states appear to depend strongly on experimental conditions and sample preparation protocols. Studies of oligomeric species indicate that their secondary structure may vary among predominantly disordered, α-helical, and mixed conformations, with some reports suggesting antiparallel β-sheet arrangements distinct from fibrillar structures. These discrepancies likely reflect differences in oligomer preparation, environmental factors (e.g., membrane presence), and methodological sensitivity [66,83,84]. Notably, interactions with lipid membranes further complicate structural interpretation. Although smaller species, such as monomers and dimers, readily adopt α-helical conformations upon membrane binding, larger oligomers tend to remain structurally heterogeneous and less adaptable. This size-dependent structural plasticity may contribute to differences in biological activity, including membrane disruption and cytotoxicity [84,85].

Importantly, spectroscopic approaches can also provide a basis for distinguishing between oligomeric species with different structural and functional properties. For instance, studies have identified distinct α-syn oligomer types (e.g., type A and type B) that differ in both secondary structure and biological activity [84]. FTIR spectroscopy revealed that type A oligomers display predominantly disordered conformations similar to those of monomers, whereas type B oligomers exhibit

Table 1
Overview of solution-based, microscopic, and surface techniques applied to α-syn oligomers research.

Technique	Measurement	Strengths	Limitations	Application to α-syn	Refs
Solution-based techniques					
Fourier-transform infrared (FTIR)	Secondary structure, conformational changes	Fast, easy to perform, sensitive to β-sheet conformations	Low resolution	Monitoring structural transition during oligomerization and fibrillization. Monitoring structural changes in α-syn following interaction with lipid membranes, structural differences between A and B oligomers, and distinguishing between different types of β-structures (parallel vs. antiparallel sheets).	[66,84,91]
Circular dichroism (CD)	Secondary structure, conformational changes	Nondestructive, fast, and suitable for real-time analysis	Sensitive to light scattering and noises, limited to one protein	Secondary structure of α-syn oligomers, structural changes in α-syn following interaction. with lipid membranes	[82,83,91]
Nuclear magnetic resonance (NMR)	Atomic structure, conformational dynamics	Very high resolution and detailed structural information	Classical NMR is limited mainly to monomers and fibrils, often requires isotope-labeled sample, and is expensive	Conformations of monomers and fibrils	[86,87,91,120]
Native mass spectrometry (nMS)	Native protein and protein-complex structures, noncovalent interactions	High sensitivity and ability to detect heterogeneous populations	Lack of detailed information on secondary structure. Requires special volatile buffers	Analysis of higher-order structures, including oligomeric states (monomer, dimer, and tetramer) in near-physiological conditions, detection of early α-synuclein oligomers	[105,121]
Ion mobility spectrometry (IMS)	Separating one or several species on the basis of the ions rotationally averaged collision cross section (CCS), measurement of protein size and shape	High resolution, complements native MS by adding structural information on shape and compactness	Limited information on secondary structure	Separation of α-synuclein monomers, dimers, and trimers with identical m/z ratios, providing information on amyloid aggregate size and morphology, understanding the conformational changes during aggregation	[106,107]
Single-molecule Forster resonance energy transfer (SmFRET)	Distance and conformational dynamics of individual protein molecules	Very high resolution with nanometer precision, real-time analysis of conformational dynamics, enables real-time monitoring of aggregation kinetics	Requires labeling, limited to a small number of intermolecular distances	Real-time tracking of intramolecular distance changes for monitoring folding, conformational transitions, and the dynamic behavior of oligomers, monitoring individual α-syn oligomers. differing in size and structural type, aggregation kinetics	[47,78,96,97]
Optical tweezers	Structure and folding processes	Single molecule analysis, allows observation of dynamic, short-lived states	Lack of direct information on secondary or tertiary structure and providing only mechanical parameters, requires complex sample preparation and functionalization	Monitoring unfolding pathways of α-syn oligomers	[98,99]
Surface-sensitive and microscopy techniques					
Transmission electron microscopy (TEM)	Size, shape, and morphology	High resolution, ability to analyze population heterogeneity	Difficult and time-consuming sample preparation, including drying and staining, possibility of artifacts	Visualization of oligomers and their transition into fibrillar forms	[83,91]
Cryo-EM	Atomic-resolution structure	Atomic-level structure of α-syn species, ability to distinguish between different amyloid polymorphs, 3D reconstruction capability	Analysis of small, heterogeneous, and dynamic oligomeric species of α-synuclein remains technically challenging, and requires high-end instrumentation	Characterizing recombinant polymorphic forms of α-syn fibrils produced *in vitro*	[93,94]
Atomic force microscopy (AFM)	Size, height, volume, and surface topography	3D topography imaging, allows the analysis of amyloid growth over time, can be performed under near-native conditions, requires low sample amounts	Preparation requires immobilization of the protein on the surface, possibility of artifacts	Structural features and three-dimensional molecular architecture of lyophilized α-syn oligomers	[19,83,91]
Atomic force microscopy-infrared spectroscopy (AFM-IR)	Secondary structure at nanoscale resolution	Combines morphology from AFM with structural spectra from IR, provides nanometer-scale resolution, enables analysis of heterogeneity and different polymorphs within a single sample	Technically demanding, risk of protein conformation change after adsorption	Monitoring of the structure of α-syn oligomers present at different stages of aggregation, determining the relationship between structure and morphology at the single-oligomer level, and the evolution	[46,118,119]

(*continued on next page*)

Table 1 (*continued*)

Technique	Measurement	Strengths	Limitations	Application to α-syn	Refs
				of secondary structure of individual oligomers	
Surface plasmon resonance (SPR)	Molecular interactions, binding affinities, adsorption and interaction kinetics	Highly sensitive method for studying molecular interactions of α-syn, requires lower sample amounts than QCM-D, enables real-time analysis of adsorption and interactions kinetics	Requires immobilization of the sample on the surface, which may affect its native conformation, and poses difficulties in working with large, heterogeneous amyloid aggregates (which may clog the sensor surface)	Designing biosensors selective for toxic α-syn species, studying the affinity of α -syn to neuronal proteins, studying kinetics and interactions with lipids	[108,112,113]
Quartz crystal microbalance with dissipation monitoring (QCM-D)	Molecular interactions, viscoelastic properties	Provides real-time adsorption measurements, provides information on layer stiffness and viscosity in addition to mass, suitable for studying interactions with lipid membranes	Requires immobilization of the sample on the surface, which may affect its native conformation, adsorbed mass contains water, which makes interpretation more difficult, requires larger sample volumes	Adsorption of oligomers on model membranes, distinguishing differences in the structure and properties of the adsorbed α-syn layer depending on the surface	[109,116]
Surface-enhanced Raman spectroscopy (SERS)	Protein identification and structure	Provides information on secondary structure and conformational changes, very high sensitivity, including detection at nanomolar protein concentrations, enables tracking of early stages of aggregation	Interpretation of spectra requires advanced analysis; results may be highly dependent on the quality and homogeneity of metallic nanostructures; potential risk of artifacts related to protein interaction with the metal surface (protein may change conformation).	Characterization of α-synuclein at physiological concentration (1 μM), direct monitoring of conformational changes of α-syn in response to different membrane regions	[108,110,117]

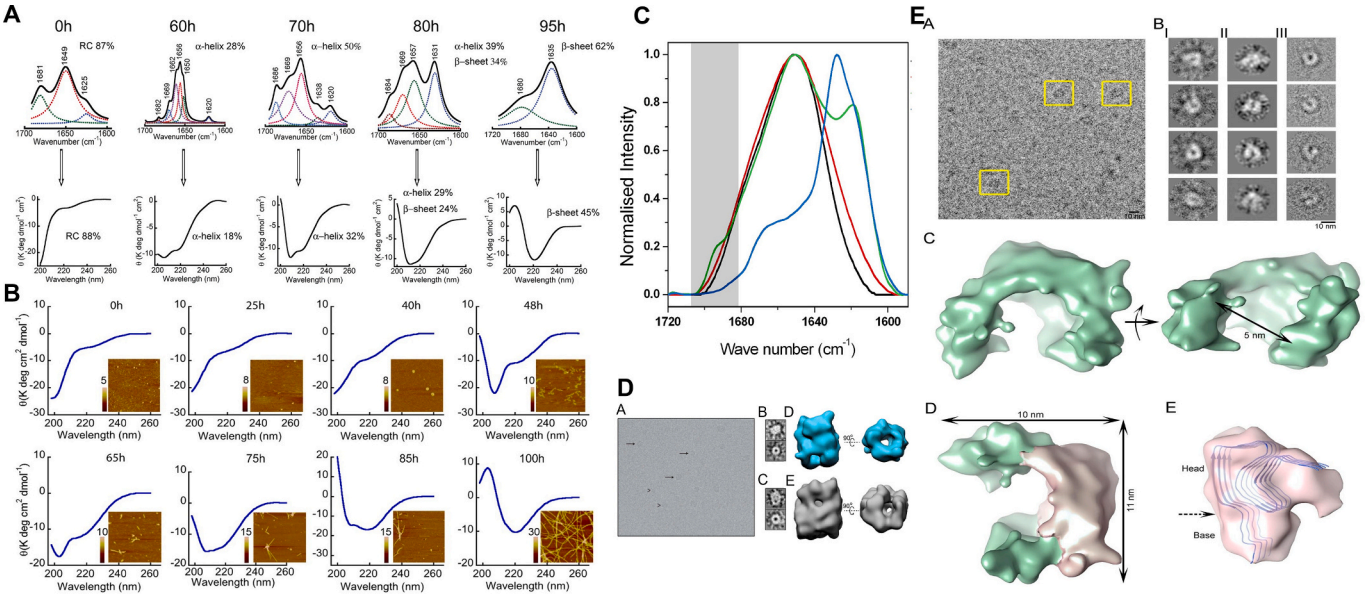


Fig. 4. Overview of spectroscopic (CD, FTIR) and microscopic (AFM, cryo-EM) characterization of α-syn oligomers. **A**. Combined FTIR and CD spectroscopy reveals stage-dependent secondary structure transitions of α-syn species during aggregation. Curve-fitted FTIR spectra in the amide I region indicate an initial random coil conformation, followed by α-helical intermediates and the formation of β-sheet–rich structures. **B**. CD spectra and AFM images of α-synuclein at various stages: early oligomers and amorphous structures, helix-rich intermediates, and mature β-sheet fibrils (100*h*). AFM images 2.5 μm; height scales indicated (A and B reproduced from Ghosh et al., *Scientific Reports*, 5, 9228 (2015), licensed under CC BY 4.0). **C**. FTIR spectra of type-A (red) and type-B (green) α-syn oligomers compared with monomer (black) and fibril (cyan). The differences between the spectra show the overlap of the bands of the non-toxic oligomer type A with those of the disordered-structure monomer. In contrast, the spectrum of type B oligomer is characterized, in addition to the same band corresponding to disordered areas, by a characteristic absorption band for the β-sheet structure and for the anti-parallel β-sheet structure (reproduced from Fusco et al., *Science*, 2017, 358(6369), 1440–1443, Copyright © 2017 AAAS). **D**. Cryo-EM images and 3D reconstructions of purified α-syn oligomers show two main subgroups: small 10S and large 15S species, with representative side and end-on views for each (reproduced from Chen, S. W. et al., *Proc. Natl. Acad. Sci. U.S.A.* 2015, 112(16), E1994–E2003). **E**. Cryo-EM and single-particle reconstruction of early α-syn oligomers formed, revealing a prominent semi-annular species. 3D density maps and docking of the tetrameric Greek key motif indicate it as the fundamental unit of oligomerization (reproduced from Chakraborty et al., *Communications Biology,* 4, 518 (2021), licensed under CC BY 4.0). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

additional β-sheet and random coil contributions, indicative of increased structural organization (Fig. 4C). These structural differences correlate

with functional outcomes, as type B oligomers show enhanced membrane-disrupting activity and increased cytotoxicity compared with type A species. This example highlights that, despite their limited resolution, CD and FTIR techniques can capture meaningful structural distinctions that are directly linked to oligomer toxicity. At the same time, it underscores the importance of careful interpretation, as such classifications may depend on preparation protocols and experimental context [84].

Taken together, CD and FTIR studies provide strong evidence for structurally diverse and dynamic oligomeric intermediates. However, their precise characterization remains challenging because of the sensitivity of α-syn assemblies to experimental conditions and the limited resolution of these techniques in distinguishing heterogeneous conformational ensembles.

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful techniques for probing the structural and dynamic properties of α-syn at residue-level resolution. Early studies demonstrated that solution NMR can detect oligomeric species and distinguish them from monomers based on signal broadening and attenuation [86]. The observed spectral changes are attributed to the presence of flexible regions within oligomeric structures, which resemble those of intrinsically disordered monomers. This behavior reflects increased conformational heterogeneity, which is readily captured by solution NMR. However, the application of solution NMR to α-syn oligomers is intrinsically limited by the transient, heterogeneous, and often sparsely populated nature of these species. As a result, NMR measurements tend to preferentially detect more dynamic and soluble conformations, potentially introducing a bias toward less aggregated oligomeric states. This limitation is further exacerbated by the short-lived nature of oligomers, which often necessitates the use of stabilization strategies, such as lipid vesicles or specific buffer conditions [87]. Although these approaches facilitate detection, they may also alter the native conformational ensemble, complicating the interpretation of structural data. To address these challenges, solid-state NMR (ssNMR) has been increasingly applied to study larger and less soluble assemblies. This technique provides detailed insights into β-sheet-rich core structures and has revealed differences between oligomeric species with distinct toxic properties, including variations in rigidity and β-sheet organization [84]. Studies of a toxic, lipid-induced prefibrillar α-syn oligomer (I1) revealed an antiparallel β-sheet structure that can reorganize into a β-arch, consistent with an early intermediate in amyloid formation [87]. This structural transition has been linked to membrane-disruptive activity and toxicity, although its characterization relies on stabilized samples because of the transient nature of the oligomer. More broadly, ssNMR provides detailed insights into β-sheet-rich core structures but typically requires highly concentrated or immobilized samples, which may favor more ordered conformations and limit the detection of dynamic or heterogeneous regions [87,88].

In-cell NMR spectroscopy provides a unique framework for studying protein structure and dynamics directly within the cellular environment, enabling the observation of proteins under physiologically relevant conditions that are not fully recapitulated by *in vitro* techniques, such as cryo-electron microscopy, X-ray crystallography, or mass spectrometry [89]. In contrast to many other structural approaches, in-cell NMR does not rely on chemical fixation or extensive modification of the cellular context and can be performed with minimal external perturbation, allowing proteins to be probed in a minimally perturbed state. These features make it particularly valuable for assessing protein conformational behavior and interactions directly in living systems [89,90]. At the same time, the application of in-cell NMR to more complex protein states remains technically demanding. The intracellular environment imposes constraints on spectral resolution and sensitivity, and the resulting data often reflect only a subset of accessible conformational states. In this context, the characterization of heterogeneous or transient assemblies remains challenging, and their structural properties in cells remain difficult to define using this approach alone. Nevertheless, ongoing methodological developments continue to expand the capabilities of in-cell NMR and are expected to improve its applicability to increasingly complex protein systems [90].

