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Investigating protein conformational ensembles by native mass spectrometry and complementary biophysical techniques—the case of α -synuclein

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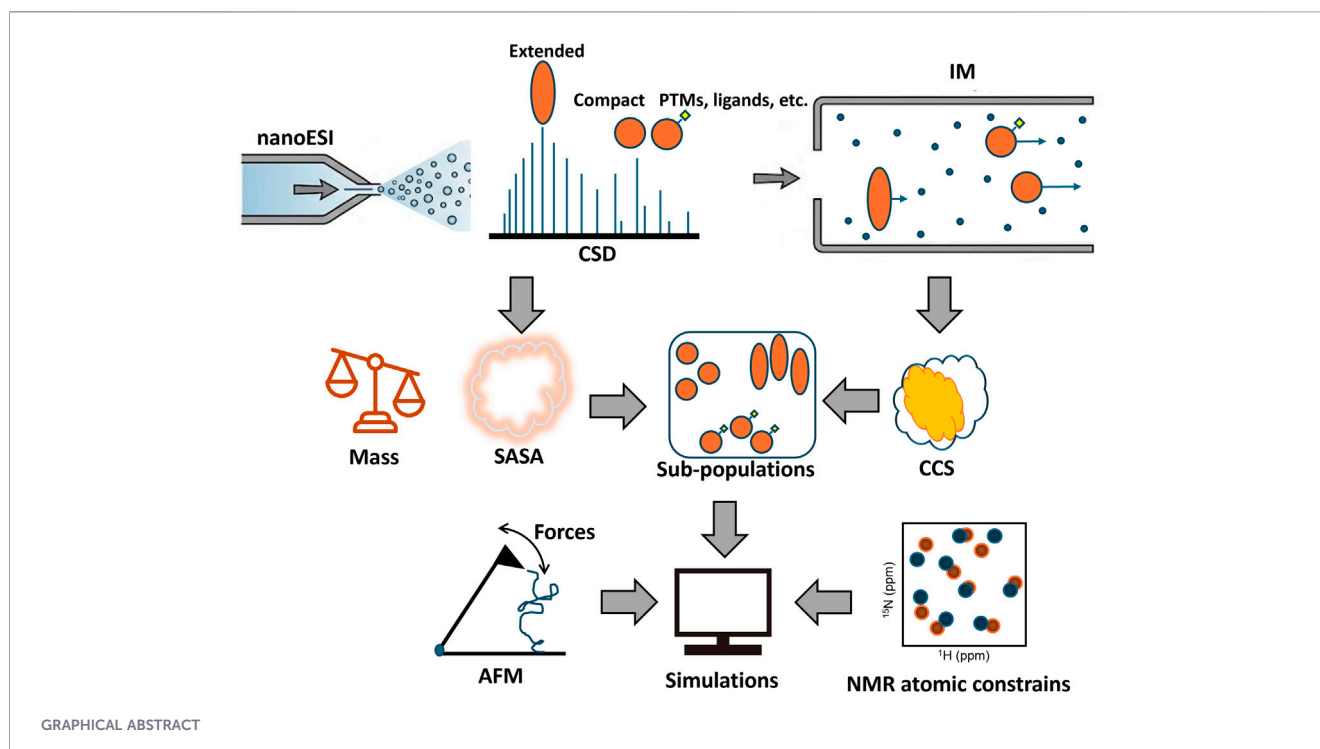
One of the challenges of modern structural biology is to account for protein structural dynamics. Intrinsically disordered proteins (IDPs), which do not fold spontaneously into a defined conformation, existing, instead, as highly heterogeneous conformational ensembles, provide an extreme and challenging example of protein dynamics. Native mass spectrometry (nMS) allows dissecting the molecular population in its main conformational components of different compactness, assessing structural parameters, covalent modifications, and binding states for each resolved species. This review summarizes the work performed by our research group in investigating the conformational states of human α -synuclein (α -syn), an IDP involved in Parkinson's disease (PD) and other neurodegenerative conditions, by applying nMS. This case study is taken as an example, highlighting the complementarity of nMS to other biophysical techniques, such as circular dichroism (CD), Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), atomic force microscopy (AFM), and molecular-dynamics (MD) simulations. This multi-technological approach is useful in analyzing the response of α -syn conformational ensembles to covalent modifications and noncovalent interactions with metal ions, small ligands, and other proteins.

KEYWORDS

atomic force microscopy, hybrid modeling, intrinsically disordered proteins, ion mobility, ligand binding, post-translational modifications, protein–protein interactions, solvent-accessible surface area

1 Introduction

Accounting for protein dynamics is one of the central challenges in modern structural biology. From enzymatic activity to amyloid aggregation, an understanding of the structural basis of protein function and dysfunction can no longer disregard conformational fluctuations (Sun et al., 2023; Niazi, 2025). This necessity has shifted the scientific focus from single, static structures to malleable conformational ensembles. Intrinsically disordered proteins (IDPs), lacking a well-defined, three-dimensional structure under physiological conditions, represent the most extreme example of this generally valid



principle (Lazar et al., 2025; Liu et al., 2025; Nussinov, 2025; Gusev et al., 2026; Stachowski and Fischer, 2026). Structural disorder confers specific advantages on the biological functions of IDPs. Indeed, it facilitates interactions with multiple partners (Alterovitz et al., 2020), rapid association kinetics (Wu and Zhou, 2019), rapid switching among multiple binding sites (Wang and Wang, 2019), independent tuning of affinity and specificity (Sharma et al., 2015), sensing of environmental variables (Devi et al., 2025), phase separation (Abyzov et al., 2022; Vendruscolo and Fuxreiter, 2022), regulation by modifications (Kulkarni et al., 2021) and intracellular degradation (Guharoy et al., 2016). Structural dynamics is also relevant to the functional role of the emerging class of proteins represented by microproteins (Chen et al., 2026).

Among IDPs, α -synuclein (α -syn) has attracted considerable attention due to its role in the pathogenesis of Parkinson's disease (PD), Alzheimer's disease (AD), and other neurodegenerative conditions (De Giorgi et al., 2022; Karamanos et al., 2022; Natalello et al., 2023; Ranjan and Kumar, 2026; Siebner and Uversky, 2026). α -Syn is a 140-residue protein, predominantly localized to the presynaptic terminals of neurons. It plays a role in neurotransmission by regulating vesicle trafficking (Wang et al., 2026) and promoting the assembly of the SNARE complex (Gao et al., 2023). The inherent plasticity of α -syn enables exploring a complex conformational landscape, with conformational distributions that are sensitive to both physiological and pathological stimuli. α -syn has been characterized in a soluble, monomeric, highly disordered state (Robustelli et al., 2018; Ch  rot et al., 2025), a helical membrane-bound state (Jacob et al., 2021; Kragelj et al., 2025), diverse oligomeric states (Ivanova et al., 2026; Jin et al., 2026), and a polymorphic, amyloid, fibrillar state enriched in β structure (Frey et al., 2024; Buell, 2026). While interconversion of α -syn between its soluble and membrane-

bound forms characterizes its physiological role (Das and Eliezer, 2019; Ch  rot et al., 2025), aggregation into oligomers, protofibrils, and fibrils can generate toxic species, leading to cell damage and disease (Rana et al., 2025; Vendruscolo, 2025). The bias toward different trajectories is dictated by rearrangements in the conformational ensemble. According to the thermodynamic hypothesis of protein aggregation, the protein native state is metastable, and even minor perturbations can push proteins toward amyloid aggregation (Vendruscolo, 2025). Therefore, it is of utmost importance to dissect disordered ensembles in their main conformational sub-populations and to understand how they rearrange in response to stimuli. Characterizing these dynamic states remains a significant hurdle as no single technique can fully capture the structural heterogeneity of an IDP. Consequently, biophysical methods have seen significant development, aiming to provide quantitative metrics—such as compactness, surface accessibility, strength of interactions, and secondary structure—that define the structural properties of the sub-populations (Karamanos et al., 2022; Lantz et al., 2023; Wagner and Gross, 2024; Byrd et al., 2025; Maciel et al., 2025; Nakandakari et al., 2025). In this context, the integration of experimental data with advanced computational modeling has emerged as an effective strategy to achieve the atomistic-level description of α -syn behavior in solution and within the cellular environment (Ghafouri et al., 2026).