Spectroscopic techniques provide key insights into α-syn oligomer structure and dynamics. However, their interpretation is inherently limited by ensem17ble averaging and sensitivity to experimental conditions. In the case of NMR, additional constraints arise from molecular mobility and sample heterogeneity. Moreover, these approaches often require protein labeling, and the characterization of oligomers frequently relies on their stabilization, which may affect their native properties. Importantly, variations in oligomer preparation protocols can significantly influence their structural properties, complicating direct comparisons across studies and making it difficult to draw unambiguous conclusions. Consequently, no single method can fully resolve the structural diversity of oligomeric species. Nevertheless, combining complementary spectroscopic techniques enables a more comprehensive characterization, allowing the limitations of individual methods to be mitigated. Ultimately, integrating these approaches with advanced imaging techniques, such as cryo-EM and AFM, provides a more complete structural and morphological understanding of α-syn oligomers.

2.3.2. *Imaging of α-syn oligomer structures using TEM, AFM, and cryo-EM*

A range of advanced microscopic techniques, including transmission electron microscopy (TEM), cryogenic electron microscopy (cryo-EM), and atomic force microscopy (AFM), is employed to visualize oligomers, fibrils, and higher-order aggregates of α-syn. These methods provide complementary insights into the structure, size, aggregation state, and morphology of oligomeric species [91]. TEM and AFM studies consistently indicate that oligomeric species predominantly exhibit spherical or globular morphologies, in contrast to the elongated filamentous structures observed in fibrils [83]. However, more detailed analyses combining TEM and cryo-EM have revealed additional structural heterogeneity, including distinct oligomer populations, such as “donut-like” and cylindrical forms, suggesting different organizational states along the aggregation pathway (Fig. 4D) [83]. Imaging studies further support the existence of structural transitions during aggregation. For example, AFM combined with Raman spectroscopy has demonstrated the conversion of spherical, α-helical-rich oligomers into β-sheet-enriched fibrillar structures, highlighting the dynamic and cooperative nature of α-syn assembly [19]. Recent advances in liquid-cell transmission electron microscopy (LC-TEM) have extended conventional imaging by enabling the visualization of biological specimens in a hydrated, near-native environment. By encapsulating liquid samples within microfabricated cells, LC-TEM allows direct observation of dynamic processes, such as oligomer formation and growth in solution [92]. This represents a key advantage over conventional TEM, which is limited to dehydrated and static samples. However, the spatial resolution of LC-TEM remains lower than that of solid specimens using TEM because of electron scattering in the liquid layer and beam-induced effects, which can limit the level of structural detail obtained [92]. Despite these constraints, LC-TEM offers a promising complementary approach for probing the dynamic behavior of protein assemblies, including α-syn oligomers, and is expected to play an increasingly important role in future studies of aggregation processes.

In recent years, cryo-EM has advanced significantly in both achievable resolution and image analysis methodologies. Cryo-EM has enabled high-resolution three-dimensional (3D) reconstruction of α-syn fibrils in their native, frozen-hydrated state. Studies over the past decade have revealed a wide variety of polymorphic filamentous forms of α-syn, including recombinant fibrils and fibrils extracted from human brain tissue [93,94]. Cryo-EM is widely used to study mature fibrils. However, analyzing small, heterogeneous, and dynamic α-syn oligomers remains technically challenging and requires further methodological advances. Nevertheless, recent cryo-EM studies have begun to resolve oligomeric intermediates, suggesting that specific structural motifs, such as ring-like or half-ring assemblies, may represent early stages of fibril

formation [95]. Beyond morphological classification, cryo-EM combined with single-particle reconstruction enables 3D structural analysis of α-syn oligomers (Fig. 4E). Using this approach, half-ring oligomeric forms have been proposed as precursors to ring-shaped assemblies, and a tetrameric Greek key motif, in which core residues form parallel β-sheets arranged in a Greek key topology at the C-terminus, has been suggested to represent a fundamental structural unit of α-syn oligomerization [95].

Imaging techniques provide essential information on the morphology, size, and structural organization of α-syn assemblies, enabling the direct visualization of oligomeric and fibrillar species. However, their interpretation is limited by sample preparation procedures, potential artifacts, and, in some cases, restricted resolution or sensitivity to dynamic and heterogeneous states. In particular, the characterization of transient oligomeric species remains challenging because of their structural variability and instability. Consequently, no single imaging approach can fully capture the complexity of α-syn oligomers. Combining complementary microscopy techniques allows these limitations to be mitigated by linking morphological observations with higher-resolution structural information. Integrating these methods with spectroscopic techniques is therefore essential for achieving a comprehensive characterization of α-syn oligomers.

2.3.3. Single-molecule tools: smFRET, optical tweezers

Resolving the structural diversity and formation kinetics of α-syn oligomers requires approaches that can overcome the limitations of ensemble averaging. Recently, single-molecule techniques have been developed to enable the observation of individual molecular species in solution, providing new insights into the fundamental processes underlying α-syn oligomerization [78]. In particular, single-molecule Förster resonance energy transfer (smFRET) provides nanometer-scale sensitivity to conformational changes and enables real-time monitoring of intramolecular distance fluctuations [96]. This capability makes smFRET particularly well suited for investigating protein folding, aggregation, and the emergence of transient, structurally heterogeneous intermediates at different stages of the aggregation process [47,78]. smFRET studies have revealed that α-syn oligomers exist as structurally distinct populations rather than a single homogeneous species [78]. Multiple oligomer types differing in size and conformation have been identified, supporting the concept of a heterogeneous oligomer ensemble. Moreover, kinetic analyses indicate the presence of distinct aggregation pathways, including sequential transitions between oligomer populations, suggesting that structurally defined intermediates may play specific roles in fibril formation [78]. These findings are further supported by studies demonstrating that different α-syn variants exhibit distinct oligomerization behaviors, particularly in the early stages of aggregation. Two major FRET populations were consistently observed, corresponding to type A and type B oligomers. The larger structures can be distinguished by their FRET signatures. In wild-type α-syn, the species observed during the lag phase were identified as type A oligomers. In contrast, the A53T and A30P variants predominantly formed dimers and type A oligomers, with later emergence of type B oligomers. The A30P variant displayed slower conversion to type B, whereas A53T converted more rapidly. These differences suggest distinct early aggregation mechanisms among variants, potentially influencing PD pathogenesis [47]. Moreover, fluorescence-based single-molecule approaches have been applied not only to investigate the behavior and dynamics of α-syn oligomers but also to enable their selective detection, highlighting their potential in diagnostic applications. For example, a FRET-based aptasensor has been developed for the detection of α-syn oligomers with nanomolar sensitivity [97]. Despite their many advantages, smFRET approaches also present limitations, including the requirement for site-specific labeling and the potential perturbation of protein structure or interactions. In addition, data interpretation remains complicated by fluorophore behavior and the need for careful calibration of FRET efficiencies. In complex biological environments, fluorescence signals may also originate from other protein species. Therefore, careful discrimination of background signals and contributions from other components is required for accurate data interpretation. Although these limitations highlight the challenges associated with fluorescence-based approaches, other single-molecule techniques offer complementary insight into α-syn oligomers by probing their mechanical properties. Optical tweezers represent a single-molecule approach for probing the mechanical properties of α-syn oligomers by enabling the manipulation of individual protein molecules using focused laser beams. This facilitates the measurement of forces associated with unfolding, folding, and intermolecular interactions. Studies using this technique indicate that even small oligomers can adopt multiple metastable conformations, reflecting their structural heterogeneity. Moreover, the mechanical stability of these assemblies increases with oligomer size, and their formation occurs rapidly in a size-dependent manner [98]. Integrative approaches combining single-molecule experiments with computational modeling have provided additional structural insights. In particular, β-sheet-rich dimeric conformations have been identified as potential early intermediates in the aggregation pathway, representing defined structural motifs that may serve as targets for therapeutic intervention [99].

Because of their ability to distinguish between heterogeneous and transient oligomeric states of α-syn, single-molecule techniques are not only a valuable complement to conventional methods but also represent essential tools in modern aggregation research. In recent years, these approaches have gained increasing prominence in the scientific literature. They offer unique insights into the structure, dynamics, and toxicity of early aggregation intermediates that are inaccessible using classical methodologies.

2.3.4. Nanopore technologies for detection of α-syn oligomers

Nanopore-based detection of protein oligomers provides precise, fast, and scalable measurements comparable to those obtained using the most advanced single-molecule methods. Its full potential becomes evident when it is combined with innovations such as DNA-labeled nanostructures, machine learning-assisted signal analysis, and next-generation detection platforms [100]. Integration of these components can significantly accelerate the development of nanopore-based diagnostics, making this approach a practical tool for early detection of diseases such as PD by identifying toxic α-syn oligomers. Nanopore detection can be performed using label-free or label-based approaches. In the case of α-syn oligomers, label-free detection allows direct analysis of individual molecules based on their inherent physicochemical properties, such as mass, size, or conformation. This approach enables the study of aggregate heterogeneity. Label-based approaches involve chemical or biological modification of the analyte to improve sensitivity and selectivity, typically by adding fluorescent tags, enzymes, or nanoparticles. However, labeling α-syn monomers may alter their conformation and aggregation kinetics [7]. Recent advances demonstrate that nanopore-based approaches enable the detection of α-syn oligomers at low concentrations in complex biological fluids, including cerebrospinal fluid [7]. Strategies combining nanopores with DNA nanocarriers functionalized with α-*syn*-specific aptamers have further improved selectivity toward soluble oligomeric species. Functional modifications of solid-state nanopores, such as silicon nitride (SiN) systems functionalized with surfactants, allow stable and continuous translocation of α-syn without the need for labeling (Fig. 5A). These approaches enable the identification of multiple oligomer populations based on differences in residence time and blockade amplitude, reflecting variations in size and conformation (Fig. 5B). As a result, nanopore sensing allows quantitative monitoring of oligomerization processes in real time and provides insight into factors influencing aggregation, such as lipid interactions [101]. Furthermore, advanced nanopore platforms have demonstrated the ability to resolve multiple oligomer subpopulations with distinct structural properties, enabling the size-based identification and quantification of discrete α-syn species (Fig. 5C, D). Analysis of ionic current signals enabled the identification and quantification of toxic

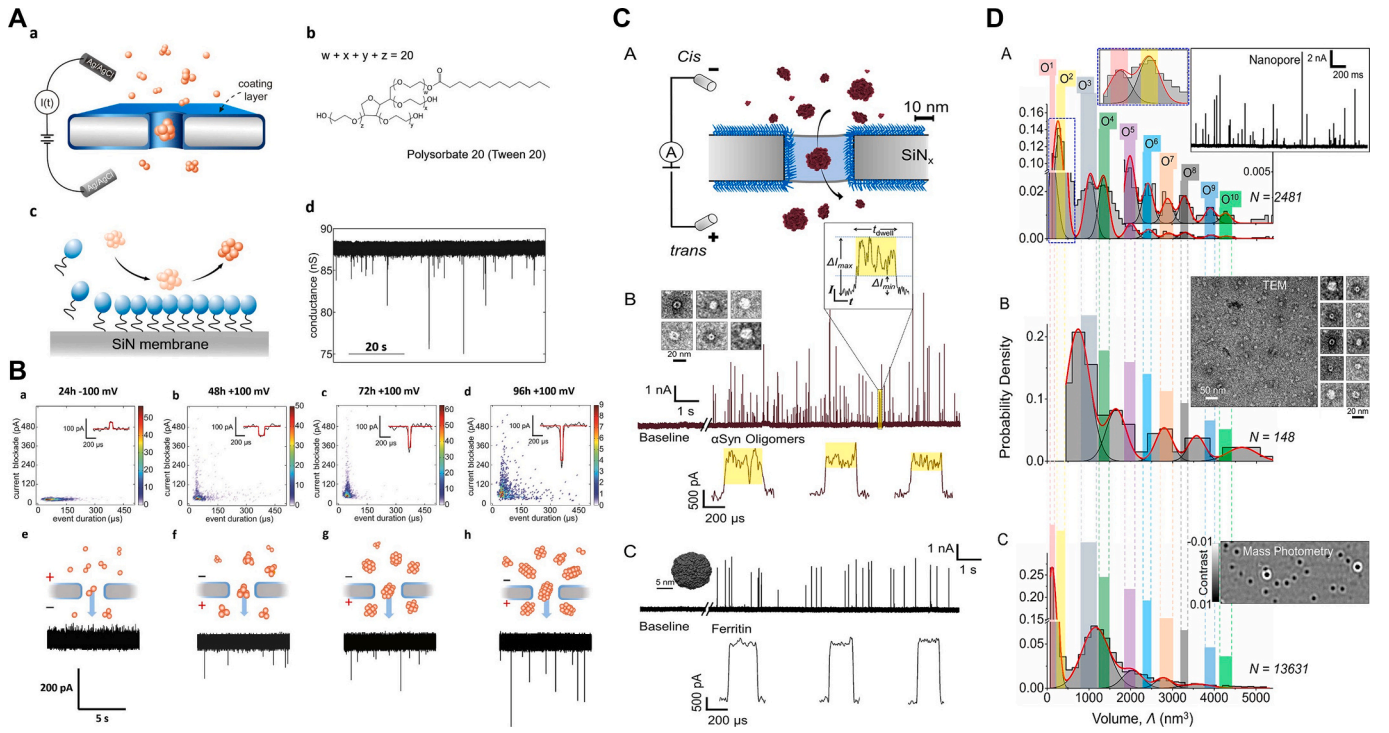


Fig. 5. Representative applications of nanopore technology for the detection of α-syn oligomers. **A**. Schematic illustration of the experimental setup for α-syn detection. The flow cell is divided by a silicon nitride membrane containing a single nanopore. To minimize nonspecific adsorption of α-syn oligomers, the membrane surface is passivated with a Tween 20 layer that adsorbs onto the hydrophobic silicon nitride surface, forming a compact coating. Upon the application of a 100 mV voltage, the electric field drives α-syn translocation through the nanopore, resulting in characteristic transient blockades in the ionic current traces. **B**. Time-dependent incubation density histograms for translocation events of samples containing α-syn. As a result of the aggregation of α-syn monomers over time, deeper translocation events occur, corresponding to an enhanced signal attributed to oligomeric species (A and B adapted from Hu et al., *Scientific Reports*, 6, 20776 (2016), licensed under CC BY 4.0). **C**. Schematic illustration of the experimental setup for detecting single α-syn oligomers during their translocation through polymer-coated nanopores. The recorded current traces, before and after oligomer addition, show characteristic resistance pulses corresponding to translocation events, whose amplitudes are proportional to the particle volume. Current modulations within individual events reveal the shape and orientation of non-spherical oligomers, as confirmed by TEM imaging. **D**. Distribution of α-syn oligomer sizes determined by nanopore recording, TEM imaging, and single-particle mass analysis using mass photometry (C and D adapted from Awasthi et al., *ACS Nano*, 17, 13 (2023), licensed under CC BY 4.0).

α-syn oligomers (10S, 15S) at ultralow concentrations, namely, in the pico- to nanomolar range. The ability to rapidly detect such species in solution without labeling underscores the potential of this technology as a sensitive, efficient, and accessible diagnostic platform [102]. The successful detection of α-syn oligomers at extremely low concentrations confirms the high sensitivity of solid-state nanopores. However, biological nanopores also offer key advantages, including consistent structural reproducibility and reduced fouling or clogging by analytes [100]. However, biological pores typically have narrow diameter ranges (1–4 nm), which can limit their applicability for detecting larger oligomers [103]. For instance, α-hemolysin-based biological nanopores exhibit transient current blockades when β-amyloid monomers translocate through the pore. By contrast, larger oligomers exceeding the pore diameter generate non-translocation "bumping" events (characterized by short durations and shallow blockade amplitudes), providing a basis for detection [104]. Thus, although biological nanopores currently have more limited applicability for α-syn oligomer detection than solid-state systems, their high reproducibility, resistance to clogging, and engineering flexibility make them a promising platform for future studies of protein aggregation.

2.3.5. Mass spectrometry: native MS and ion mobility analysis for structural and compositional profiling

Mass spectrometry (MS) is gaining increasing importance in bioanalysis because of its high resolution and sensitivity, even when only small sample volumes are available [105]. In conventional MS, samples are typically analyzed under denaturing conditions, such as high voltage, elevated temperature, low pH, or organic solvents. These conditions lead to the disruption of noncovalent interactions and protein denaturation [105]. In contrast, native mass spectrometry (nMS) employs near-physiological conditions, preserving native protein structures and non-covalent interactions with ligands, cofactors, or metal ions. As a result, nMS enables the analysis of higher-order structures, including oligomeric states, protein complex composition, protein–protein and protein–ligand interactions, and conformational dynamics and complex stability under varying environmental conditions. Ion mobility spectrometry (IMS) provides complementary structural information by separating ions based on their shape and charge, enabling the distinction between α-syn species with similar mass-to-charge ratios. The combination of nMS and IMS (IM-MS) therefore allows the resolution of structurally distinct oligomeric forms and provides insights into aggregate size, morphology, and conformational heterogeneity. These approaches have been successfully applied to investigate ligand binding and its influence on α-syn aggregation. Metal ion interactions have been shown to modulate aggregation kinetics, whereas binding of small molecules can induce conformational changes associated with inhibition of oligomer and fibril formation. In particular, ligand-induced compaction of α-syn structures has been observed, suggesting a potential mechanism for modulating aggregation pathways [106,107]. Overall, native MS and IM-MS provide a powerful platform for analyzing α-syn oligomerization and its modulation by external factors while maintaining near-native conditions. These techniques offer valuable insights into the molecular basis of amyloid formation. Moreover, these approaches provide a useful platform for designing and evaluating therapeutic strategies to prevent α-syn aggregation, including both fibril formation and the generation of toxic oligomer intermediates.

2.3.6. Interface-sensitive techniques: SPR, QCM-D, SERS, and AFM-IR

Surface-sensitive techniques, such as surface plasmon resonance (SPR), quartz crystal microbalance with dissipation monitoring (QCM-D), surface-enhanced Raman spectroscopy (SERS), and atomic force microscopy-infrared spectroscopy (AFM-IR), provide complementary insights into α-syn oligomer interactions at interfaces. These approaches enable real-time monitoring of binding events, structural transitions, and membrane-associated processes that are not accessible through bulk measurements [108–111]. SPR is widely used for quantitative analysis of the binding affinity and kinetics of α-syn oligomers toward ligands, receptors, and antibodies [108]. These studies demonstrate that oligomer binding is strongly conformation-dependent and influenced by surface immobilization, which can modulate interaction strength compared with solution conditions. In particular, aptamer-based SPR assays have shown selective recognition of oligomeric species, with high sensitivity reaching the picomolar range, highlighting their potential for detecting structurally distinct α-syn assemblies (Fig. 6A) [112]. SPR has also been applied to assess the affinity of α-syn oligomers toward neuronal proteins, identifying potential interaction partners involved in oligomer-mediated toxicity [113], while receptor-targeted studies further emphasize the complexity of cellular uptake mechanisms [114]. Complementary to SPR, QCM-D provides additional information on mass adsorption and the viscoelastic properties of interfacial layers, enabling differentiation between rigid and soft protein layers [115]. The combined use of SPR and QCM-D is particularly valuable, as these techniques probe distinct aspects of interfacial interactions, including differences in hydration and the mechanical properties of the adsorbed layer. QCM-D studies demonstrate that α-syn interacts preferentially with negatively charged lipid membranes, inducing structural rearrangements and membrane remodeling (Fig. 6B) [109]. These measurements further reveal that protein conformation, orientation, and interaction strength depend strongly on surface chemistry and charge [109,116]. To date, most QCM-D studies of α-syn have focused on monomers and fibrils. Analysis of oligomers remains limited because of their instability, heterogeneity, and the need for relatively large sample amounts, which hinder reproducibility. QCM-D could be combined with advanced methods for stabilizing and isolating oligomers to enable more detailed studies of oligomer interactions with surfaces and improve understanding of toxicity mechanisms in neurodegenerative diseases. Extending these insights, SERS enables highly sensitive detection of α-syn and provides structural information on transient oligomeric species at low concentrations [110]. Importantly, SERS-based platforms allow the investigation of protein-membrane interactions in model lipid systems, revealing environment-dependent conformational transitions, including α-helix formation and β-sheet enrichment, depending on lipid composition [117]. These findings highlight the role of membrane interfaces in modulating early aggregation events and oligomer structure. At higher spatial resolution, AFM-IR enables nanoscale mapping of secondary structure, providing direct insights into the structural heterogeneity of α-syn oligomers at the level of individual assemblies. Studies using this approach reveal progressive conformational changes during aggregation, including transitions from α-helical and disordered

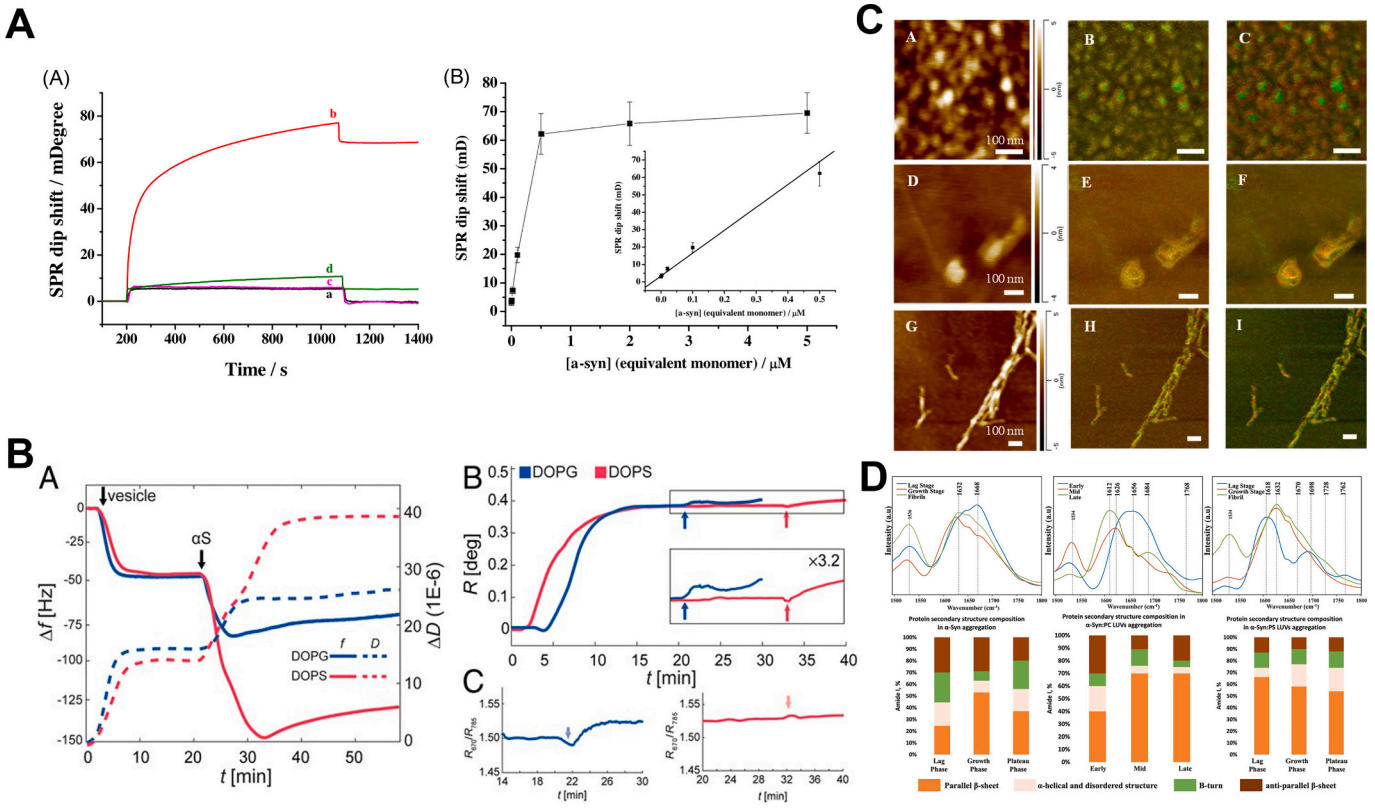


Fig. 6. Overview of surface-sensitive techniques (SPR, QCM-D, and AFM-IR) used to characterize interactions of α-syn oligomers. **A**. SPR sensograms after injection of α-syn monomer, oligomer, and fibril before and after sonication, and the dependence of SPR dip shift on α-syn oligomer concentration. Curve c) corresponds to the α-syn oligomeric form and proves its selective binding to the thiolated aptamer immobilized on SPR sensor chips (reproduced with permission from Sun et al., *Sensors and Actuators B: Chemical,* 2017, 245, 87–94. Copyright © 2017 Elsevier). **B**. Time-resolved changes in mass and structural properties induced by the binding of α-syn to surface-bound DOPG and DOPS vesicles, monitored in real time using QCM-D and SPR (reproduced from Hannestad et al., *Biophysics and Computational Biology*, 117(25), (2020), licensed under CC BY 4.0). **C**. Monitoring of α-syn aggregation during the delay phase (A–C), growth phase (D–F), and late stage (G–I) using the AFM-IR technique. **D**. AFM-IR spectra and amide I analysis of α-syn aggregates with or without PC/PS lipids (α-syn, left; α-syn:PC, center; α-syn:PS, right). Results represent changes in secondary structure, revealing contributions of parallel β-sheets, α-helix/unordered regions, β-turns, and antiparallel β-sheets and how lipids influence α-syn organization (reproduced from Dou et al., *ACS Chemical Neuroscience*, 14, 17 (2023), licensed under CC BY 4.0).

structures to β-sheet-rich arrangements, with both parallel and antiparallel β-sheet organizations observed at different stages [46,111]. Notably, AFM-IR data indicate that antiparallel β-sheet structures are more prevalent in early oligomeric forms, whereas later-stage aggregates are dominated by parallel β-sheet configurations, reflecting structural maturation during fibril formation. Importantly, AFM-IR has demonstrated that lipid environments strongly modulate these structural transitions. Differences in phospholipid composition, such as phosphatidylcholine (PC) versus phosphatidylserine (PS), influence oligomer size, morphology, and secondary structure, with negatively charged lipids promoting the formation of larger, more ordered aggregates (Fig. 6C, D). In addition, the protein-to-lipid ratio plays a critical role in determining oligomer structure and aggregation pathways [118,119]. These findings highlight the sensitivity of α-syn oligomers to interfacial conditions and underscore the importance of membrane composition in shaping their structural and functional properties. Interface-sensitive techniques provide unique insights into α-syn interactions at surfaces, enabling real-time monitoring of binding events, membrane-associated aggregation, and associated structural transitions under controlled conditions. Importantly, the outcomes of these measurements are highly dependent on experimental parameters, including oligomer preparation protocols, surface functionalization, and lipid composition. Variations in membrane charge, curvature, and physicochemical properties can significantly influence α-syn behavior, making direct comparisons between studies challenging and emphasizing the need for careful standardization of experimental conditions (refer to Fig. 7).

3. Interface science: oligomer interactions with nanostructures and lipid membranes

Interactions of α-syn with biological and synthetic interfaces can profoundly affect its structure, stability, and propensity to aggregate. Such interfaces may induce conformational changes, sequester specific regions of the protein, or shift the equilibrium among its monomeric, oligomeric, and fibrillar forms. Understanding how these surfaces modulate α-syn behavior is essential for elucidating the molecular mechanisms underlying its toxicity and for advancing both diagnostic and therapeutic strategies.