A wide range of biophysical techniques have been used to describe the conformational ensemble of monomeric α -syn in solution, *in vitro*, and even *in cellula*. For instance, surface-enhanced Raman scattering (SERS) revealed a broad distribution of random coil, α -helix, and β -sheet conformations (Dai et al., 2021), while the integration of small angle X-ray scattering (SAXS) with the ensemble optimization method (EOM) and molecular-dynamics

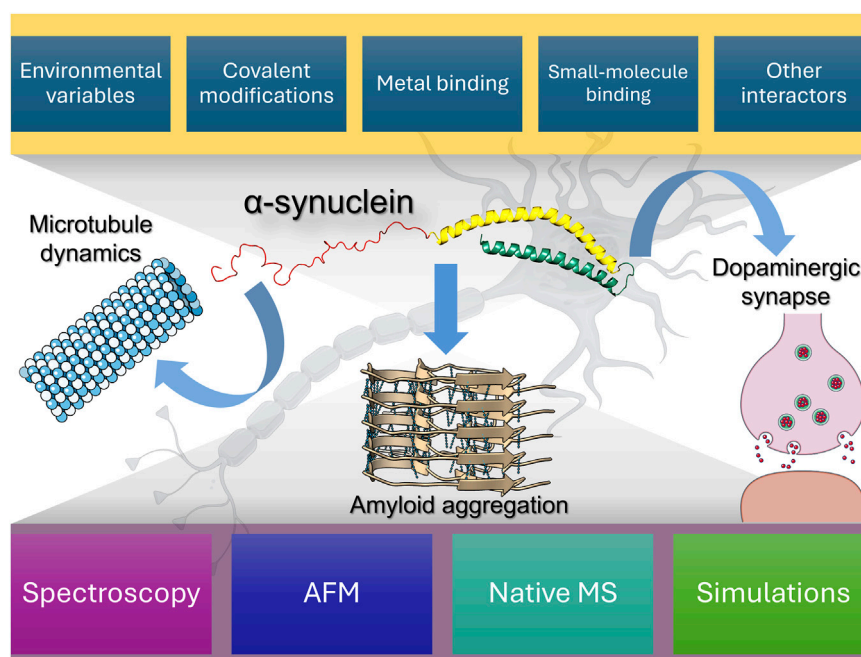


FIGURE 1
Schematic representation of the biological system and the biophysical methods discussed in this review.

(MD) simulations has revealed semi-extended and twisted conformations consistent with the experimental data (Dey et al., 2025).

Nuclear magnetic resonance (NMR) spectroscopy is a central tool in this field, offering a high-resolution view on structural changes in response to mutations, post-translational modifications, and binding (Dedmon et al., 2005; Kang et al., 2013; Masaracchia et al., 2020; Dyson and Wright, 2021; Gonzalez-Garcia et al., 2023; Brücke et al., 2025; Levorin et al., 2025). NMR methods specifically focusing on protein side chains deliver atomic information on protein–ligand complexes, mapping binding sites, and highlighting the nature of the interactions (Rodella et al., 2026; Turchi et al., 2026).

The integration of NMR with SANS, hydrogen–deuterium exchange (HDX), and MD simulations has shown that salt concentrations (Na^+ , Ca^{2+} , and K^+) significantly influence the accumulation of aggregation-prone conformations (Zacharopoulou et al., 2025). NMR has also been applied to studies on α -syn conformational states *in cellula* (Theillet and Luchinat, 2022). Pioneering experiments in bacterial (Waudby et al., 2013) and mammalian cells (Theillet et al., 2016) have shown that α -syn maintains a compact but disordered state inside the cytoplasm. This intracellular state appears to be functional to the cell as it shields aggregation-prone regions from the environment (Theillet et al., 2016). More recently, dynamic nuclear polarization (DNP)-assisted solid-state NMR on intact cells has added a spatial dimension to this description, identifying α -helix-rich sub-populations that accumulate at the cellular periphery (Kragelj et al., 2025). Furthermore, this technique has been applied for comparing the α -syn (A53T) fibril seeding process *in vitro*, in cellular lysates, and inside cells (Ansari et al., 2024). The results depict structural details, concerning not only the cross- β core of the

fibrils but also the surrounding flexible regions and reveal conformational selection peculiar to the in-cell propagated samples.

Mass spectrometry (MS) provides many different methods to IDP studies, with a major distinction between native MS (nMS) (Beveridge and Calabrese, 2021; Reid et al., 2023; Báez Bolívar et al., 2026) and conformation-dependent labeling (footprinting) approaches (Lermyte, 2020; Britt et al., 2022; Castel et al., 2023). Intact molecules and supramolecular complexes are analyzed by nMS, retaining noncovalent interactions *in vacuo* (Gavriilidou et al., 2022; Lawrence et al., 2025). In footprinting approaches, solvent-accessible regions of a protein are selectively modified in solution, and the location and extent of these modifications are subsequently identified by conventional MS methods (Wagner and Gross, 2024). Footprinting techniques include HDX (Pierangelini et al., 2025), fast photochemical oxidation of proteins (FPOP) (Wagner and Gross, 2024), cross-linking MS (XL-MS) (Ubbiali et al., 2022; Dollar and Webb, 2026; Yang et al., 2026), and limited proteolysis coupled with MS (LIP-MS) (Serdiuk et al., 2025) and are rapidly evolving toward *in-vivo* investigation (Lin et al., 2022; Piersimoni et al., 2022; Cheng and Gross, 2025; Balakrishnan et al., 2026). All these methods, collectively referred to as “structural MS”, exploit the unique advantages of MS in dissecting complex samples but, at the same time, suffer intrinsic limits in the resolution achievable in structural description. Therefore, a multitechnological approach is ideal to exploit the potential of structural MS (Britt et al., 2022).

Computational methods by MD and artificial intelligence (AI) offer an important complementary approach to experimental studies on IDPs. Improved force fields and sampling methods allow simulating entire conformational ensembles by MD and have been applied to α -syn and benchmarked against experimental results (Ahmed et al., 2021; Robustelli et al., 2022; de Bruyn et al., 2024; Semenyuk, 2024; Bera and Mondal, 2025; Lv

TABLE 1 Overview of the main biophysical techniques applied to the characterization of α -syn conformational ensemble, highlighting their specific advantages and limitations.

Technique	Observables	Structural information	Strength	Limitation
Native IM-MS	Mass-to-charge ratios and arrival-time distributions	Solution SASA and gas-phase CCS	Describing heterogeneous populations and transient intermediates	Limited structural resolution and gas-phase structural rearrangements
NMR	Chemical shifts, spin couplings, and relaxation parameters	Solution or solid state structure and conformational dynamics	Atomic resolution	Limitations on system size and species discrimination
AFM	Pulling force	Strength of molecular interactions	Single-molecule description	Limited structural resolution and sampling efficiency
CD/FTIR	Circularly polarized light absorption and infrared absorption	Solution or solid-state conformational features	Fast conformational assessment and chemical readout	Ensemble averaging and limited structural resolution
Computational simulations	Atomic coordinates and trajectories	Solution- and gas-phase structures and dynamics	High spatiotemporal resolution	Limitations on system size and conformational sampling

et al., 2025). AI methods predict well cooperatively folded proteins, but IDP ensembles pose limitations. The integration of metapredictor FiveFold with the Protein Folding Variation Matrix (PFVM) serves as an interesting approach to explore conformational space and account for structural heterogeneity (Niazi and Yang, 2025). MD simulations and ensemble docking procedures can also predict binding modes and binding affinities of ligands, guiding drug discovery and shedding light on recognition mechanisms of IDPs (Menon et al., 2024; Dhar et al., 2025). A recently developed Bayesian framework integrates structural constraints derived from AlphaFold2 with MD simulations to generate atomistic ensembles of IDPs (Schnapka et al., 2026).

This review summarizes the work performed by our group and collaborators on the characterization of α -syn conformational transitions and intermolecular interactions. α -syn is considered here as a case study and as an example of the application of complementary biophysical methods, with a focus on the interplay between experimental and computational approaches. This study does not serve as a comprehensive overview of nMS applications to IDPs, nor to α -syn itself. The following sections discuss quantitative descriptors of the α -syn conformational ensemble provided by nMS, atomic force microscopy (AFM), and solution spectroscopy (Section 2); its modulation by external factors, including covalent modifications, metal ions, small molecules, and protein–protein interactions (Section 3); and the integration of experimental and computational methodologies (Section 4). These contents are schematically represented in Figure 1. Table 1 provides a schematic, comparative overview of the biophysical methods discussed in this review and used to characterize α -syn conformational ensemble.