3.1. Interaction of oligomeric α-syn with nanomaterials

Nanoparticles can significantly modulate the aggregation behavior of α-syn, depending on their physicochemical properties, including size, charge, concentration, and chemical composition. Some nanoparticles mimic the action of lipid membranes, whereas others disrupt membrane functions by influencing the kinetics and mechanisms of protein aggregation. Understanding α-syn nanomaterial interactions is crucial not only for clarifying the protein's behavior but also for developing novel diagnostic and therapeutic approaches for synucleinopathies. Collectively, available studies indicate that nanoparticles modulate protiens aggregation through surface-dependent interactions that alter protein conformational equilibria, including adsorption-driven structural rearrangements, stabilization of aggregation intermediates, and modulation of β-sheet formation [122]. The resulting effects depend strongly on nanoparticle physicochemical properties such as surface charge, hydrophobicity, curvature, and chemical composition. Certain nanoparticles, such as gold nanoparticles (AuNPs) and silica nanoparticles (SiO_2-NPs), can accelerate aggregation [123]. By contrast, other nanoparticles, such as charged graphene oxide (GO) and graphene quantum dots (GQDs), exhibit inhibitory effects and can even disassemble preformed α-syn fibrils [124–126]. Further, SiO_2-NPs, widely used in diagnostics and therapies targeting the central nervous system, can penetrate dopaminergic neurons. These NPs can disrupt protein degradation pathways, including the autophagy-lysosome pathway (ALP) and the ubiquitin–proteasome system (UPS). These disruptions lead to oxidative stress and impaired proteasomal function, thereby promoting pathological α-syn aggregation and neurotoxicity. and ultimately PD pathogenesis [127]. Similarly, AuNPs can enhance α-syn aggregation through binding to the protein's N-terminus [125]. This binding leaves the hydrophobic NAC region exposed and promotes fibril formation. However, surface modification of AuNPs with polyphenolic compounds (such as naringenin) can suppress α-syn conformational fluctuations and inhibit its transition into a cross-β-sheet-rich structure [128]. Mohammad-Beigi et al. [124] investigated the concept of disrupting α-syn self-assembly, primarily by electrostatic interactions. Both positively and negatively charged surfaces, including graphene layers and superparamagnetic iron oxide nanoparticles, first interact with the charged termini of α-syn, followed by interaction with its hydrophobic NAC region. These nano-objects effectively inhibited all stages of fibril formation and disassembled preexisting amyloid fibrils. Their inhibitory efficacy can be attributed to high surface charge density and strong affinity for highly charged α-syn [124]. The anti-aggregation activity of GO was further confirmed by Malik et al. [125]. In this case, GO strongly interacts with α-syn by electrostatically and hydrophobically sequestering the NAC region [125]. This process reduces the availability of free monomer in solution, thereby impeding fibril elongation. A similar monomer-sequestering effect and fibrillation delay were observed with zinc oxide nanoparticles (ZnO NPs) [124]. Surface potential studies revealed that positively charged ZnO NPs primarily interact with the N-terminal region of α-syn. By contrast, negatively charged ZnO NPs functionalized with amino acids (such as tryptophan or tyrosine) preferentially target the NAC domain. This shift in binding interface significantly suppresses fibril formation by stabilizing the protein in a nanoparticle-bound monomeric state [129]. Overall, these findings identify nanoparticle surface properties as key determinants of α-syn aggregation. Nanoparticles can promote or inhibit fibrillation through diverse mechanisms, including electrostatic interaction, hydrophobic binding, and conformational stabilization. Future research should focus on designing functional nanointerfaces with controlled surface charge and selective affinity for defined protein domains [130]. Such precision surface engineering holds promise not only for elucidating aggregation mechanisms but also for developing targeted therapeutic strategies. However, a critical challenge remains in terms of enabling efficient nanoparticle transport across the BBB, which is a prerequisite for any potential therapeutic application.

3.2. Interaction of oligomeric α-syn with lipid membranes

Neurodegenerative disorders, such as PD, are associated with disrupted lipid homeostasis. Moreover, abnormal lipid signaling disrupts key neuronal pathways in dopaminergic neurons, including synaptic transmission and receptor activation. Furthermore, elevated levels of α-syn are associated with disturbances in lipid metabolism [55]. The interaction of α-syn with lipid membranes is governed by both electrostatic and hydrophobic forces and involves significant conformational changes in the protein. Notably, the N-terminal segment of α-syn adopts extended or interrupted α-helical structures depending on the lipid-to-protein ratio and membrane curvature. These interactions appear to play a critical role in the physiological regulation of α-syn function at synapses. However, the precise role of this protein in cellular membranes remains unclear. Importantly, α-syn's ability to bind lipid membranes is now considered a major factor influencing its pathological aggregation and associated toxicity. Lipids can participate in the aggregation of misfolded α-syn and changes in membrane chemical composition [131,132]. The physical properties of membranes clearly promote their conversion into amyloid fibrils. Moreover, α-syn aggregates, including oligomers, can bind lipid membranes, leading to membrane disruption. The harmful effects of oligomers, which underlie neurotoxicity, are attributed to their direct interaction with cellular membranes. Interaction with lipid membranes not only initiates α-syn aggregation and oligomer formation but also damages cellular

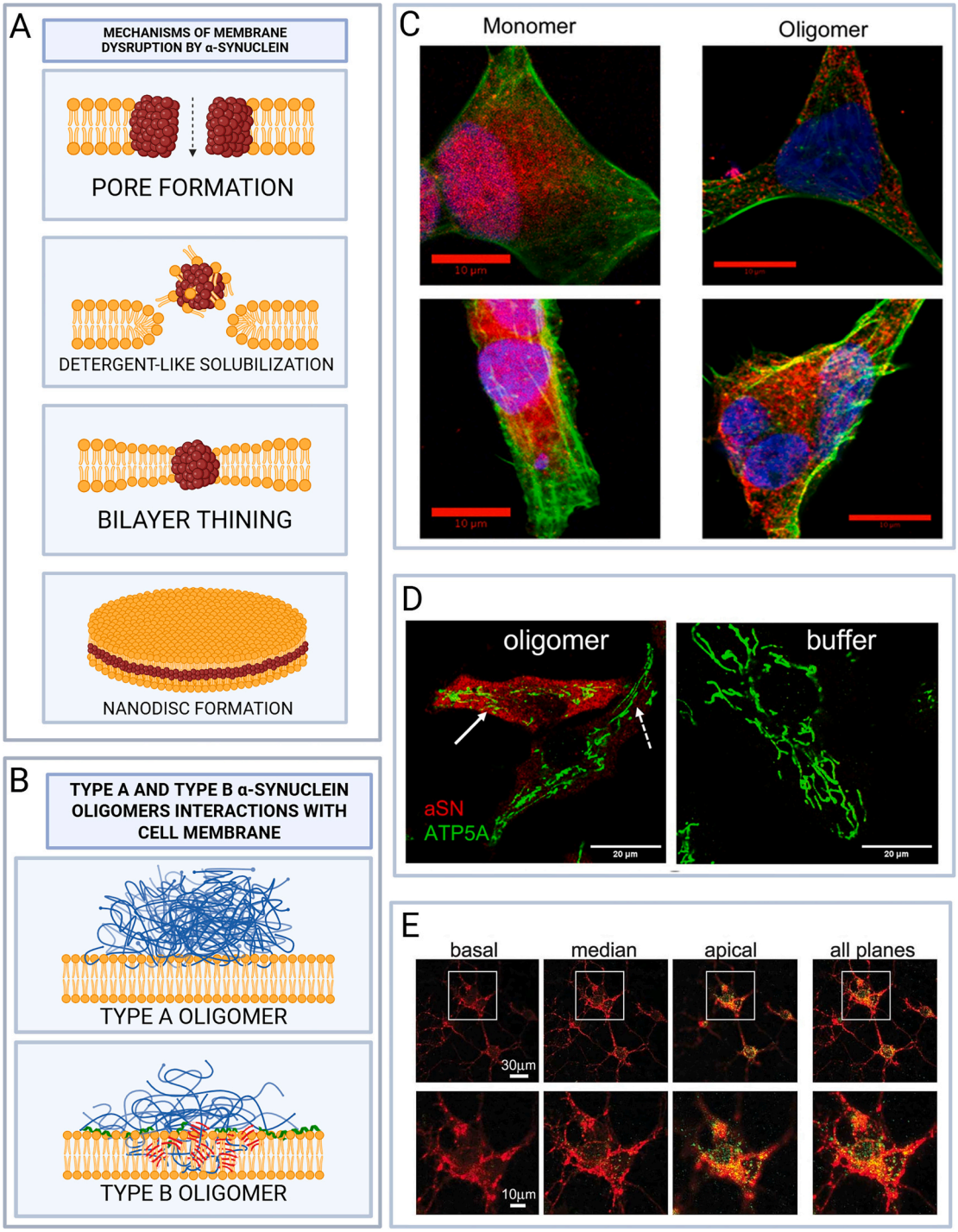


Fig. 7. Mechanisms of α-syn oligomer interaction with lipid membranes and cellular distribution. **A**. Schematic representation of membrane disruption mechanisms (based on [62]). **B**. Schematic representation of type-A and type-B α-syn oligomer interactions with biological membranes, showing surface binding of largely disordered type-A species and partial insertion of more structured type-B oligomers that disrupt bilayer integrity (based on [84]). **C**. Confocal microscopy images of SH-SY5Y neuronal cells with α-syn monomers and oligomers. The α-syn monomer is uniformly distributed throughout the cell, including the nucleus. In contrast, the oligomer is mainly located in the cytoplasm and near the cell membrane. **D**. Analysis of mitochondrial fragmentation in the presence of α-syn oligomers or buffer. A comparison of images of cells treated with oligomers or buffer revealed a significant increase in mitochondrial fragmentation (C and D reproduced with permission from Andersen, C. B. et al., *Biochemistry* 2023, 62, 2417–2425). Copyright © 2023 American Chemical Society). **E**. Confocal microscopy images of basal, middle, and apical focal planes and their merged views of primary cortical neurons after 15 min incubation with type-B α-syn oligomers. Red and green fluorescence indicate the cell membrane and oligomers, respectively. Type-B oligomers show stronger binding compared with type-A species and colocalize with the plasma membrane (reproduced with permission from Fusco et al., *Science*, 2017, 358(6369), 1440–1443, Copyright © 2017 AAAS). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structures. This process leads to the loss of membrane integrity, disruption of homeostasis, and eventually neuronal cell death [62,133].

Understanding and investigating the binding of α-syn oligomers to lipid membranes is complex because of the diversity of oligomeric forms. These forms exhibit varied structures, including differing β-sheet content, and therefore interact with membranes through distinct mechanisms. To date, one of the key factors determining the destructive interaction between oligomers and membranes is the solvent exposure of the lipophilic N-terminus. It facilitates the insertion of the oligomeric core into the hydrophobic interior of the membrane. The exposure of α-syn's N-terminus enables both monomer and oligomer binding to membranes and their insertion and anchoring in lipid bilayers. Strong neurotoxicity has been attributed to oligomer-induced membrane permeabilization. The structured β-sheet core encompassing residues, such as 70–88 in the NAC region of the oligomers, penetrates the lipid bilayer. It destabilizes the bilayer and increases its permeability [62]. Conducting experimental research on oligomer binding to lipid membranes remains challenging, mainly because of their transient nature, structural heterogeneity, and low abundance [12]. Furthermore, monomer binding can be easily monitored by spectroscopy, as evidenced by the strong increase in α-helical structure upon membrane association [62]. By contrast, the secondary structure changes in oligomers upon binding with the membrane are minimal. Numerous experimental studies using monolayer lipid vesicles as model biological membranes have confirmed that oligomer binding can be identified when the liposome composition contains at least 20% negatively charged lipids. Therefore, many studies have confirmed that oligomer binding is mediated by positively charged lysine residues [62]. These residues are located at the membrane boundary when the N-terminal region of the protein is organized into an amphipathic α-helix. Using microfluidic methods, such as microfluidic diffusional sizing (MDS) and microfluidic free-flow electrophoresis (μFFE), researchers have quantitatively characterized these interactions between monomeric and oligomeric forms of α-syn and lipid membranes. The oligomeric forms of α-syn bind selectively to negatively charged lipid bilayers with significantly higher affinity, up to 150-fold greater, than monomeric protein forms. This higher-affinity binding allows oligomers to compete with monomers for membrane-binding sites and may disrupt the functional interaction of monomeric α-syn with the membrane. The increased binding strength of α-syn oligomers is explained by their high avidity, resulting from the exposure of multiple N-terminal regions participating in membrane anchoring. Such multipoint interactions significantly enhance overall membrane binding [12].

3.2.1. *Impact of membrane composition and curvature on oligomer binding and insertion*

The influence of lipid composition and the membrane's physicochemical properties, such as curvature and charge, is critical for the binding and incorporation of α-syn oligomers. The diversity of lipids and local changes in membrane topology can significantly modulate protein-membrane interactions, affecting the stability, conformation, and functionality of the oligomers [134,135]. The composition of lipids plays a key role in interactions with α-syn, which binds preferentially to lipids with acidic head groups [136,137]. The presence of certain lipids, such as phosphatidylinositol-3,4,5-trisphosphate (PIP3) or palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), promotes α-syn aggregation, which may lead to synucleinopathy [138,139]. Numerous studies have shown that α-syn exhibits preferential binding to membranes that contain negatively charged lipids and have high curvature [135–137,140,141]. Van Rooijen et al. [140] used confocal and fluorescence microscopy to observe the preferential binding of α-syn oligomers to negatively charged giant unilamellar vesicles (GUVs) composed of negatively charged lipids. They did not observe any interactions between the oligomers and amphoteric 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) GUVs. The affinity of α-syn oligomers also depended on lipid composition, likely because of structural differences among the main lipid groups. Another significant factor that affects the affinity of α-syn oligomers is the degree of lipid packing. Studies have shown that oligomers do not bind to denser bilayer regions with elongated lipid tails and reduced surface area per head group [140]. α-Syn shows particular affinity for highly curved synaptic vesicles, which may be related to its function in these structures. The shape of the membrane influences the modulation of the helical region conformation of α-syn, which likely contributes to its preferential binding to surfaces with high curvature [141]. Middleton et al. [141] demonstrated the significant effect of lipid composition and membrane curvature on α-syn binding. In this study, fluorescence correlation spectroscopy provided quantitative measurements of the binding energy and the protein's molar partition coefficient. The presence of anionic lipids significantly increased the affinity of α-syn for the membrane. Membrane curvature also plays a key role; for example, α-syn binds to small, highly curved lipid vesicles approximately 15-fold more strongly than to larger, less curved structures. These results emphasize the importance of the membrane's physical properties, especially its shape, in modulating α-syn interactions with membranes. Moreover, membrane curvature can modulate α-syn conformation, favoring its transition to an α-helical structure. This structural transition of α-syn is important for its membrane function and for pathological processes associated with neurodegeneration. The lipid composition of the membrane also significantly affects its interaction with α-syn. The presence of anionic lipids increases α-syn's affinity for the membrane, further promoting its aggregation that causes the formation of toxic oligomers. The presence of lipids with positive spontaneous curvature, such as cardiolipin, can influence the preferential binding of α-syn in regions of high membrane curvature [135].

3.2.2. *Bridging interface interactions to membrane-induced cellular toxicity*

Interactions between α-syn oligomers and lipid membranes play a significant role in the pathological processes that lead to α-syn aggregation and toxicity. Based on many years of research, PD and related synucleinopathies have been considered not only proteinopathies but also lipidopathies [142,143]. There is strong scientific evidence supporting the physiological and pathogenic interactions between α-syn and lipids, particularly fatty acids and lipid membranes [143]. Disrupted lipid homeostasis, or lipidopathy, may occur early in PD and may promote α-syn misfolding and assembly through changes in membrane lipid composition, suggesting a contributory role in the upstream pathogenesis of PD [67,144]. Moreover, lipids can co-aggregate with misfolded α-syn. Lipid membranes can initiate the formation of α-syn fibrils and oligomers. Membrane-disrupting species expose an N-terminal region rich in positively charged lysine residues. These species interact with the negatively charged membrane, anchoring the oligomers and enabling their penetration into the hydrophobic core of the bilayer [62,145]. An additional factor contributing to membrane disruption is the β-sheet-rich core, which increases membrane permeability. Three main mechanisms of interaction between α-syn oligomers and membranes have been identified. The first involves the formation of pore-like structures that embed themselves in the bilayer and increase its ion permeability. Alternatively, α-syn oligomers can act in a detergent-like manner, disrupting lipid order and leading to local disintegration or dissolution of membrane fragments. The third mechanism involves lipid extraction and the formation of nanodiscs. In this case, oligomers stabilize detached fragments of the bilayer, causing lipid depletion and further weakening of membrane integrity. All three mechanisms ultimately compromise membrane integrity and cellular homeostasis, forming the core of α-syn oligomer toxicity. Pore formation drives uncontrolled ion fluxes, particularly Ca^{2+} overload. By contrast, detergent-like disruption and lipid extraction weaken the bilayer, leading to leakage, loss of membrane potential, and organelle dysfunction [62]. All these effects induce persistent cellular dysfunction and stress, leading to neuronal impairment and contributing to the progression of neurodegenerative diseases. Lashuel et al. [146] demonstrated that α-syn can form ring-

shaped protofibrils that integrate into the cell membrane. Further, the resulting pore-like structures can disrupt membrane integrity and increase permeability. Such disruptions may lead to cellular dysfunction or even cell death. Additionally, the protofibrillar form of α-syn induces liposome permeabilization. Further, its α-helical structure can form highly conductive ion channels. Tosatto et al. [47] showed that monomeric α-syn can form reversible pores in lipid bilayers through oligomerization at the membrane surface. These structures, which exhibit three characteristic levels of conductance, are formed with the involvement of the N-terminal region of the protein in an α-helical conformation. In contrast to β-sheet oligomers formed along the aggregation pathway, these pore-forming oligomers are dynamic and dependent on membrane interaction. Studies by van Rooijen et al. [140] and Stefanovic et al. [147] examined the impact of α-syn oligomers on the permeability of model membranes with simple compositions (e.g., DOPG and POPG) and more complex compositions mimicking plasma and mitochondrial membranes. The interaction of the oligomers with the membranes did not disrupt all membrane types, suggesting that binding and disruption are separate processes determined by membrane composition. The addition of α-syn oligomers to vesicles with a composition mimicking mitochondrial membranes resulted in a significantly slower loss of content, whereas no leakage was observed in artificial plasma membranes. Unlike complex plasma membrane models, model mitochondrial membranes are more susceptible to oligomer-induced damage over time. This effect is associated with the presence of cardiolipin, which plays a crucial role in the interaction between α-syn and the mitochondrial membrane [147]. Confocal microscopy [148] confirmed mitochondrial fragmentation in the presence of α-syn oligomers. High-resolution imaging using stimulated emission depletion microscopy (STED) revealed that α-syn oligomers appear as regular, circular structures. This finding may suggest their enclosure in endocytic vesicles, which do not colocalize with mitochondria in STED images [148]. Another aspect examined in the context of membrane toxicity is the occurrence of mutations in the α-syn structure, which can lead to its oligomerization. Using computational modeling and cellular studies, researchers demonstrated that both wild-type and A53T α-syn can penetrate membranes by transitioning from α-helical to coiled conformations and forming oligomers. These oligomers have been reported to permeabilize lipid bilayers and form pore-like structures in model membrane systems. This process occurred more rapidly and with more favorable interaction energy in the A53T mutant compared with the wild type [149]. Pore formation by α-syn in cellular membranes represents a key mechanism leading to cellular dysfunction. It also contributes to the development of neurodegeneration in PD. Collectively, these findings support a structure-interface-toxicity framework in which specific conformational properties of α-syn oligomers determine their mode of membrane interaction and, consequently, their neurotoxic potential. In particular, oligomers exposing flexible hydrophobic interfaces and partially structured β-sheet cores appear especially effective in destabilizing lipid bilayers, inducing ion dysregulation, and promoting downstream cellular stress. Across biophysical and membrane-focused studies, membrane interactions emerge as a central mechanistic link connecting oligomer structure with pathological toxicity.

4. Neurobiological implications

Building on the structural and interfacial mechanisms discussed above, this section examines the neurobiological consequences of α-syn oligomer formation, with particular emphasis on cellular and animal models used to interrogate oligomer-driven toxicity.

Synucleinopathies involve a cascade of biochemical disruptions, such as mitochondrial dysfunction, oxidative stress, increased apoptosis, and defects in protein degradation systems [150]. Although large inclusions were once widely studied, attention has shifted toward soluble oligomers, which are increasingly recognized as potent mediators of neurotoxicity. Oligomers can be released from larger aggregates or formed *de novo*. Consequently, the neurobiological implications of α-syn oligomers reflect a dynamic toxicological flux rather than static pathological events, driven by the continuous transition between different aggregation states with size-dependent neurotoxicity [151,152].

4.1. Cellular and animal models for studying α-syn oligomer and fibril toxicity

While the biochemical pathway of α-syn aggregation is well-characterized, the transition from monomeric protein to proteotoxic species requires robust experimental frameworks to dissect the resulting cellular dysfunction. The heterogeneity of α-syn aggregates—ranging from small, soluble oligomers to insoluble amyloid fibrils—requires a multi-tiered modeling approach to capture distinct biophysical properties, kinetic behaviors, and cellular interactions of each species [153]. Many *in vitro* and *in vivo* experimental models mimicking α-syn pathology, such as oligomerization, toxicity, and neuronal propagation, have been generated in recent years [154,155]. The primary challenge in modeling synucleinopathy lies in the transient nature of oligomeric intermediates. Although mature fibrils are thermodynamically stable and easily delivered as preformed fibrils (PFFs), oligomers exist in a fragile dynamic equilibrium with monomers and larger aggregates. Moreover, the distinction between these species is frequently blurred in biological environments because of continuous interconversion and the coexistence of multiple conformational states [156]. Recent evidence indicates that mature fibrils can act as catalytic reservoirs [152]. In this context, the fibril surface facilitates the generation of toxic oligomers via secondary nucleation, a process that is significantly accelerated at the lipid-water interface of cellular membranes. Consequently, studying fibril toxicity is not a diversion from oligomer research but a prerequisite for understanding the continuous flux of toxic species in the brain. To dissect these complex interactions, a variety of experimental frameworks have been developed, ranging from simplified cell cultures to complex transgenic organisms. Each of these frameworks, however, captures distinct aspects of the aggregation process and its pathological consequences. The individual limitations of these frameworks must be considered when interpreting their results.

One of the basic approaches to studying disease pathogenesis is based on tracking α-syn oligomer aggregation in selected *in vitro* models [157]. α-Syn labeling enables visualization of aggregation dynamics. Thus, different imaging approaches have been developed, including: i) coexpression and fusion of the target protein to GFP using FRET, which assesses synuclein aggregation based on energy transfer between donor and acceptor fluorophores [158,159], ii) detection of dimeric or higher-order oligomeric species using protein complementation assay (PCA), in which synuclein is tagged with either N- or C-terminal portions of a split reporter [160]; and iii) proximity ligation assay (PLA), which uses proximity-dependent rolling circle amplification of oligonucleotide-labeled antibodies to generate a signal marking α-syn aggregates [161]. All these tracking and visualizing methods can be used in different *in vitro* models. The relevant models must first be generated or adopted.

Several models help elucidate the mechanisms of α-syn oligomer toxicity and facilitate the development of therapeutic strategies. General categories include cellular and animal models. These models can employ different α-syn perturbations, including overexpression of wild-type or mutant α-syn (A53T, A30P, or E46K) or treatment with exogenous oligomers/fibrils.

4.1.1. Cellular models: From Yeast to Human iPSCs

Cellular models provide scalable platforms for interrogating oligomer-related mechanisms across evolutionary and phenotypic complexity. Because of conserved protein quality control machinery, yeast (*Saccharomyces cerevisiae*) is considered one of the basic models for human metabolism studies. Specifically, detailed molecular studies of yeast have revealed mechanisms related to protein folding and

degradation that are involved in prion disease. However, these microorganisms have limited translational relevance [162].

An alternative approach employs easy-to-handle cell lines, including immortalized human non-neuronal cell lines, such as human H4 neuroglioma (H4) and human embryonic kidney 293 (HEK293) lines. These cells can be easily transfected with stable and transient overexpression of human wild-type or mutated α-syn [157,163,164]. However, these cells do not exhibit a dopaminergic phenotype, which makes the assessment of the role of α-syn oligomers in nervous system functionality more challenging and limited.

Primary dopaminergic neurons prepared from embryonic or early postnatal rodents represent a more advanced model. These cultures are used to observe the specific neurobiological consequences of α-syn accumulation in dopaminergic systems, including disruption of synaptic vesicle recycling and the "dying-back" axonal degeneration pattern [165,166]. In addition to primary neurons, immortalized dopaminergic-like cell lines (PC12, SH-SY5Y) offer scalable alternatives. Transient and constitutive (stable) overexpression of wild-type or mutant α-synuclein in these cells induces cytotoxicity and affects cell survival [167,168]. In a stable PC12 cell line, expressing non-toxic levels of wild-type human α-syn or A30P mutant, impaired dopamine release has been observed [169]. The addition of extracellular α-syn from PD brain lysates to differentiated SH-SY5Y cells can trigger the formation of α-syn aggregates [170]. Finally, extracellular α-syn has been observed to be internalized by neighboring cells via endocytosis and to form Lewy body–like inclusions [171]. Overall, these studies demonstrate the importance of dopaminergic neuron-like cell lines as a predictive tool to mimic a neurodegenerative-like phenotype *in vitro*.

The emergence of patient-derived induced pluripotent stem cells (iPSCs) has created new possibilities for creating physiologically relevant disease models *in vitro*. They offer broad opportunities to understand disease mechanisms and determine novel molecular targets in personalized therapies [172]. Skin fibroblasts constitute a person-specific system, in which PD-relevant genes, such as *PINK1* and *Parkin*, exhibit functional expression. Moreover, these cells can be reprogrammed into iPSCs to generate patient-specific neurons with the donor's genetic landscape, enabling the study of synuclein pathology toxicity in a physiologically relevant human context [173]. An alternative approach involves the direct conversion of fibroblasts into induced neurons (iNeurons), which retain the same developmental age and epigenetic alterations. Compared with iPSCs, iNeurons are relatively simple and shows high reprogramming efficiency. The transformation is based on the down-regulation of a single RNA-binding polypyrimidine-tract-binding protein 1 (PTBP1) in fibroblasts [174,175].

4.1.2. Animal models

Animal models enable the extension of findings to the level of neural networks, focusing on the spatiotemporal spread of toxicity and behavioral outcomes.

Drosophila melanogaster (fruit fly) and *Caenorhabditis elegans* (roundworm) are widely used invertebrate models for studying α-syn aggregation and toxicity in the context of neurodegeneration. These organisms can be genetically engineered to express human α-syn, which mimics key pathological features, such as dopaminergic neuron loss and motor deficits, seen in human PD [176,177].

The Danio rerio model offers a vertebrate central nervous system (CNS), optical transparency, straightforward genetic manipulation, and defined structural and compositional organization [178]. Zebrafish models of PD can be used to assess the degradative capacity of α-syn (both wild type and mutant A53T) in intact neurons *in vivo* [179]. Seeding and spreading models involve injecting preassembled α-syn fibrils into the animal brain to trigger the misfolding and aggregation of the brain's own α-syn protein. This process causes the formation and widespread accumulation of pathological α-syn, neuronal dysfunction, and the formation of Lewy-like inclusions. Consequently, critical features of human PD are recapitulated [180]. This model system enables monitoring the dynamics of α-syn aggregation from early formation to its spread throughout the neuron and, ultimately, to neuronal death. Additionally, the use of microfluidic systems enables the separation of neuronal somata from axonal projections and the monitoring of intercellular trafficking of internalized α-syn seeds in primary neurons. This approach, when supported by genetic engineering, enables the identification of biomarkers, optimization of dosing, and compliance with regulatory requirements [181]. Most recently, models utilizing direct intrastriatal injection of stable oligomers (OSyn) have successfully recapitulated early synaptic dysfunction and non-motor symptoms before cell loss, offering a refined platform for targeting the prodromal phase of synucleinopathies [182].

Transgenic mice and adeno-associated virus-mediated (AAV) models provide complementary strategies to stabilize or visualize the transient oligomeric species. Transgenic mice expressing "oligomer-prone" structure-based mutants, such as E57K, have established that the accumulation of nonfibrillar, stable oligomers leads to neurodegeneration independently of fibril formation [183]. Recent "knock-in" models (e.g., hαSyn^{TP}) have further validated oligomer toxicity at physiological expression levels, thereby circumventing potential artifacts associated with massive overexpression [184]. Biochemical analyses of established A53T lines continue to serve as a benchmark by identifying specific detergent-soluble oligomeric species (e.g., 53 Å) that precede overt pathology [185]. Recombinant AAV vectors targeting nigral expression of oligomer-promoting variants (E57K, E35K) have confirmed the heightened toxicity of these species to dopaminergic neuron variants [186]. Innovative AAV-based Bimolecular Fluorescence Complementation (BiFC) assays have evolved to visualize oligomerization in the living brain [187], although recent studies caution that BiFC fragments themselves may influence aggregation kinetics [188].

4.1.3. Organs-on-chip

Complex pathological processes observed in neurodegenerative diseases, related to abnormal accumulation of α-syn aggregates (i.e., neuronal cytotoxicity, gliosis of the substantia nigra, and loss of dopaminergic neurons), require more complex and advanced models to study these multicellular interactions [189]. For modeling α-syn pathology, organs-on-chip systems employ a human brain chip representing the substantia nigra region of the brain. The model contains dopaminergic neurons, pericytes, microglia, astrocytes, and microvascular brain endothelial cells, cultured under fluid flow. This model recapitulates several key aspects of PD, such as accumulation of phosphorylated α-syn (pSer129-αSyn), compromised barrier function, neuroinflammation, and mitochondrial impairment [190]. The most important advantage of the system is its capacity to recapitulate the dynamics of cell-cell interactions in human synucleinopathies. This system serves as a testing platform for target identification and validation of novel therapeutics [191–194]. The system enables the recreation of a more physiological microenvironment, including the coculture of relevant cells on tissue-specific extracellular matrices (ECM), continuous flow exposure, and *in vivo*-relevant mechanical forces, such as fluidic shear stress. This system can support the development and validation of future pharmacological strategies [157].

4.2. Mechanisms of oligomer-induced neurotoxicity

From an interface-science perspective, oligomer toxicity can be explained as a cascade initiated at negatively charged and highly curved lipid membranes, which subsequently amplifies across organelles and cells (Fig. 8) [84]. The final pathological burden is shaped by both oligomer state and intrinsic neuronal susceptibility [195]. The following subsections detail these mechanisms, which form an interconnected, self-amplifying cascade.