2 Main components of the α -syn conformational ensemble in solution

This and the following sections describe monomeric α -syn in solution as depicted by nMS, focusing on quantitative biophysical parameters, such as solvent-accessible surface area (SASA), compactness, and collisional cross-section (CCS), characterizing

the different conformers. Native MS allows conformational analysis of proteins, thanks to the dependence of protein ionization under electrospray conditions on fold state (Chowdhury et al., 1990; Chowdhury et al., 1991). Although many other factors, such as solvent components and instrumental parameters, affect the final net charge of protein ions during electrospray (Konermann et al., 2019; Sharif et al., 2025), substantial evidence indicates that protein conformation has, by far, the most impact (Abzalimov et al., 2012; Hall and Robinson, 2012; Kaltashov et al., 2013; Natalello et al., 2017; Vallejo et al., 2022). Most importantly, while performing comparative studies, operating under well-controlled conditions can reduce and even abrogate the interference of the environmental factors, highlighting the effects due to intrinsic features (Báez Bolívar et al., 2026).

Any protein conformer analyzed by electrospray ionization (ESI) yields a bell-shaped distribution of charge states (Figure 2A). Disordered conformational ensembles result in broad, multimodal charge-state distributions (CSDs) that can be deconvoluted by Gaussian fitting upon transformation to the $x = z$ abscissa axis, dissecting the heterogeneous population into sub-populations (Figure 2B) (Dobo and Kaltashov, 2001; Mohimen et al., 2003; Borysik et al., 2004; Santambrogio et al., 2022). Each component represents one of the main protein conformational states and can be characterized by its average charge state and relative intensity. The ESI process is achieved by nebulizing a liquid sample through a capillary in the presence of an electric field (Kearle, 2000). nMS is normally performed using capillaries with micrometric, or even sub-micrometric emission tip (nano-electrospray ionization, nESI), allowing for mild settings of the instrument interface (Elliott et al., 2017; Susa et al., 2017; Österlund et al., 2025; Byrd et al., 2026b; Byrd et al., 2026a). Under such conditions, the noncovalent interactions responsible for structure and for binding can be preserved and may manifest themselves in the spectra by affecting ion charge states and/or masses. CSD analysis probes the global structural compactness of the ionized proteins. The higher the structural compactness, the lower the average charge (Chowdhury et al., 1991; Mirza et al., 1993; Grandori, 2003; Santambrogio et al., 2022). These effects can be

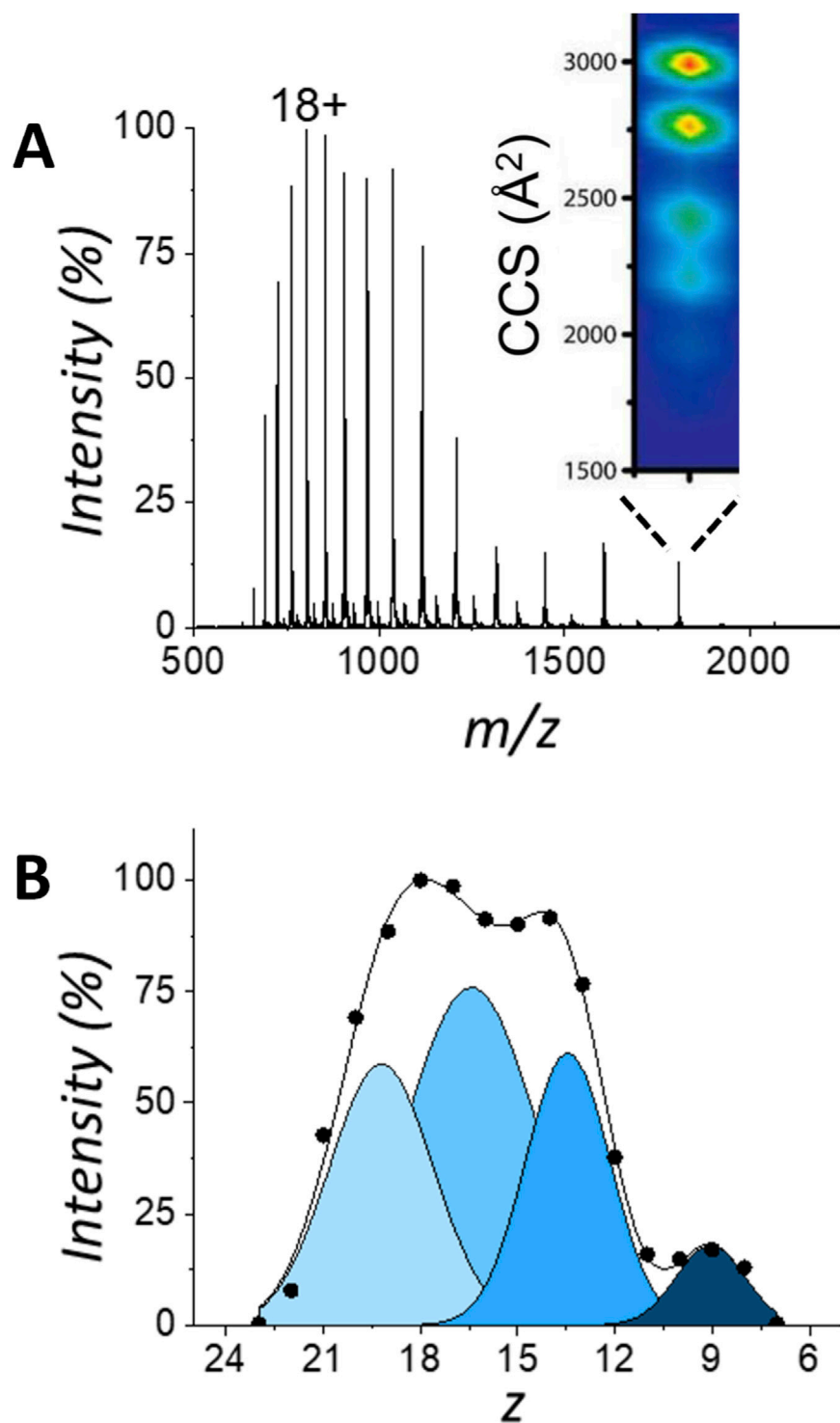


FIGURE 2
CSD analysis. **(A)** nMS spectrum of human α -syn in the positive-ion mode. The most intense peak is labeled by the corresponding charge state. The inset shows the CCS values for the conformers detected in the $8+$ charge state by IM-MS. **(B)** Multi-Gaussian deconvolution of the spectrum shown in panel **(A)**. Adapted from Santambrogio et al., 2022; Ponzini et al., 2019.

exploited in either positive- or negative-ion mode. Other experimental parameters such as sample-source conditions (Gabelica et al., 2019), buffer composition (Stuchfield et al., 2018), nonvolatile salts (Báez Bolívar and McLuckey, 2025), supercharging effects and agents (Zhang et al., 2025), and in-

source dissociation energy (Lakshmanan and Loo, 2019) are critical for IDP measurements. Controlling these factors improves the interpretation and reproducibility of nMS data.

This approach was initially applied to the investigation of α -syn conformational ensemble in the negative-ion mode at neutral

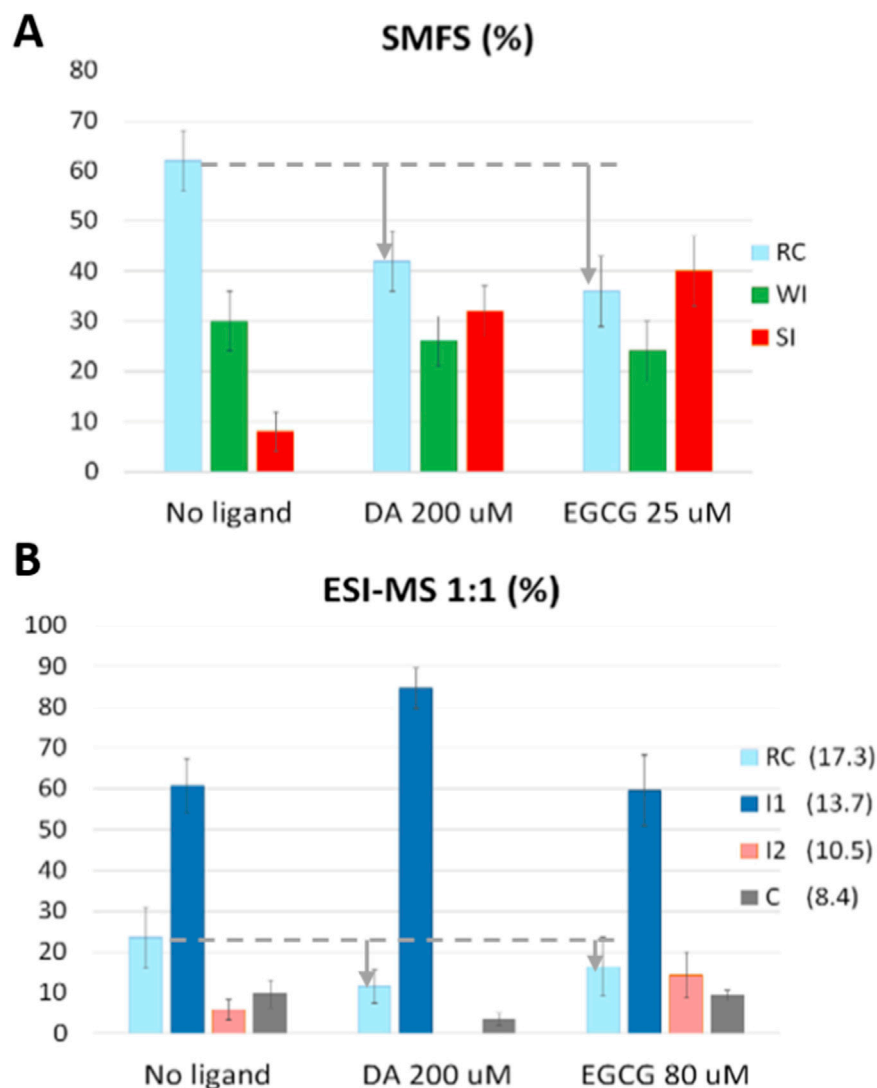


FIGURE 3
Comparison between nMS and AFM. α -syn species distributions in the presence of DA (200 μ M) or EGCG (25 μ M for AFM, 89 μ M for nMS), as obtained by (A) AFM and (B) nMS. The intensity-weighted average charge of the peak envelopes is reported in brackets for each component. Error bars in panel (A) represent the standard deviation calculated from the normal distribution used to fit the histograms of AFM plots. Error bars in panel (B) represent the standard deviation from three independent experiments. Adapted from Corti et al., 2019.

pH since α -syn is a slightly acidic protein (Natalello et al., 2011). At least three components - compact, fully unfolded, and partially folded - could be detected by Gaussian fitting by titrations with denaturants and cosolvents. Different folding intermediates accumulate under different solvent conditions. Thus, it is possible to visualize how varying the solvent conditions reshapes the conformational ensemble. Similar results were then obtained in the positive-ion mode, resolving two distinct intermediates in the partially folded state (Konijnenberg et al., 2016), in agreement with data reported by other groups (Frimpong et al., 2010). Although absolute quantitation can be biased by differences in ionization efficiency (Aliyari and Konermann, 2020) and instrument polarity, this method can effectively identify the main conformers and also provide relative quantitation and describe transitions by comparative analyses. These studies pointed out the existence of partially folded states of pure α -syn in solution under non-

denaturing conditions, a conclusion that is consistent with other evidence in the literature reported at that time and accumulated afterward. Based on the good agreement between the negative- and positive-ion mode, the latter was selected for further studies. Analyzing a protein in an ionization polarity opposite to its solution-phase net charge ("wrong-way around" ionization) should always be subjected to such a comparison since it can destabilize the structure (Grandori, 2003; Samalikova and Grandori, 2003; Edwards et al., 2026).

The identification of partially folded intermediates of IDPs is of relevance for basic studies on protein folding and also for functional studies. Even minor, transient states could elicit biologically relevant processes such as molecular recognition or amyloid aggregation. Therefore, it is of general interest to detect the distinct conformational components and monitor how they switch between states under changing environmental conditions. In

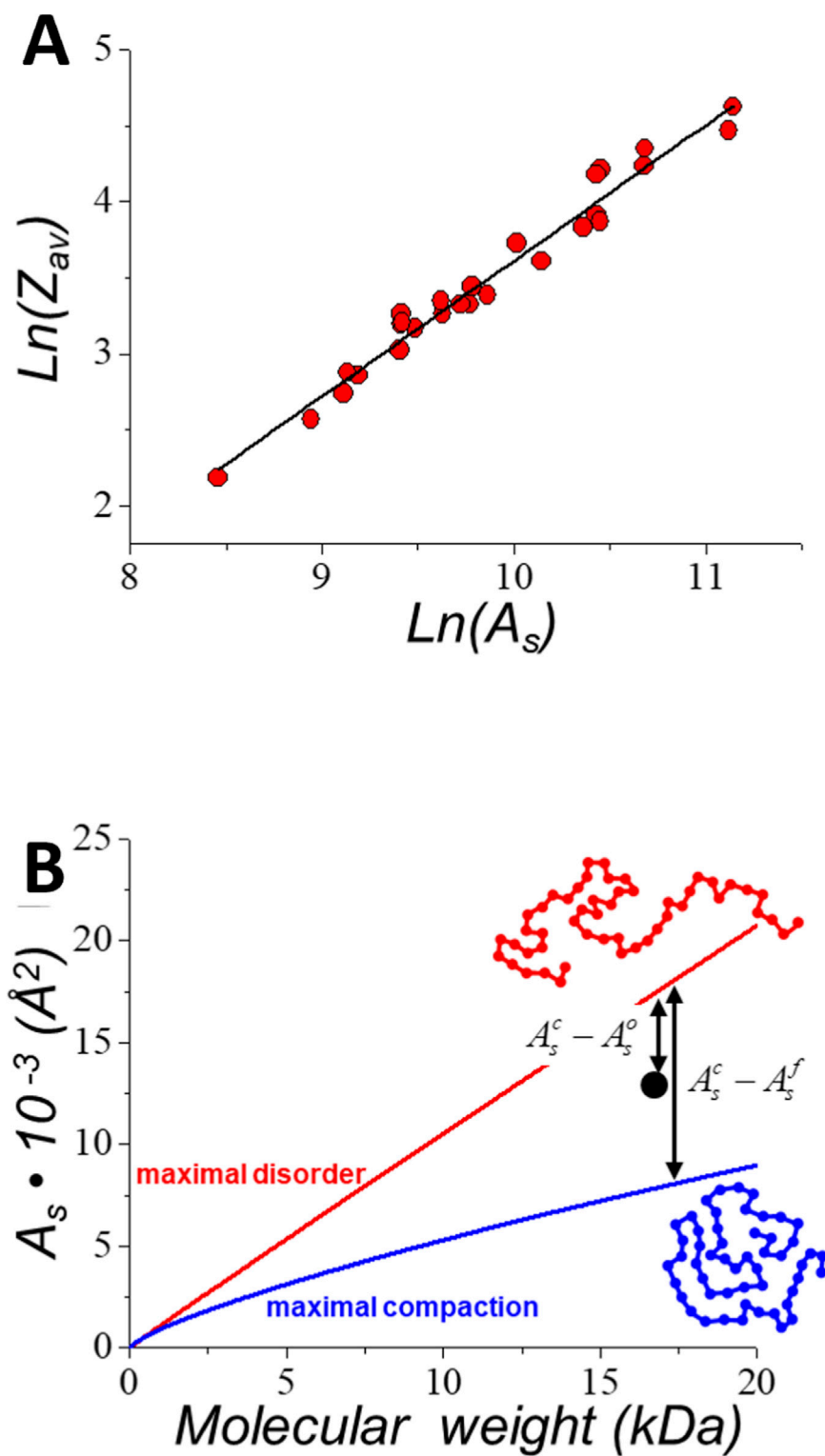


FIGURE 4

Extracting structural parameters by nMS. (A) Logarithm of the average charge obtained by nMS, against the logarithm of the calculated SASA value, for a set of fully disordered proteins. The line represents the linear fitting of the results. (B) Relationship between SASA and molecular weight for globular proteins (blue curve) and fully disordered proteins (red curve). Adapted from Santambrogio et al. (2019).

particular, species that accumulate under amyloidogenic conditions could be important actors of the pathogenic process and can be further characterized by low-resolution and high-resolution methods. Native MS studies have identified α -syn conformational components that accumulate under conditions triggering amyloid aggregation (Natalello et al., 2011; Moons et al., 2020; Byrd et al., 2023; Byrd et al., 2025; Báez Bolívar et al., 2026) and represent promising candidates for computational modeling. It is interesting to note that these conformational intermediates are quite specific for each tested condition. Folding intermediates (of either ordered or disordered proteins) are also targets of innovative drug design strategies (Biasini and Faccioli, 2025). Furthermore, transient conformational states could correlate with transient exposure of cryptic sites, an intriguing aspect in the biology of IDPs and post-translational modifications (PTMs) (Das et al., 2012; Das et al., 2016; Gasparotto et al., 2025).