4.2.1. α-Synuclein-induced mitochondrial impairment

Mitochondrial dysfunction is a central mechanism of neuronal death

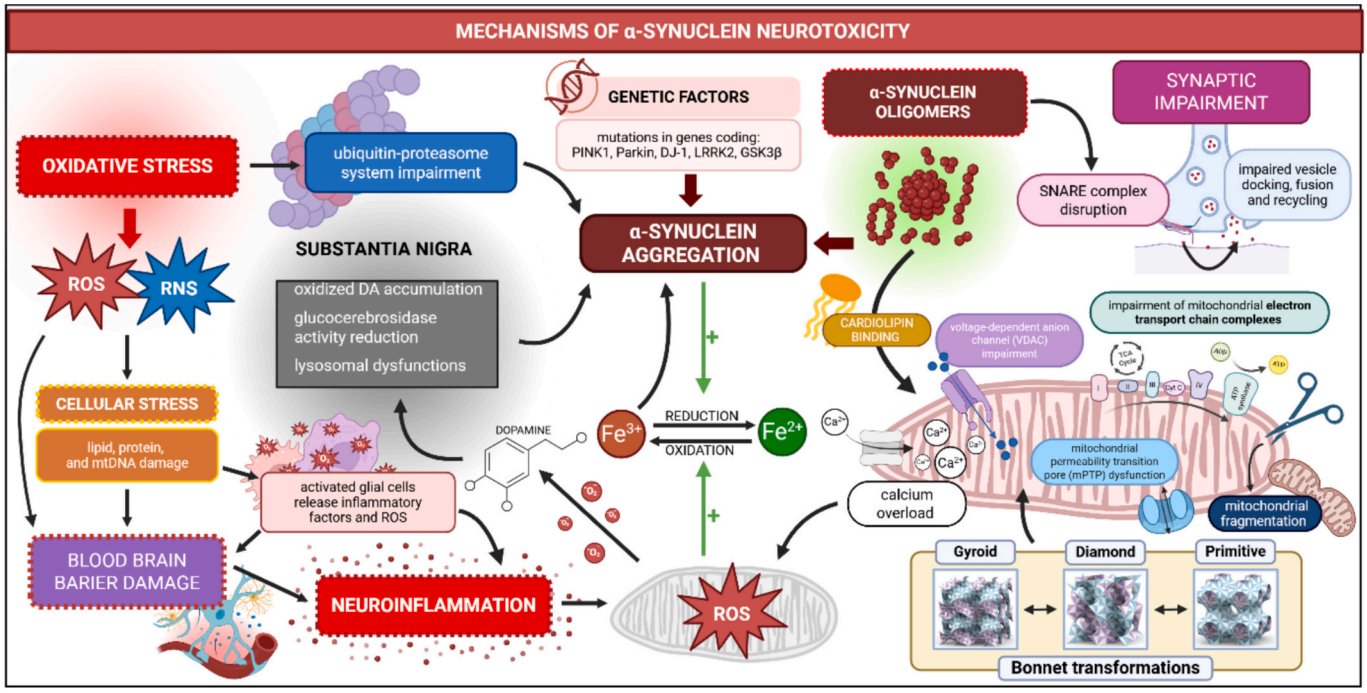


Fig. 8. Mechanisms of α-synuclein (α-syn) neurotoxicity. Genetic mutations (e.g., SNCA, PINK1, PRKN/Parkin, DJ-1, LRRK2, and GSK3β) and ubiquitin–proteasome system impairment promote α-syn misfolding, aggregation, and accumulation of toxic oligomers. Oxidative/nitrosative stress (ROS/RNS), amplified by Fe^{3+}/Fe^{2+} redox cycling and dopamine oxidation, drives lipid/protein/mtDNA damage and reinforces aggregation. α-Syn oligomers interact with mitochondrial membranes (including cardiolipin binding), disrupt electron transport chain complexes, and trigger permeability transition, fragmentation, and Ca^{2+} overload, leading to further ROS generation. Activated glia sustain neuroinflammation and further ROS production, while blood–brain barrier damage creates a permissive inflammatory milieu. At synapses, oligomer-mediated SNARE complex disruption impairs vesicle docking, fusion, and recycling. Together, these interconnected pathways form self-amplifying loops that culminate in neuronal dysfunction and degeneration, particularly in the substantia nigra. The schematic also illustrates Bonnet transformations, representing reversible transitions between primitive, diamond, and gyroid mitochondrial membrane architectures associated with mitochondrial membrane remodeling (adapted from Ref. [220] with permission of the International Union of Crystallography).

in synucleinopathies. α-Syn oligomers disrupt mitochondrial integrity through direct membrane interaction, triggering a cascade of pathological events [196,197].

4.2.1.1. Membrane permeabilization. α-Syn oligomers interact with mitochondrial membranes, disrupting their integrity [197]. Membrane phospholipids with high curvature, net negative charge, and loose packing strongly favor α-*syn*-membrane interactions [198]. These interactions are mediated by the N-terminal and NAC regions, which adopt an amphipathic α-helical conformation [199]. The significant role of cardiolipin (CL) in α-syn-mitochondria interaction has been revealed by several studies [55,200,201].

4.2.1.2. Calcium dysregulation. Binding of α-syn oligomers to membranes disrupts ion homeostasis, particularly Ca^{2+}, via excessive mitochondrial uptake and impaired efflux. This calcium overload triggers mitochondrial stress and ROS generation, producing a self-amplifying cycle of oxidative damage and further membrane permeabilization [202].

4.2.1.3. Mitochondrial dynamics. Consequently, α-syn pathology induces mitochondrial fragmentation by shifting the organelle's dynamic balance toward fission and inhibiting fusion processes [203]. Additionally, α-syn targets the voltage-dependent anion channel and disrupts mitochondrial permeability transition pore function, further exacerbating organellar dysfunction [204]. α-Syn accumulation within mitochondria and resulting dysfunction have been demonstrated in animal models, isolated rat brain mitochondria [205], and postmortem substantia nigra (SN) neurons from patients with PD [206]. Simultaneously, the impairment of mitochondrial electron transport chain complexes has been reported to induce PD symptoms [206].

Emerging structural data indicate that the mitochondrial inner membrane may adopt bicontinuous cubic-phase geometries (Fig. 8), which are sensitive to ion composition, including the potassium-sodium balance, and to isotopic composition, including the local deuterium-to-hydrogen ratio. These factors regulate the timing and coherence of curvature transitions within the hydrated polymeric intermembrane domain. Electrolyte imbalance, loss of curvature coherence, and isotopic order disruption may therefore represent an underappreciated mechanism of mitochondrial energy failure in PD, distinct from, and not fully accounted for by, the protein-mediated membrane permeabilization described by classical models [207].

4.2.2. Synaptic impairment and vesicle dysregulation

Neuronal communication relies on a Ca^{2+}-triggered synaptic vesicle fusion at active zones of nerve terminals, a process tightly regulated by Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor (SNARE) proteins and Ca^{2+} nanodomains [208,209]. Pathological α-syn oligomers disrupt this machinery at multiple steps. Monomeric α-syn normally facilitates SNARE complex assembly via its cysteine-string protein α (CSPα)-chaperoning function [210], but oligomerization shifts α-syn away from its membrane-bound, SNARE-supportive state toward aggregation-prone soluble species, coupling a loss of normal presynaptic support with a toxic gain of function [211]. Large soluble oligomers sequester the SNARE complex by binding synaptobrevin-2, thereby inhibiting vesicle docking and trans-SNARE assembly [212]. This interference is amplified by the cooperative effects of monomeric α-syn and oligomers, which synergistically enhance vesicle clustering, thereby potentiating fusion inhibition even at low oligomer concentrations [213]. Consistently, exposure to nonnative α-syn assemblies reduces the pool of docked synaptic vesicles at active zones, linking

oligomer-driven SNARE interference to early vesicle dysregulation [214].

In addition, disruption of the perisynaptic interfacial environment may contribute to synaptic dysfunction. Reduced serum albumin, consistently reported in PD patients, may promote oligomer adsorption and aggregation at air-water interfaces generated by nanobubble fusion at synaptic junctions; van der Waals dispersion forces, stronger in air than in aqueous environments, may drive this process, thereby accelerating depletion of the functional synaptic α-syn pool and amplifying oligomer-driven toxicity [207].

4.2.3. *Oxidative stress and neuroinflammation*

Oxidative stress and neuroinflammation form a self-amplifying cycle in which ROS and reactive nitrogen species (RNS) activate microglia and astrocytes, subsequently triggering the release of pro-inflammatory cytokines and additional ROS. These processes result in lipid, protein, and mitochondrial DNA damage and compromise BBB integrity [215]. ROS promote α-syn aggregation through multiple mechanisms, such as the oxidation of dopamine to toxic quinones that covalently modify α-syn and the oxidation of Fe^{2+} to Fe^{3+} [68,216]. Notably, α-syn functions as a ferrireductase, reducing Fe^{3+} to Fe^{2+} and contributing to the formation of a self-perpetuating cycle of iron-induced aggregation [217]. Moreover, nitrosative and oxidative stress can impair the ubiquitin-proteasome system, causing abnormal protein accumulation [218]. In dopaminergic neurons, oxidized dopamine additionally reduces glucocerebrosidase activity, causing lysosomal dysfunction and further exacerbating α-syn accumulation [219].

4.3. *Seeding mechanisms: role of oligomers in templating endogenous α-syn misfolding*

Misfolded α-syn released from damaged cells can propagate pathology to neighboring neurons through prion-like seeding mechanisms [221].

Pathological α-syn offers prion-like biochemical properties, e.g., protease resistance and detergent-insolubility, reflecting its β-sheet-rich amyloid structure and conformational stability [222]. This prion-like process involves the conformational templating of the misfolded protein's β-sheet structure, leading to the autocatalytic amplification of α-syn aggregates. Several mechanisms mediate the spread of these aggregates, including extracellular vesicles and tunneling nanotubes (TNTs), which are transient tubular connections between distant cells that enable direct transfer of α-syn pathology [223,224]. After TNT-mediated transfer, α-syn fibrils induce the aggregation of soluble endogenous α-syn in acceptor cells, likely via escape from the lysosome [225]. Lysosomal membrane permeabilization induced by α-syn assemblies may facilitate this escape [226,227].

4.4. *Diagnostic strategies targeting α-syn assemblies and seeding activity*

Several mechanistically distinct approaches have emerged for detecting and characterizing pathological α-syn species, including oligomer-selective platforms, aggregate-responsive biosensors, and seed amplification assays. Each class targets a different aspect of α-syn pathology — the identity, conformational state, or seeding competence of misfolded assemblies — although, in practice, emerging platforms increasingly combine elements of molecular recognition with label-free physical detection. Accurate interpretation of experimental readouts requires a clear conceptual separation of these categories, which is frequently overlooked in the literature.

Oligomer-selective approaches employ conformation-specific bioreceptors to recognize soluble, nonfibrillar α-syn assemblies and primarily report on the abundance and physicochemical heterogeneity of putatively toxic oligomeric species. The conformation-specific quantification of α-syn oligomers in CSF can be achieved using a label-free plasmonic biosensing system that employs the amphipathic peptide PSMα3 as a conformation-specific bioreceptor [228]. This biosensing platform offers a detection limit of approximately 0.13 nM and a time-to-result of approximately 30 min, with high reproducibility across independent cohorts. An alternative oligomer-selective approach offers aptamer-based electrochemiluminescent biosensors that have demonstrated rapid, noninvasive detection of α-syn with detection limits below 1 femtomolar in serum [229].

In contrast, aggregate-responsive biosensors detect α-syn species largely based on size, charge, or total aggregate burden, rather than conformational identity, and typically do not resolve oligomeric stoichiometry or distinguish short fibrillar fragments from discrete oligomeric species. For instance, solid-state nanopore platforms report events corresponding to the hydrodynamic volume and net charge of the transiting species, enabling label-free, single-molecule detection of small oligomeric assemblies [230–232]. To date, this technique has been successful *in vitro* in artificial buffers, which may not fully recapitulate clinical sample conditions. Furthermore, the method lacks selectivity because single-protein identification is confounded by the presence of heterogeneous analytes, including proteins with similar sizes and shapes. This limitation was partially addressed by Liu et al. [7], who employed an aptamer-modified DNA carrier to selectively capture and detect soluble α-syn oligomers directly in clinical samples by using a quartz nanopore. The DNA carrier application offers a wide range of options for engineering and tailoring specific protein recognition sites. Such an approach enables quantitative assessment of the size distribution and relative concentration of the α-syn oligomeric species in clinical biofluids. Performing such detection at the single-molecule level highlights the future promise of a nanopore-based strategy for patient stratification, disease progression monitoring, and evaluation of treatment efficacy [7]. Complementary advances in single-molecule imaging, including aptamer DNA-PAINT and confocal fluorescence microscopy, have extended detection sensitivity for α-syn assemblies in CSF and serum [233]. However, this method cannot recognize oligomers smaller than 20 nm, which may be highly cytotoxic if they are transferred across lipid membranes due to inflammation initiation [234].

Seed amplification assays constitute a mechanistically distinct category that does not directly detect oligomers but instead quantifies the seeding behavior of misfolded α-syn species capable of templating amyloid fibril growth; therefore, these assays predominantly reflect fibril- or protofibril-associated catalytic activity rather than the native pool of soluble oligomers. Detection of pathogenic α-syn species by seed amplification assays has shown unprecedented diagnostic sensitivity and specificity for synucleinopathies, even in prodromal phases years before the onset of Parkinsonian signs or dementia [235].

Another diagnostic test used for detecting α-syn assemblies in the brain and CSF of patients with synucleinopathies is based on prion-like seeding activity of α-syn. This assay (real-time quaking-induced conversion (RT-QuIC)) is an ultra-sensitive, protein misfolding assay used to detect and quantify abnormal, misfolded proteins, such as prions, α-syn, and tau, in biological samples. In this system, recombinant proteins undergo templated misfolding and aggregation in the presence of seeds derived from the biological sample. The kinetics of this fibrillization are tracked in real-time using Thioflavin T, which serves as a fluorescent reporter of amyloid formation. This technique allows for the early and accurate diagnosis of various neurodegenerative diseases, including prion diseases, such as Creutzfeldt-Jakob disease (CJD), and synucleinopathies, such as PD [236].