Protein conformational analysis by nMS, however, should be performed with caution, particularly in the case of IDPs. Indeed, the peculiar conditions of electrospray ionization could denature protein structures, as well as induce collapse of unfolded chains, creating artifacts in either direction of non-native rearrangements. Furthermore, the different mechanisms that might control the ESI process of folded and unfolded proteins could introduce additional uncontrolled effects (Aliyari and Konermann, 2020). Nonetheless, studies by temperature-variable nMS show good agreement with calorimetry in assessing thermodynamic parameters for protein stability (Wang et al., 2018; Laganowsky et al., 2022). In order to test the reliability of the results described above, the α -syn conformational ensemble has been investigated using an orthogonal biophysical method, such as AFM (Corti et al., 2019). By this approach, the protein is immobilized on a surface soaked by the desired solvent, and individual molecules are engaged and pulled by the tip of a cantilever, measuring the force required to achieve mechanical unfolding. In analogy with the ion-sorting process in MS, surface scanning by a high number of events by AFM can depict a conformational ensemble, dissecting its main sub-populations. Thus, AFM probes the strength of intramolecular interactions in solution at the single-molecule level.

Comparative analysis of nMS and AFM (Corti et al., 2019) has shown that AFM detects structural heterogeneity of pure α -syn at neutral pH, including highly structured and partially folded states, in agreement with other single-molecule analyses of this protein (Brucalé et al., 2012; Dai et al., 2021). Although the precise quantitation of the conformational components by AFM and nMS differs to some extent, this comparison shows that compact α -syn conformations at neutral pH and in the absence of interactors are not artifacts of ESI-MS (Figure 3). Direct quantitative comparison should be performed with caution since the two techniques measure different structural properties.

2.1 Solvent-accessible surface area

The effect of structural compactness on protein ionization during electrospray has been systematically evaluated with reference to experimental structural models of folded proteins and *in-silico* simulated conformational ensembles of unfolded

proteins or IDPs (Kaltashov and Mohimen, 2005; Testa et al., 2011; Hall and Robinson, 2012; Li et al., 2016). SASA emerges as the structural feature that correlates with the acquired net charge during electrospray, regardless of the conformational state (Figure 4A). Charge dependence on mass, instead, is strongly influenced by the folding state. Thus, each detected conformational component can also be quantitatively characterized by calculating the SASA from the established empirical equations. In the case of α -syn, the values fall in the ranges of 72–80, 105–150, 175–235, and 265–310 nm² for the four components resolved in the positive-ion mode. It is interesting to point out that the value obtained for the conformer of highest compactness approximates the average value of globular proteins of the same size, indicating a high degree of compaction. These estimates will provide better accuracy, along with the accuracy of the computational models of disordered ensembles used to build the correlation function.

2.2 Structural compactness

The SASA values obtained as described above can be used in relation to molecular weight in order to evaluate the degree of protein compaction. A normalized parameter, the compaction index (CI), measures the extent of compaction relative to the whole possible excursion from unfolded to compact states for a chain of a given size (Figure 4B) (Brocca et al., 2011; Uversky et al., 2012; Santambrogio et al., 2019). Such a parameter could be calculated from any structural feature linked to compactness (i.e., gyration radius or SASA) based on empiric or theoretical reference curves describing the dependence of that particular structural parameter on protein size for folded and unfolded proteins. The CI ideally varies between 0 and 1, respectively, for minimal and maximal compaction. The normalization introduced by the CI allows for comparison of the compaction degree among proteins of different lengths and offers a tool for IDP classification on a *continuum* scale. The CI value can be computed on each conformational component detectable by deconvolution of the nMS spectra or as the weighted average over the whole conformational ensemble.

The ensemble value for pure α -syn in aqueous solution at neutral pH is 0.33, consistent with a highly disordered chain. However, the individual values of the four distinct components are 0.99, 0.76, 0.32, and -0.1, suggesting that the protein exists as a heterogeneous and dynamic conformational ensemble spanning different degrees of structural compactness (Santambrogio et al., 2019).

2.3 Collisional cross-section

Additional structural characterization by nMS can be obtained by ion mobility (IM) measurements (Webb, 2022; Naylor and Nagy, 2025). A dedicated module of the instrument separates ions based on their mobility through a buffer gas. Different instrumental platforms are available: drift tube ion mobility spectrometry (DTIMS), traveling wave ion mobility spectrometry (TWIMS), trapped ion mobility spectrometry (TIMS), differential mobility analyzers (DMA), and field asymmetric waveform ion mobility spectrometry (FAIMS) (Butalewicz and Brodbelt, 2025). Upon selection of adequate sets of calibrants, accurate charge-state matching, and calibration procedures (Gabelica et al., 2019;

Krasnova et al., 2025), IM-MS yields the rotationally averaged CCS for every ion detected, providing an additional structural descriptor besides mass, charge, CI and SASA (Christofi and Barran, 2023). IM measurements can resolve slightly different conformations even within the same charge state. Such species are characterized by similar overall compactness but slightly different geometry. This two-dimensional separation of protein ions in the gas phase is a particularly powerful approach in describing heterogeneous molecular ensembles (Mitra, 2019; Beveridge and Calabrese, 2021; Britt et al., 2022; Lantz et al., 2025).

For α -syn at neutral pH, four distinct conformers can be resolved by TWIMS within the compact form (8+ ion) (Figure 2A), and two conformers can be identified within the partially folded intermediates (11+/13+ ions) (Konijnenberg et al., 2016; Daniels et al., 2019; Ponzini et al., 2019). IM-MS was used to compare the conformational ensembles of modern human α -syn with ROOT α -syn, the predicted ancestral sequence of α -, β -, and γ -synucleins (Sam et al., 2025). The results indicate that the ancestral sequence has a more limited conformational heterogeneity, suggesting that α -syn conformational freedom has expanded along the evolution. This acquired feature goes along with increased aggregation propensity.

In conclusion, nMS and IM-MS depict conformational ensembles by identifying the main components and estimating their relative abundance and by providing quantitative structural descriptors on each component. This information can be used to guide computational modeling by structural constraints or to filter results obtained by MD simulations. This approach can be applied to investigate changes in the ensemble, in response to solvent conditions, mutations, covalent modifications, and binding. Most importantly, all these effects can be described in a species-specific manner, giving insights into the functional properties of the identified conformational components.

In exploiting these powerful aspects of the method, we should always be aware of the risk of triggering non-native protein conformations (Cheung See Kit et al., 2023). This concern is even stronger in IM-MS than CSD analysis. While CSDs carry a footprint of the preexisting structures in solution, IM directly measures gas-phase protein features (Cheung See Kit et al., 2023). Thus, comparison with other experimental methods and combination with computational simulations is of utmost importance. On the other hand, the peculiar conditions of IM-MS help investigate the role of solvents on protein conformational ensembles, as recently described for α -syn (Cheung See Kit et al., 2023). This study shows an expansion of the gas-phase vs. solution conformational landscape of α -syn, suggesting that the ions observed under these conditions do not simply represent kinetically trapped conformers of the original solution.

3 Modulation of α -syn conformational ensemble by external factors

We examine in this section how the α -syn conformational distribution is modulated by external perturbations, including covalent modifications, metal ions, small molecules, and protein–protein interactions.

3.1 Covalent modifications

Modifications affecting chain mass can obviously be accurately determined by MS. nMS offers the unique advantage of monitoring protein conformation and modification distinctly, simultaneously, and relating conformers to proteoforms (Zhou et al., 2020; Reid et al., 2023; Saikusa, 2024; Lawrence et al., 2025). Thus, it becomes possible to investigate the conformational effects of the modification, not only by comparing the fully modified and unmodified proteins but also dissecting heterogeneous ensembles derived from sub-stoichiometric modifications.

Thanks to the high accuracy and resolution of MS instrumentation, the oxidation state of cysteine residues can be monitored easily, by the loss of 2 Da in protein mass for each disulfide bridge formed, for example, the α -syn double-Cys mutant (V71C and T92C), designed to introduce an intramolecular disulfide bridge, stapling together the ends of the NAC region and mimicking the contact observed in the Greek-key motif of mature α -syn fibrils (Carija et al., 2019). While mass deconvolution yields the extent of oxidation in the molecular population, CSD depicts the two distinct conformational ensembles of the reduced and oxidized proteins. This analysis shows that formation of the disulfide bridge induces an overall shift of α -syn ensemble toward more compact conformations. In particular, there is an accumulation of the more compact intermediate at the expense of the less compact one and the fully extended state. These changes in the conformational ensemble described by nMS are redox-sensitive and in agreement with 2D NMR data, showing that the oxidized proteoform populates distinct and more compact conformational states (Carija et al., 2019). The oxidized mutant protein also shows a much lower propensity for aggregation, indicating that a flexible and unconstrained NAC region is important to promote the formation of α -syn amyloid structures and that the α -syn conformational ensemble can be biased toward conformations that are not competent for aggregation. Consistently, the disulfide-containing protein displays reduced neurotoxicity. Furthermore, it inhibits aggregation of wt α -syn even at sub-stoichiometric concentrations, indicating that the mutation interferes with the nucleation and/or elongation steps.