Furthermore, the distinct fluorescent profiles of α-syn PMCA reactions could be used to discriminate α-syn seeds in CSF samples from PD and MSA cases [237]. Studies have also shown the applicability of α-syn RT-QuIC to olfactory mucosa samples, with sensitivities of 55.5% for PD and 81.8% for MSA, and a specificity of 84.4% [238]. Two independent RT-QuIC studies detected seeding activity in the postmortem skin samples from different synucleinopathies, such as PD, DLB, and MSA, with 93–94% sensitivity and 93–98% specificity [239,240]. A recent study has revealed seeding activity in post-mortem

submandibular gland samples from PD cases using α-syn RT-QuIC with an overall sensitivity of 100% and a specificity of 94% [239]. Thus, these findings suggest that α-syn seeds could also be detected in saliva samples from patients with PD [241]. This method was modified by Groveman et al. [242] by using a mutant human recombinant α-syn (K23Q Hu) as a substrate. This modification allowed much more rapid detection of seeds within 1–2 days compared with the PMCA assays, which required 5–13 days. Initially, the testing of CSF samples from PD and DLB cases showed high sensitivities, i.e., 92% and 94%, respectively, along with 100% overall specificity. Further, the testing of CSF samples from PD [243–246], DLB, isolated REM sleep behavior disorder (iRBD), and pure autonomic failure patients revealed sensitivities of 93-100% and specificities ranging from 84 to 98% [246]. Altogether, RT-QuIC appears to be a promising solution in detecting α-syn aggregates in the skin, which is highly important because of the low invasiveness of this approach. However, detailed studies are required to unequivocally confirm its correlation with symptoms and disease progression before exploring its potential use as a peripheral tissue test. Another prion detection assay is protein misfolding cyclic amplification (PMCA) and was adapted for α-syn identification by Shahnawaz et al. [247]. The α-syn PMCA assay offers detection of α-syn seeding activity in CSF samples from patients with different synucleinopathies. This process achieved sensitivities of 88.5% in a cohort of 76 patients and 95.4% in a cohort of 153 patients for PD, sensitivities of 80% and 85% for MSA cases, and specificity between 94 and 100% [237,247]. Furthermore, the distinct fluorescent profiles of α-syn PMCA reactions can be used to discriminate α-syn seeds in CSF samples from PD and MSA cases [237].

Altogether, α-syn kinetics aggregation assays, also referred to as α-syn seed amplification assays (α-syn SAAs), support the diagnostic differentiation of PD from other neurodegenerative diseases. These assays show high sensitivity and specificity and can also provide prognostic value, as quantitative measures, such as time to threshold, can predict the risk of cognitive decline in PD. While less established for MSA and DLB, these assays show promise for diagnosing these synucleinopathies, with ongoing research focusing on optimizing accuracy and establishing definitive prognostic markers [248]. Promising results were demonstrated using peripheral samples such as skin, olfactory mucosa, and blood, which could lead to less invasive diagnostic methods as alternatives to CSF sampling. Combining multiple peripheral tissue specimens could further increase the diagnostic accuracy [248]. These assays can differentiate synucleinopathies. For example, distinct kinetics can be observed in progressive supranuclear palsy samples compared with PD or in individuals with REM sleep behavior disorder (RBD) [249]. All these studies suggest the potential of these assays to predict phenoconversion to a clinical synucleinopathy.

5. Integrative approaches and future perspectives

An interdisciplinary approach combining data from structural biology, nanoscience, and neurobiology enables a deeper understanding of the complex, interface-driven pathomechanisms of α-syn oligomerization. This integration not only facilitates the rational evaluation of novel therapeutic targets but also paves the way for advanced diagnostic platforms capable of detecting early-stage biomarkers across the spectrum of synucleinopathies [7,154].

Advanced techniques enable the identification of diverse protein forms, or proteoforms, and the observation of their dynamic structural changes in response to potential inhibitors. This capability is crucial for understanding pathological mechanisms and developing new therapies. Techniques such as native top-down mass spectrometry (nTDMS) and IM–MS allow identification of different forms of α-syn and the study of changes in its conformation upon binding to CLR01 [107]. Studies have shown that the “molecular tweezer” CLR01 binds to α-syn proteoforms, including species phosphorylated at Ser129 and metalloforms [107], Lantz et al. conducted IM-MS analyses demonstrating that CLR01 binding leads to compaction of α-syn under gas-phase conditions. These findings support the protein's transition to a compact conformation. This conformational change, which is inferred to reflect solution-phase behavior, has been proposed as a potential mechanism that may limit oligomerization and subsequent amyloid fibril formation [107]. In a similar study using top-down ECD and IM-MS on tau protein, Nshanian et al. [250] confirmed an analogous effect of CLR01. This study demonstrated inhibitor-induced compaction. These findings suggest a common mechanism of action for CLR01 against intrinsically disordered proteins, such as α-syn and tau.

Table 2 summarizes cross-level experiments using multimodal strategies to study α-syn oligomers. The biophysical and interface-sensitive analytical approaches have been applied in complementary ways across cellular, *ex vivo*, and *in vivo* systems to provide insights into oligomer structure, dynamics, and toxicity. The gathered data underscore substantial methodological diversity adapted to the complexity of the biological system. In practice, none of these integrated approaches captures the full biological spectrum of α-syn oligomers. *In vitro* biophysical assays yield precise structural data but do not address physiological conditions. Cellular models allow investigation of intracellular pathways and toxicity, while still lacking the ability to quantify oligomeric stoichiometry in situ. Studies using human tissue increase disease relevance but provide only static snapshots. *In vivo* imaging and PCA-based strategies rely on overexpression designs and indirect readouts, which limit their interpretability. Together, the data show that multimodal integration across biological levels is essential, but a fully standardized, real-time, in-brain analytical platform for α-syn oligomers remains unavailable.

However, as new technologies develop, one of the key challenges in α-syn research remains the standardization of methods for generating its oligomeric forms. The integration of nanoscale imaging techniques and biophysical analyses with various biological models, from cell lines (SH-SY5Y, HTB-148, M17D, H4, and HEK293T) and primary neurons to animal models (C57BL6 mice and Sprague Dawley rats) and human samples, enables the synthesis of data on α-syn oligomers in various biological contexts. Super-resolution methods, such as DNA-PAINT and STED, allow the analysis of oligomer morphology and size in cells and tissues [148,251,256], while fluorescence techniques (FLIM-FRET, TR-FRET, and BiFC) enable the detection of oligomerization, short-range interactions, and subcellular localization in various cellular systems [159,164,254,255]. Ex situ biophysical analyses (AFM, FTIR, ssNMR, TEM, AUC, and CD) allow determination of the structural and physicochemical properties of oligomers, complementing in situ observations [84,254]. This combination of data from different methods and models provides a better link between the structural properties of oligomers and their potential toxicity and/or role in the pathogenesis of synucleinopathies. However, integrating such data into a single study remains limited, so developing multimodal and multimodel approaches should be an important direction for future research. Moreover, the reliable assessment of oligomer toxicity in both *in vitro* and *in vivo* models is needed [83,186]. Currently used protocols vary significantly between laboratories. Therefore, the direct comparison of research results is difficult. Various strategies for obtaining α-syn oligomers have been developed, including (a) ultrafiltration (centrifugal filtration) as a rapid separation method for aggregate mixtures [156], (b) chemical formaldehyde stabilization, which creates stable and immunoreactive calibration oligomers [259], and (c) induction in the presence of dopamine and lipid peroxidation products, such as 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) [260]. Each of these methods has advantages and limitations. The ultrafiltration approach improves the purity of the preparation, but controlling the concentrations in the mixture remains problematic [156]. Formaldehyde stabilization facilitates the creation of standards but may reduce the likelihood of preserving the natural properties of oligomers [259]. Cross-linking, for example, with HNE, ONE, or dopamine, allows the generation of stable calibrators, although their compatibility with pathological forms remains uncertain [260]. The presence of lipids, such as phosphatidylcholine and

Table 2
Integration of biophysical and neurobiological approaches for detection of α-syn oligomers.

Level	Biological system / model	Integrated methods	Outcomes	Limitations	Ref.
In vitro (biophysical + cellular)	Stabilized α-syn oligomers (A vs B) / anionic lipid SUVs / SH-SY5Y cells / primary cortical neurons	MAS ssNMR/CEST + FRET/FTIR + AFM + FCS	Imaging of oligomer co-localization with membranes/structures, associated with membrane disruption/cytotoxicity.	Synthetic/stabilized oligomers; limited native *in vivo* relevance (heterogeneity, chronicity).	[84]
In vitro (cellular/ in-cell)	HTB-148 (H4) cells / HA/V5-tagged α-syn co-expression (± Hsp70)	FLIM-FRET (HA/V5 tags; Alexa488/ Cy3) + detergent-sensitive co-IP/ Western	Intracellular oligomerization and conformation of α-synuclein	Indirect, proximity-based readout (relative—not absolute distances); fixed-cell snapshots; limited structural/stoichiometric resolution of oligomer species	[159]
	SH-SY5Y / PFFs seeding	DNA-PAINT microscopy (SMLM/SR) + conformation-specific MJFR-14-6-4-2 antibody	Super-resolution morphometry of seeded α-syn aggregates.	No direct molecular evidence that the observed nanoclusters are α-syn oligomers	[251]
	SH-SY5Y / ONE-stabilized α-syn oligomers	In cell NMR (HSQC/1D) + STED/ confocal co-localization + live MitoTracker imaging	Interactions of α-synuclein aggregates with cellular structures	Indirect, label-dependent readout; synthetic ONE-stabilized oligomers; limited native in vivo relevance.	[148]
	M17D inclusion model (αSyn-3 K:: YFP) / recombinant α-syn PICUP oligomerization	PICUP + SDS-PAGE/gel densitometry (oligomer bands) + M17D αSyn-3 K: fluorescence YFP puncta (inclusion readout)	Tested compound 46 reduces αSyn-3 K::YFP inclusion formation (anti-aggregation activity)	Indirect readout (αSyn-3 K::YFP puncta); synthetic PICUP-stabilized oligomers; limited native *in vivo* relevance	[252]
	M17D inclusion model (αSyn-3 K:: YFP) / recombinant α-syn PICUP oligomerization	PICUP + SDS-PAGE/gel densitometry (oligomer bands) + M17D αSyn-3 K:: YFP fluorescence puncta assay (inclusion readout)	Tested compounds reduce αSyn-3 K::YFP inclusion formation (anti-aggregation activity).	Indirect readout (αSyn-3 K::YFP inclusion puncta); PICUP-stabilized synthetic oligomers; limited native *in vivo* relevance.	[253]
	WT mouse primary hippocampal neurons + synthetic "kinetically trapped" recombinant α-syn oligomers.	AUC + TEM/AFM + CD/FTIR + ThT (± seeding) + A11/OC dot-blot	Oligomer uptake/ internalization	Indirect cellular readouts; no direct in-cell confirmation of oligomer identity/state; Synthetic "trapped" oligomers; limited native *in vivo* relevance	[254]
	HTB-148 (H4) cells / α-syn BiFC (GN–αSyn + αSyn–GC) ± Hsp70 / PD mutants	BiFC live-cell imaging + FACS (oligomer signal) + PCA (split Gaussia luciferase) validation + native-PAGE/ IB (high-MW oligomers)	Live-cell α-syn oligomerization detected; Hsp70 lowers oligomer/high-MW signal; oligomer signal associates with cytotoxicity	Indirect, tag-/overexpression-based readouts (BiFC/PCA) that trap/ stabilize interactions; no direct in-cell molecular/stoichiometric definition of oligomer species; limited native *in vivo* relevance.	[164]
	Dopamine-induced α-syn oligomer standard / A53T brainstem lysates (± HEK293T cell lysates for assay platform)	TR-FRET single-antibody oligomer assay (SynBa3-Tb/SynBa3-d2)	Detectable α-syn oligomer burden in A53T mouse brainstem lysates (absent for monomer controls)	Indirect readout; no size/ stoichiometry resolution or soluble-vs-fibrillar discrimination; no direct molecular validation of the detected species as α-syn oligomers	[255]
	Human post-mortem orbitofrontal cortex (PD vs control; Braak 4–5) + Line 61 (Thy1–α-syn) mouse brain	Aggregate-specific SIMOA (excludes monomer) + SiMPull single-molecule capture + DNA-PAINT/qPAINT super-resolution morphometry (length/ area/eccentricity; localization counts as mass proxy) + Bayesian/kinetic modeling of size distributions (production vs removal; "diseased-cell" fraction)	Aggregate morphometry/ mass quantification; model-inferred oligomer/aggregate clearance	Indirect, extraction/capture-biased; oligomer vs short fibril indistinguishable; *ex vivo* snapshot (no real-time)	[256]
Ex vivo	Human post-mortem anterior cingulate cortex (PD vs healthy controls; Braak stages incl. late-stage cohorts)	ASA–PD: autofluorescence quenching (Sudan Black) + high-NA single-molecule fluorescence microscopy (pS129 α-syn IF) + computational puncta detection/quantification; orthogonal validation: PLA + SEC-ELISA + seed amplification assay (SAA/ThT) + PK resistance; spatial mapping vs cell markers (neurofilament/P2RY12/GFAP/Olig2)	Large-scale single-molecule imaging quantifies nanoscale α-syn assemblies in human brain tissue	Indirect (antibody/threshold-dependent) readout; no stoichiometry; oligomers vs short fibril fragments indistinguishable; *ex vivo* snapshot (no real-time)	[257]
	Human CSF (PD vs healthy controls)	Oligomer-selective aptamer–DNA capture + quartz nanopore single-molecule ionic-current sensing	single-molecule detection of α-syn oligomers in minimally processed CSF	Indirect (aptamer/capture-biased); size inferred from current blockade (no stoichiometry); oligomers vs small fibrils not fully discriminated	[7]
	C57BL/6 J mouse striatal injection / Stabilized, kinetically trapped β-sheet α-syn oligomers	AUC + TEM/AFM + CD/FTIR (oligomer characterization) + pSer129 IHC/IF (α-syn pathology) + TH stereology (SNc dopaminergic neuron loss)	Oligomer uptake and spreading inclusion pathology in the brain	Probe-biased localization; no in situ oligomer identity/stoichiometry (oligomer vs short fibril undistinguished); Synthetic "trapped" oligomers; limited native *in vivo* relevance	[254]
In vivo	AAV8 co-expression of human α-syn split-VenusYFP (V1S + SV2): Sprague–Dawley rat SNpc (nigrostriatal pathway) + C57BL/ 6 mouse cortex (cranial window)	Fluorescent PCA (split-VenusYFP complementation) + *ex vivo* brain fluorescence imaging/confocal; *in vivo* two-photon imaging; PK sensitivity + α-syn IF (4B12) support	Visualization of α-syn oligomerization in the brain (split-Venus PCA puncta; in vivo two-photon and *ex vivo* mapping)	Indirect (tag-/overexpression-based) readout; no size /stoichiometry resolution; limited discrimination of oligomers vs other aggregate states in situ; no direct molecular validation of the detected species as α-syn oligomers	[258]