Another example is offered by α -syn methionine oxidation. This modification is known to take place *in vivo*, under oxidative stress conditions, and can be reproduced *in vitro* by incubation with agents promoting the formation of reactive oxygen species (ROS) (Binolfi et al., 2016; Rodríguez et al., 2016; Carmo-Gonçalves et al., 2018; Ramis et al., 2021; Uceda et al., 2023). This is the case with either dopamine (DA) or epigallocatechin-3-gallate (EGCG) (Ponzini et al., 2019). These compounds, in aqueous solutions and in the presence of oxygen, lead to production of H_2O_2 and eventually to accumulation of $HO\cdot$ by Fenton chemistry.

α -syn contains four methionine residues, mapping at positions 1, 5, 116, and 127. The oxidation of these side chains to sulfoxide in different combinations has been shown to redirect the aggregation pathway of the protein from amyloid fibrils to SDS-resistant oligomers with variable degrees of toxicity (Leong et al., 2009). The overall *in vitro* oxidation kinetics in the presence of DA or EGCG has been monitored on the full-length protein under denaturing conditions (Ponzini et al., 2019). The site-specific oxidation kinetics has been determined by applying a bottom-up

approach. Comparison among the four Met-containing peptides shows very similar profiles, with a slightly lower susceptibility of Met 127 relative to the other target sites. This difference is likely due to the intrinsic structure of the protein, which has been shown to promote bent conformations of the C-terminal region (Appadurai et al., 2023). This intrinsic structure surrounding Met 127 has been implicated in the emergence of ligand-binding sites involving a cluster of tyrosine side chains corresponding to positions 125, 133, and 136 (Appadurai et al., 2023; Dhar et al., 2025).

The intact protein has been analyzed by nMS showing that the fully oxidized form has similar conformational properties as the non-oxidized species under conditions that stabilize either highly disordered or highly ordered states of α -syn (Ponzini et al., 2019). Nonetheless, circular dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopies show that α -syn oxidation inhibits the intrinsic propensity of the protein to acquire an ordered secondary structure (either α or β) in response to alcohols and dehydration (Ponzini et al., 2019). This effect could be related to the impact of methionine oxidation on α -syn aggregation properties and aggregation pathways. It should be pointed out that nMS secondary structure is not directly affected by protein content. Therefore, integration by methods describing the secondary structure, even at low resolution as CD and FTIR, can provide useful complementary information, as exemplified by this work.

3.2 Metal binding

Metal ions are known to affect α -syn aggregation properties, exerting variable effects, depending on the concentration and oxidation state (Moons et al., 2020; Byrd et al., 2025). Cu^{2+} binds with high affinity and stimulates α -syn oligomerization and fibrillation. nMS was employed to investigate the conformational effects of this metal ion on the monomeric protein in solution (Natalello et al., 2011). Formation of the 1:1 complex induces a drastic conformational change, shifting the α -syn ensemble toward the compact state. A comparison between the extracted conformational components of the free and bound protein indicates an increase in the compact state from ~10% for the free form to ~50% for the 1:1 complex, at neutral pH. Although higher stoichiometries can be observed, only the first binding event seems to be driven by specific and high-affinity interactions. Furthermore, it accounts by itself for most of the conformational effects observed in the presence of copper. These results are in agreement with IM-MS and NMR data, showing compaction of the protein tertiary structure upon Cu^{2+} binding (Miotto et al., 2017; Moons et al., 2020).

This study exemplifies one of the most relevant aspects of binding analysis by nMS, that is, retrieving species-specific information from complex molecular ensembles generated by linked equilibria of folding and binding transitions. Since mass reports on binding and charge reports on folding, the combined data can dissect the population in the different binding states of each resolved conformational component. This detailed information is important to describe conformational selectivity of ligands, relative affinity, and conformational effects, besides, of course, stoichiometry, of each resolved conformer. More examples are described in the following paragraphs, concerning interactions with small molecules and other proteins.

3.3 Small-molecule binding

DA and EGCG are known to inhibit α -syn fibrillation and redirect its aggregation pathway toward the oligomeric state. However, the oligomers that accumulate in the presence of each ligand are different, being toxic in the presence of DA and nontoxic in the presence of EGCG (Ehrnhoefer et al., 2008; Martinez-Vicente et al., 2008; Choi et al., 2013). The binding of these molecules to α -syn in solution at neutral pH has been investigated by nMS (Konijnenberg et al., 2016).

Titration at increasing ligand concentrations show opposite conformational selectivity of the two compounds. While EGCG binds preferentially to α -syn in the compact state, DA binds to open conformations, with highest apparent affinity for the less structured intermediate. In addition, the ligands also shift the conformational ensemble toward the compact or disordered state. Results consistent with this conformational effect of DA binding are reported by other nMS studies on α - and β -synuclein (Illes-Toth et al., 2013; Luise et al., 2021), whereas structural compaction has been observed upon α -syn interaction with cyclized nordihydroguaiaretic acid (NDGA), another polyphenolic inhibitor of α -syn aggregation, analogously to the EGCG effect (Daniels et al., 2019). These distinct conformational selectivities and conformational effects of the two ligands are likely at the basis of the different structures of the oligomers that accumulate under the two conditions. However, the relationship between compactness and aggregation propensity in amyloid proteins is nuanced as compact conformations are not universally protective nor extended states universally amyloidogenic. Furthermore, overlapping factors, such as solubility, likely play a role, as shown for the α -syn-EGCG complex (So et al., 2021). In α -syn, compact states driven by the interaction between the N- and C-terminal regions are considered to be protective (Bertoncini et al., 2005; Dedmon et al., 2005; Ubbiali et al., 2022). Nonetheless, partially collapsed states might generate kinetically trapped intermediates that promote aggregation (Nadwa et al., 2025). The universal threat of amyloid aggregation has been interpreted by a general mechanism of collapse under conditions promoting hydrophobic interactions and hydrogen bonding but preventing the formation of the native state (Goto et al., 2018).

This system has been analyzed also by IM-MS, revealing the same trend as described above, even within individual charge states (Konijnenberg et al., 2016). In particular, the 8+ population of the compact state redistributes itself in favor of the most compact conformer as the EGCG- α -syn ratio increases. On the contrary, the 11+ intermediate shifts toward the highest CCS along the titration by DA. The reliability of the observed complexes is supported by control experiments with ligand analogs and mock proteins, indicating that they are not artifacts generated by the ESI conditions. Furthermore, the concentrations of the analytes were all well below the empiric threshold of 100 μM , above which nonspecific complexes are easily observed by ESI-MS (Daubenfeld et al., 2006; Bui et al., 2026). Such a generally observed phenomenon is related to the ESI mechanism and can be accounted for during quantitative analysis of the data (Sun et al., 2006; Guan et al., 2015; Bui et al., 2026).

While EGCG binds without defined stoichiometry, a 1:3 model fits best the observed interactions with DA (Konijnenberg et al., 2016). The position of the binding sites in the 1:1 complex was explored by applying top-down fragmentation by electron-transfer dissociation (Webb, 2022). In spite of the limitations due to the low spatial resolution, side reactions, and possible structural rearrangements, this method can be applied to map the binding sites of noncovalent ligands (Lermyte et al., 2018; Łacki et al., 2019). Reaction with radical ions introduces random breakages in the peptide backbone, while maintaining most noncovalent interactions and preventing the dissociation of the ligand. Thus, the masses of the products reveal which fragments still contain the bound ligand and which do not, allowing to map the interaction sites by a sort of bracketing method. In agreement with NMR evidence (Jha et al., 2017; Pandey et al., 2023), EGCG has no specific binding site, interacting with the protein in variable positions along the whole peptidic chain. DA, instead, displays more specific interactions, involving preferentially the C-terminal region. In particular, the first binding event can be localized in the surroundings of the 125YEMPS129 region, as predicted by simulations (Herrera et al., 2008) and in line with NMR measurements (Pandey et al., 2023).

In a comparative study, the response of α -syn to these ligands has also been investigated by AFM. The same trend of changes has been observed in the conformational ensemble, with a reduction of structural disorder upon binding (Figure 3). However, the distinction between highly compact and partially folded states does not emerge from the AFM data. Both ligands stabilize the “strong interactions” of α -syn conformers (Corti et al., 2019). These apparent discrepancies between the two techniques could be interpreted by the application of computational methods, as described in Section 4.1.

3.4 Protein–protein interactions

α -syn is known to interact with many proteins and cellular structures, including microtubules (Baghel et al., 2026; Flores-León and Outeiro, 2026; Lougee et al., 2026; Quan et al., 2026). These interactions are relevant to its physiopathological role. Our research group has participated in a multi-technological investigation of the interplay between α -syn and tubulin (Cartelli et al., 2016). Direct binding between the two proteins has been tested *in vitro* by complementary biochemical and biophysical techniques (Cartelli et al., 2016). nMS has shown that α -syn forms a 1:1 complex with the tubulin $\alpha_2\beta_2$ tetramer and failed to detect an interaction with the $\alpha\beta$ dimer, providing direct evidence for a specific interaction with unpolymerized tubulin and defining complex stoichiometry. These findings are consistent with NMR diffusion coefficient measurements, indicating that the two proteins directly interact in solution (Cartelli et al., 2016). Moreover, the CSD observed for the complex by nMS is compatible with a compact supramolecular assembly, suggesting a structural rearrangement and induced folding of α -syn upon tubulin binding. This interpretation was further supported by far-UV CD measurements showing acquisition of α -helical structure in the complex, relative to the isolated proteins (Cartelli et al., 2016).

Collectively, these observations support a model, in which tubulin binding induces conformational reorganization of α -syn

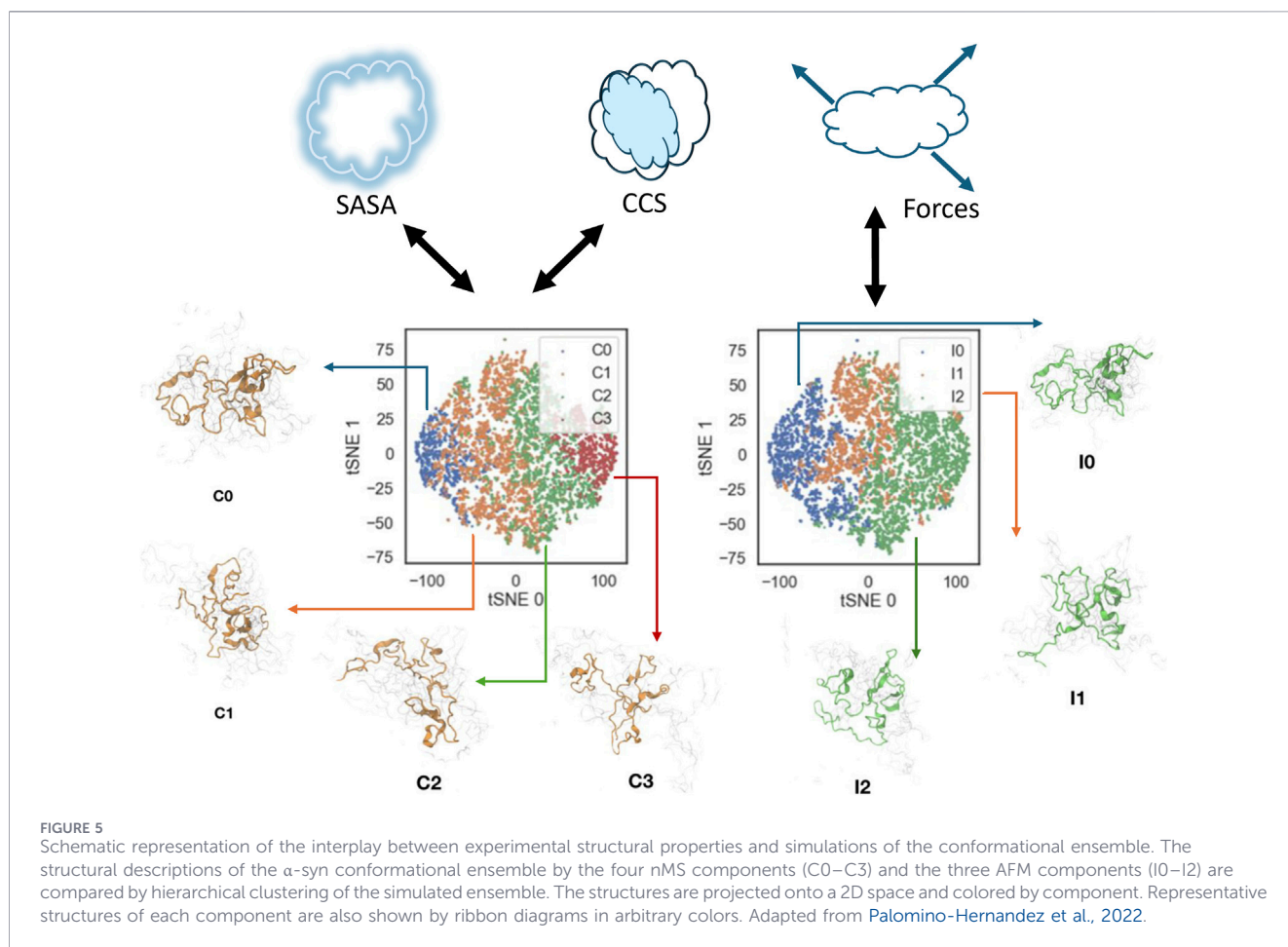
and promotes functional properties associated with modulation of microtubule dynamics. Although the translation of this mechanistic framework to physiological conditions is still evolving, several independent observations increasingly support the biological relevance of α -syn–tubulin interactions. Early co-immunoprecipitation studies from mammalian brain tissue and synaptosomal fractions (Payton et al., 2001; Alim et al., 2002), together with more recent proximity-ligation assays in primary neurons, support the spatial association of α -syn and tubulin at endogenous expression levels in murine and human brains (Amadeo et al., 2021). While direct structural characterization of the native complex *in vivo* is still lacking, these findings suggest that α -syn–tubulin interactions are not restricted to simplified *in vitro* systems and may contribute to microtubule/cytoskeletal regulation in physiological contexts.

Consistent with this view, animal models point toward a role of α -syn in microtubule organization (Amadeo et al., 2021), although the underlying mechanisms remain incompletely resolved. Genetic ablation of α -syn does not result in overt developmental or cytoskeletal abnormalities (Abeliovich et al., 2000), suggesting either compensatory mechanisms involving other synuclein family members (Cabin et al., 2002; Greten-Harrison et al., 2010) or context-dependent requirements for α -syn function (Vargas et al., 2014). At the same time, more recent studies have proposed a more important role for α -syn in cytoskeletal organization through the formation of short noncanonical microtubules involved in efficient axonal transport (Toba et al., 2017), providing a potential mechanistic link between α -syn physiology and intracellular trafficking.

Overall, the current evidence supports the existence of physiologically relevant interactions between α -syn and the microtubule system. Although important mechanistic questions remain open, the continued integration of structural data into *in vivo* studies is expected to clarify how these interactions contribute to microtubule homeostasis and PD pathogenesis (Volpicelli-Daley et al., 2014; Baghel et al., 2026).

4 The interplay with computational methods

The integration of experimental data and simulations, known as “integrative modelling,” is the fundamental approach for describing protein dynamics at an atomic level. While experimental techniques provide measurable structural constraints, computational simulations provide the physical framework necessary to interpret these data and overcome limitations in spatial and temporal resolution. As highlighted by recent reviews (Pak and Voth, 2018; Gusew et al., 2026), this approach is particularly valuable for IDPs, where the sampling of a vast conformational space requires the use of “ensemble refinement” methods to derive atomistic models from inherently low-resolution experimental evidence. In this context, nMS captures snapshots of the conformational distribution, dissecting dynamic ensembles into their main components and monitoring rearrangements in response to stimuli. This section explores this synergy through the interplay with simulations in explicit solvent (Section 4.1) and gas phase (Section 4.2) (Figure 5).



4.1 Comparison with simulations in solution

As discussed above, nMS provides an insightful description of conformational ensembles but is intrinsically a low-resolution technique. Therefore, a powerful approach is to combine it with atomic resolution models generated by computational simulations. Recently, improved force fields for MD simulations have been observed to generate reliable atomistic-level details for both ordered and disordered proteins in explicit solvents, offering unprecedented opportunities for computational modeling of conformational ensembles (Robustelli et al., 2022; Dhar et al., 2025).