A11 – oligomer-specific antibody; **AAV8** – adeno-associated virus serotype 8; **AFM** – atomic force microscopy; **ASA–PD** – Advanced Sensing of Aggregates–Parkinson's Disease; **AUC** – analytical ultracentrifugation; **BiFC** – bimolecular fluorescence complementation; **CD** – circular dichroism; **CEST** – chemical exchange saturation transfer; **coIP** – co-immunoprecipitation; **CSF** – cerebrospinal fluid; **DNA-PAINT** – DNA-based point accumulation in nanoscale topography; **ELISA** – enzyme-linked immunosorbent assay; **FACS** – fluorescence-activated cell sorting; **FCS** – fluorescence correlation spectroscopy; **FLIM-FRET** – fluorescence lifetime imaging microscopy combined with FRET; **FTIR** – Fourier-transform infrared spectroscopy; **GFAP** – glial fibrillary acidic protein; **HSQC** – heteronuclear single quantum coherence; **IB** – immunoblot; **IF** – immunofluorescence; **IHC** – immunohistochemistry; **MAS ssNMR** – magic-angle spinning solid-state nuclear magnetic resonance; **MitoTracker** – mitochondrial fluorescent dye; **MJFR-14-6-4-2** – conformation-specific alpha-synuclein antibody; **NMR** – nuclear magnetic resonance; **OC** – oligomer conformation-specific antibody; **PCA** – protein complementation assay; **PICUP** – photoinduced crosslinking of unmodified proteins; **PK resistance/sensitivity** – proteinase K digestion resistance/sensitivity; **qPAINT** – quantitative points accumulation in nanoscale topography; **SAA** – seed amplification assay; **SEC-ELISA** – size-exclusion chromatography ELISA; **SH-SY5Y** – human neuroblastoma cell line; **SiMPull** – single-molecule pulldown; **SIMOA** – single molecule array; **SMLM/SR** – single-molecule localization microscopy / super-resolution; **SNc** – substantia nigra pars compacta; **STED** – stimulated emission depletion microscopy; **SUVs** – small unilamellar vesicles; **TEM** – transmission electron microscopy; **ThT** – Thioflavin T; **TR-FRET** – time-resolved Förster resonance energy transfer; **WT** – wild type; **YFP** – yellow fluorescent protein.

phosphatidylserine, can modify the secondary structure of oligomers. However, this can potentially increase their toxicity to cells, highlighting how experimental conditions shape the biological properties observed [119]. The diversity of these techniques results in oligomers with distinct structural and functional properties. Such structural and functional differences significantly complicate comparisons across studies. The lack of uniform and standardized protocols for obtaining α-syn oligomers is a major barrier to conducting consistent and reproducible studies [261–264]. Such limitations extend beyond oligomer preparation protocols and also affect downstream analytical and diagnostic assays. Current assays are primarily qualitative and have limited ability to differentiate between the specific synucleinopathies, such as PD, MSA, and DLB. The lack of assay standardization can affect performance and comparability. Research is focused on developing quantitative methods to better correlate assay results with disease severity and progression.

Existing findings also need further validation in longitudinal studies before they can become standard clinical practice [39,265–267]. Among the emerging platforms, aptamer-enriched nanopore sensing has demonstrated the detection of α-syn oligomers in CSF with minimal sample processing, achieving a limit of detection of approximately 2.2 pM and enabling discrimination between patients with PD and healthy controls based on ionic current signals [7]. Despite these promising analytical properties, nanopore-based platforms face several class-level barriers that must be overcome before clinical translation is considered. Fabrication reproducibility is sensitive to instrument-specific parameters, including laser settings, ambient humidity, and temperature, resulting in pore-size variability that directly affects signal interpretation [7]. The measurement conditions, including the non-physiological, high-ionic-strength buffers needed to maintain an optimal signal-to-noise ratio, differ significantly from the native CSF composition, and sample dilution or processing may alter the native oligomeric distribution [7,268]. The lack of standardized protocols across laboratories is an additional challenge, as nanopore geometries, carrier designs, aptamer sequences, and signal analysis pipelines vary widely between groups, making cross-study comparisons difficult and regulatory-grade validation challenging [269]. At the clinical level, all available studies are limited to small, cross-sectional cohorts. The approach reported by Liu et al. shows both the promise and the current limitations of this strategy. Although discrimination between PD patients and healthy controls was demonstrated in a double-blinded experiment using only 1.5 μL of unprocessed CSF, the cohort included only ten age-matched individuals, and measurements were made under highly controlled laboratory conditions that have not yet been replicated at scale [7]. There are no longitudinal data to support the use of nanopore sensing as a tool to monitor disease progression, and the technology has not been benchmarked against established synucleinopathy diagnostic criteria in a large, independently recruited, and neuropathologically confirmed patient populations. Integration with AI and machine learning tools represents a promising path to improve the reliability of data analysis. Convolutional neural networks (CNNs), such as QuipuNet, have been successfully applied to nanopore signal analysis in nucleic acid sequencing and related applications and represent a promising direction for future automation of nanopore-based protein analysis [270]. Similarly, SquiggleNet is a neural network capable of real-time classification of nanopore signals. For example, the growing potential of machine learning (ML)-based methods has been reported for analyzing signals with low signal-to-noise ratios (SNRs) in DNA sequencing [271]. It should be noted, however, that these AI-driven approaches have not yet been validated for α-syn proteoform classification in clinical specimens, and their translation from nucleic acid to protein analytes introduces additional complexity related to conformational heterogeneity and the absence of ground-truth labels for oligomeric subtypes. Therefore, integrating AI-based approaches represents a promising path to accelerate and improve the reliability of nanopore signal analysis, with potential future applications in α-syn proteoform research. Another significant challenge is achieving optimal integration of the sensor element, the organ/tissue, and processing algorithms to achieve high selectivity and remote signal recording [272].

The translational and analytical challenges outlined above point to a broader issue: the need to reconsider the conceptual framework within which α-syn oligomer data are generated and interpreted. Current approaches have advanced the detection of transient oligomeric species, their conformational transitions, membrane binding, and toxic effects. Yet, the field still tends to interpret these processes, particularly *in vitro*, through a relatively restricted model of the biological interface dominated by the interaction between α-syn and the lipid bilayer [273,274]. This narrowly defined interface approach has been effective in controlled membrane-mimetic systems, but it may be insufficient to explain why oligomeric assemblies that appear structurally similar by current analytical criteria produce divergent biological outcomes across experimental models and disease contexts.

In physiological conditions, however, α-syn is exposed not only to an isolated bilayer but also to a dynamic and chemically heterogeneous interfacial environment composed of lipids, proteins, ions, hydration layers, extracellular polymers, redox conditions, and surface architecture. The endothelial surface layer–glycocalyx complex has recently been discussed as a biologically relevant nano- to mesoscale interface that may contribute to barrier function, ion regulation, cell traffic, and interfacial biochemical organization [189,275]. In the context of PD, this approach has been extended to a hypothesis-generating framework linking gut-brain communication, microbiome-derived perturbations, glycocalyx disruption, ion imbalance, and redox-active interfacial processes [207]. These concepts are not to be seen as replacing α-*syn*-centered mechanisms of synucleinopathy but rather as highlighting that oligomer formation, persistence, seeding behavior, and toxicity may be modulated by a broader physicochemical environment that deserves further exploration.

The analytical techniques used to study α-syn oligomers may not faithfully report on the native solution-state species but can impose interfacial constraints that alter the measured ensemble. The observed size, stability, and toxicity of oligomeric assemblies are affected by surface chemistry, charge distribution, confinement, hydration, curvature, buffer identity, and sample history [273,275], and conventional

descriptors, such as pH, ionic strength, buffer composition, and electrostatic screening, may function as operational rather than absolute descriptors in complex interfacial systems, especially when specific ion effects and dispersion forces are taken into account [276,277]. Discrepancies between α-syn study outcomes may therefore reflect not only biological heterogeneity but also method-dependent modulation of oligomer populations.

Changes in bubble coalescence, hydrophobic interactions, electrolyte conductivity, and phase separation have been shown to be ion-pair specific and influenced by the gas state of solution at ionic strengths close to physiologically relevant values [273,278]. These findings should not be overinterpreted as a direct mechanism of α-syn aggregation, but they highlight variables that are rarely controlled in α-syn oligomer studies, despite the frequent use of agitation, sonication, microfluidics, nanopores, biosensor surfaces, and air-liquid interfaces. These variables may be relevant for an intrinsically disordered and amphipathic protein such as α-syn because they could influence the equilibrium among monomeric, oligomeric, protofibrillar, and fibrillar states [279].

A key objective for future work is to distinguish oligomeric features that are conserved across interfacial conditions from those induced or stabilized by experimental platforms and identify which features correlate reproducibly with cellular dysfunction. This distinction is particularly important because many current models rely on stabilized, synthetic, or surface-selected oligomeric species, whereas disease-relevant assemblies are likely to be transient, polymorphic, and embedded within complex biological microenvironments. Convergence across orthogonal nanoscale and surface-sensitive methods, reproducible oligomer preparations, standardized interfacial conditions, physiologically informed interface models, and biologically anchored toxicity endpoints will therefore be required to ensure that future progress better reflects the concept that α-syn oligomer pathogenicity is not encoded by structure alone but emerges at the interface between molecular conformation, surface chemistry, and neurobiological vulnerability.

In parallel, research interest is increasingly turning toward strategies that may modulate α-syn oligomer formation or stability. New avenues for potential intervention are emerging with the use of nanostructures. Computational and experimental studies have revealed that graphene-based nanomaterials exhibit such potential [280]. Graphene quantum dots (GQDs) can inhibit α-syn fibrillation and induce the disaggregation of existing amyloid fibrils, as confirmed by *in vitro* and *in vivo* experiments [132,281,282]. However, it should be emphasized that the majority of these findings derive from *in vitro* assays using recombinant α-syn or cell-free systems, and *in vivo* evidence remains limited. Critical questions regarding biocompatibility, blood-brain barrier penetration, and dose-dependent toxicity of graphene-based nanomaterials in the human central nervous system remain unresolved. Furthermore, the biological activity of these nanomaterials is sensitive to lateral dimensions, surface functionalization, oxidation state, and dispersing medium, complicating cross-study comparison. The path from experimental proof-of-concept to therapeutic applicability therefore requires systematic preclinical evaluation, including pharmacokinetic and toxicological profiling, before meaningful claims of clinical translatability can be made. These observations align with broader evidence demonstrating that graphene-based nanomaterials possess theranostic potential for targeting and biosensing pathological α-syn assemblies and supporting regenerative strategies [258]. Although these reports are promising, the use of nanomaterials is still in the very early stages of experimental studies. Thus, future work should integrate modern technologies, including standardized biological protocols, precise spectroscopic methods, advanced AI algorithms, and safe bioactive nanomaterials. This integration can enable the creation of multi-scale, dynamic, and clinically useful platforms for detecting, monitoring, and intervening in pathological forms of α-syn. Realizing this vision will require coordinated efforts toward method standardization, regulatory-grade validation, and cross-disciplinary collaboration between analytical chemists, neurologists, and clinical trialists. These advancements represent a step toward effective management of synucleinopathies.

6. Conclusions

In this work, we demonstrate that α-syn oligomers, which are critical pathogenic species in the synucleinopathy cascade, cannot be understood within a single experimental domain. Traditional approaches, developed independently, focus on the characterization of isolated oligomer conformations, optimization of detection platforms in cell-free environments, or the study of α-syn pathology without identifying the contributing molecular species. This fragmentation prevents the systematic exploration of relationships among oligomer structure, interfacial behavior, and cellular toxicity.

The structural insights obtained from biophysical investigations have revealed the conformational plasticity of α-syn. Early oligomeric intermediates populate a continuum of partially folded states with varying β-sheet content, stability, and aggregation propensity. Advances in single-molecule techniques have allowed the quantitative analysis of oligomer dynamics. Translating these structural insights requires understanding how conformationally distinct oligomers engage with interfaces.

Membrane composition, curvature, and charge modulate oligomer binding, insertion, and pore formation, while nanostructured materials and biosensing platforms provide insight into how surface chemistry shapes oligomer structure and stability. Membranes can promote conformational rearrangements that favor toxicity, while engineered nanointerfaces offer opportunities to detect, modulate, or destabilize these pathogenic conformers. Neurobiological studies demonstrate the harmful effects of α-syn assemblies on neuronal function and survival but do not fully define how distinct oligomeric species contribute to these events. In cellular and *in vivo* models, α-syn has been shown to disrupt mitochondrial respiration, perturb synaptic vesicle cycling, induce oxidative stress, and trigger sustained neuroinflammatory responses. Importantly, seeding-competent species, which may include oligomeric conformers, can propagate between cells and seed endogenous α-syn misfolding. The recent success of ultrasensitive seeding assays, such as RT-QuIC and PMCA, indicates the translational value of misfolded α-syn species and seeding activity as biomarkers for early diagnosis and differential discrimination among synucleinopathies.

A limitation in this research framework is that oligomer preparation and characterization are not yet standardized; therefore, reference preparations, consensus characterization methods, and cross-laboratory validation are needed. The evidence summarized in Table 2 also highlights another significant limitation of the current field. Although many studies may be considered integrated, many still rely on stabilized or synthetic oligomer preparations, overexpression models with tagged α-syn, indirect proximity assays, or aggregate-sensitive readouts that may not distinguish soluble oligomers from short fibrillar or protofibrillar species. Therefore, mechanistic conclusions derived from such approaches should be considered model-dependent rather than fully representative for native disease-relevant oligomer populations. Translational claims should also remain cautious until candidate platforms are validated in standardized, reproducible, and minimally perturbing systems with explicit discrimination of native oligomer populations from other aggregated species.

This limitation is not only technical but also conceptual, because detected oligomer species may depend partly on the preparation conditions and analytical interface used to observe them. α-Syn oligomer pathogenicity is likely not a function of molecular structure alone but an emergent phenomenon arising from molecular conformation, interfacial chemistry, and neurobiological vulnerability. Future studies should therefore aim to distinguish oligomeric features that are conserved across interfacial conditions from those induced or stabilized by analytical platforms, with particular attention to variables, such as

glycocalyx architecture, redox-active species, dissolved gas phenomena, and ion effects, which define the physiological environment of α-syn *in vivo* but are seldom controlled *in vitro*.

Moving forward, multimodal sensing platforms combining real-time interfacial binding kinetics, conformational fingerprinting, and single-molecule analysis with machine learning classification represent a promising direction for studying oligomer subpopulations.

In this work, we propose the structure-interface-toxicity nexus as the organizing principle for α-syn oligomer research. This conceptual framework links oligomer conformation to the broader physicochemical interface and its downstream neurobiological consequences. This integration offers an avenue for advancing conformer-specific therapeutics, deploying interface-based diagnostics, and moving synucleinopathy research toward predictive, mechanism-based approaches for clinical application.

CRediT authorship contribution statement

Magdalena Goncerz: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Błażej Rubiś:** Writing – original draft, Investigation, Formal analysis, Data curation. **Marta Szukalska:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ewa Totoń:** Writing – original draft, Methodology, Investigation. **Monika Nehra:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Ida Malesza:** Writing – review & editing, Visualization, Methodology, Investigation. **Patrycja Sujka-Kordowska:** Writing – review & editing, Methodology, Investigation, Data curation. **Barbara Jachimska:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Ewa Florek:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Edyta Mądry:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Sandeep Kumar:** Writing – review & editing, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Małgorzata Kujawska:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

This is a review article and the data reported in this manuscript is already available in public domain

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