The experimental data described here for α -syn in solution in the absence of ligands have been compared with the results of long-time-scale, all-atom MD simulations of α -syn, as described by Robustelli et al. (2022). The goal was to retrieve *bona fide* atomic models for the conformational components detected by nMS and AFM so as to guide the interpretation of the comparative analysis performed by these two techniques (Palomino-Hernandez et al., 2022). A pool of structures randomly sampled from a 75- μ s simulation was analyzed by hierarchical clustering using the Ward's linkage algorithm, according to the calculated values of SASA and CCS with four partitions for comparison with the four components detected by nMS (Corti et al. 2019). Analogously, hierarchical clustering was performed according to intramolecular interactions with three partitions for comparison with the three classes detected by AFM (Corti et al. 2019). This

procedure generates sub-populations of the computational ensemble that represent the main components detected by experimental methods.

A comparison between MD and nMS shows good agreement in terms of the statistical weight and structural features of the four components, particularly the intermediate species. The main discrepancies are in the most compact and most extended components, which appear to be, respectively, slightly more compact and more extended by nMS than by MD. The comparison between MD and AFM also shows substantial agreement, particularly concerning the intermediate component. Instead, the “random coil” seems slightly underestimated and the “strong interactions” component is slightly overestimated by AFM compared to MD. Nonetheless, this study reinforces the hypothesis that these two orthogonal experimental techniques capture conformational transitions of the structural ensemble in a consistent way, despite the different structural features investigated and the peculiar conditions imposed by their respective experimental settings.

The overlap between the nMS and AFM sub-populations was evaluated using principal component analysis. It emerges that the “random coil” component by AFM overlaps quite well with the sum of the fully extended component and the first (more extended) intermediate by nMS, sharing ca. 75% of the conformations and covering ca. 30% of the total conformational space. Instead, the second intermediate by nMS overlaps only 50% of the

conformations with the “weak interactions” observed by AFM. The same applies to the comparison between the compact form by nMS and the “strong interactions” by AFM. Thus, the two techniques capture the highly disordered component with similar sensitivity, while their description of the highly structured components differs significantly.

4.2 Comparison with simulations in the gas phase

For the interpretation of nMS data, it would be desirable to compare experimental results with computational models generated not only in solution but also in the gas phase. However, in simulation of proteins under ESI conditions, the problem of positioning ionized residues inside the protein structure is encountered, with the only constraint of the experimentally determined net charge. This is a highly challenging task because of the high number of ionizable side chains and zwitterionic states. Indeed, it has been hypothesized and directly shown in some cases that the peculiar features of native protein structures can stabilize opposite charges during electrospray ionization (Grandori, 2003; Bonner et al., 2017). Thus, the specific and favorable intramolecular interactions of the native state could compensate for the energetic penalty of charge separation *in vacuo*.

In order to simulate protein structures under ESI conditions, a dedicated computational protocol has been developed, consisting of alternating cycles of Monte Carlo and MD simulations, to explore the protomer and conformer space of desolvated proteins in a combined manner and identify the most probable (lowest energy) states (Marchese et al., 2010). This procedure has been applied so far only to folded proteins, considering a set of structures with different shapes, sizes, and isoelectric points (Marchese et al., 2012). The results show that in most cases, zwitterionic states are conserved, new hydrogen bonds are formed, and the radius of gyration slightly decreases, compared to the original structure in solution. Therefore, the general trend is that proteins preserve native-like features, but the structure “shrinks” slightly, owing to the collapse of side chains onto the protein backbone. These structural rearrangements can be interpreted using a self-solvation mechanism triggered by the lack of an external solvent, in agreement with another study by computational simulations (van der Spoel et al., 2011).

This protocol has not been applied to IDPs yet. Structural rearrangements may be more pronounced in the case of disordered proteins due to larger conformational freedom and amplification of electrostatic effects *in vacuo*. Indeed, gaseous structures detected by IM-MS appear to be largely governed by gas-phase electrostatics (Cheung See Kit et al., 2023). Consistently, simulations by ensemble methods suggest that IDPs undergo an expansion of the conformational landscape upon desolvation (Borysik et al., 2015). It should be pointed out that both repulsive and attractive electrostatic interactions can play a role (Grandori, 2003; Breuker et al., 2011; Bonner et al., 2017; Hu et al., 2019; Pitts-McCoy et al., 2019). In addition to Coulomb expansion at high charge states, the persistence of zwitterionic states at low values of net charge can lead not only to stabilization of globular structures but also to collapse of flexible structures in the gas phase (Marchese et al., 2010). It has also been shown that charge

permutations of a disordered protein domain display sequence-specific and salt-dependent expansion or collapse, as captured by IM-MS and gas-phase simulations (Beveridge et al., 2019). It would be important to develop more models of conformational ensembles in the solution and gas phase to support the interpretation of nMS data on IDPs. This challenging task should also account for proton mobility under ESI conditions (Li et al., 2017; Bashyal et al., 2019).

Another topic of computational simulations is the ESI mechanism itself. Several different models have been proposed and are related to the protein conformational state: the charged-residue model (CRM) for globular proteins (Fernandez de la Mora, 2000), the ion-evaporation model (IEM) mixed to CRM for tightly folded and highly charged ions (Aliyari and Konermann, 2020), the chain-ejection model (CEM) for disordered polymers (Konermann et al., 2013), and a hybrid model based on a bead ejection mechanism (BEM) for multidomain proteins containing intrinsically disordered regions (Khristenko et al., 2023). The experimental charge state of proteins is usually interpreted as dictated by which mechanism is in action. An alternative (or complementary) interpretation based on gas-phase basicity has been proposed (Marchese et al., 2010; 2012). All these points will require further comparative studies.

Nonetheless, a multi-technological approach integrating nMS data to the description of IDPs and their transitions is highly promising (Srivastava et al., 2020; Atilgan and Atilgan, 2022; Ghafouri et al., 2026). Such a hybrid approach to ensemble modeling can benefit from the population-resolved restraints derived from nMS, assisting computational simulations by forward models and by independent validation.

5 Future directions

α -syn is heavily modified by PTMs *in vivo*. More than 50 modifications have been identified on soluble α -syn from diseased and control brains, with carboxymethylation, acetylation, and methylation as the most abundant (Zhang et al., 2023). Interrogating α -syn structural ensembles in response to PTMs could shed light on important regulatory and pathogenic mechanisms. Like ~80% of the eukaryotic proteins, α -syn is predominantly acetylated at the N-terminus in mammalian cells (Kellie et al., 2014). Although this modification does not induce major structural changes *in vitro* and in intact cells (Fauvet et al., 2012), it affects α -syn secondary structure propensity, aggregation behavior, membrane binding, and metal binding (Mason et al., 2016; Rossetti et al., 2016; Wang et al., 2024; Calis and Gevaert, 2025). It has also been shown to shift species distributions in IM-MS toward more extended conformations (Mason et al., 2016). Most studies on recombinant α -syn, including those discussed in this review, employ the unmodified protein. Future experiments and computational efforts should systematically compare modified and unmodified forms to better capture the structural features of endogenous α -syn.

More broadly, future advances in the structural characterization of α -syn and other IDPs and amyloid proteins will likely depend on the integration of emerging MS-based and hybrid methodologies (Biehn and Lindert, 2022; Ghafouri et al., 2026; Wu et al., 2026) capable of resolving heterogeneous and dynamic conformational ensembles. At the same time, integration with MD simulations and

AI-assisted, ensemble-refinement approaches may improve the interpretation of sparse experimental restraints and lead to experimentally grounded ensemble models (Nadwa et al., 2025). On-line separation platforms will allow analysis under increasingly physiological conditions (Ivanov et al., 2024; Kaltashov et al., 2024; El-Baba et al., 2026) and progress toward the “omics” scale (Reid et al., 2023). Together with membrane-native MS (Jung et al., 2025; Deedwania et al., 2026), these approaches are expected to bridge the current gap between biophysical structural characterization and the endogenous cellular environment. Developments in charge-detection MS (Hampton et al., 2025; Rider et al., 2025) and single-particle mass analysis (Lai et al., 2025) will expand access to highly heterogeneous and high mass samples (Kaltashov et al., 2024), while the increasing sensitivity and versatility of native top-down MS will provide direct characterization of proteoforms, supramolecular assemblies, and PTM-dependent structural changes (Rogawski et al., 2021; Bennett et al., 2025; 2026). Time-resolved nMS (James et al., 2026) and, more generally, time-resolved structural MS (Nkyaagye et al., 2026; Overby et al., 2026) may capture transient conformational transitions and ligand-induced remodeling across multiple timescales. Future integrative frameworks will see a constantly increasing contribution by MS, not only to structural description but also to the mechanistic understanding of IDP function and dysfunction. These developments promise to increase the druggability of these elusive pathogenic systems (Qin et al., 2025; Sun et al., 2025).

Author contributions

SB: Investigation, Writing – original draft. EP: Visualization, Writing – review and editing. CS: Conceptualization, Supervision, Visualization, Writing – original draft. RG: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review and editing.

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