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Current Opinion in Structural Biology

Advances in the determination of disordered protein ensemble

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Intrinsically disordered proteins (IDPs) play essential roles in regulation, signaling, and phase separation, yet their structural complexity cannot be captured by a single conformation. Instead, they populate dynamic ensembles that encode a context-dependent function. Recent advances in experimental techniques coupled with physics-based simulations, coarse-grained models, and machine learning, have transformed our ability to generate and interpret IDP ensembles. Integrative frameworks now combine complementary data with computational approaches to refine ensembles at both local and global levels. Nevertheless, challenges remain in benchmarking, error estimation, and modeling assemblies involving protein–protein and protein–nucleic acid interactions. We highlight recent progress and outline the emerging directions that will shape the next generation of ensemble determination methods.

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Current Opinion in Structural Biology 2026, **96**:103198

This review comes from a themed issue on **Folding, Binding and Protein Design (2026)**

Edited by **John Christodoulou** and **Michele Vendruscolo**

For complete overview of the section, please refer the article collection - Folding, Binding and Protein Design (2026)

Available online 11 December 2025

https://doi.org/10.1016/j.sbi.2025.103198

Introduction

Approximately 70 % of human proteins contain at least one segment of 30 or more amino acids lacking stable secondary or tertiary structure, and ∼5 % are entirely disordered [1]. These intrinsically disordered proteins and regions (IDPs/IDRs) play central roles in regulation, signaling, and phase separation, yet their behavior cannot be captured by a single static structure. Instead, they populate dynamic conformational ensembles that encode functional versatility and context-dependent interactions [1]. While state-of-the-art predictors such as AlphaFold [2] excel for folded proteins, they fail to represent IDP heterogeneity, underscoring the need for dedicated approaches. Ensemble determination requires an integrative workflow that combines experimental techniques, computational generation, and validation (Figure 1) [3]. Advances in biophysical methods, physics-based simulations, knowledge-driven strategies, and machine learning (ML) now allow ensembles constrained at multiple scales. By merging data integration frameworks with artificial intelligence (AI), the field is moving toward quantitative and predictive descriptions of disordered protein behavior. Here, we review recent progress in experimental and computational methodologies for IDP ensemble determination.

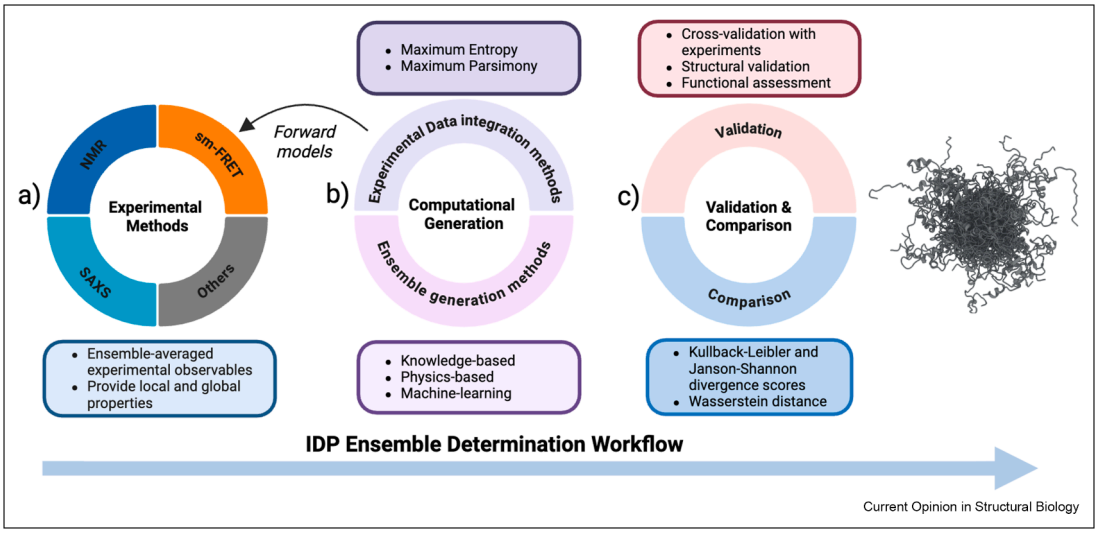
Recent advances in experimental techniques

The structural characterization of IDPs relies on experimental approaches that capture the broad conformational heterogeneity and dynamic nature of these systems. In recent years, methodological advances in nuclear magnetic resonance (NMR) spectroscopy, single-molecule Förster resonance energy transfer (smFRET), and small-angle scattering (SAS), complemented by other biophysical techniques, have significantly expanded our ability to determine conformational ensembles with higher resolution, and physiological relevance [3] (Figure 2).

NMR remains the gold standard for atomic-level insight into IDPs, capturing ensemble-averaged features such as secondary structure propensities, backbone torsion angles, and long-range contacts with minimal perturbation [3]. Recent developments in relaxation dispersion and paramagnetic techniques (e.g. PRE) have enhanced detection of sparsely populated or transiently folded states [4]. Moreover, in-cell NMR continues to mature, extending studies to physiologically relevant environments [5] (Figure 2b).

smFRET offers nanometer-scale distance measurements and uniquely resolves subpopulations and

Figure 1



Roadmap for the determination of IDP conformational ensembles. Experimental data (e.g. NMR, smFRET, SAXS) provide observables that guide computational ensemble generation. Integration methods refine ensembles using forward models, while comparison and validation ensure consistency with independent data. IDP, intrinsically disordered protein; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; smFRET, single-molecule Förster resonance energy transfer.

dynamic transitions that are obscured in ensemble-averaged methods. Applied to IDPs, smFRET has characterized global chain dimensions, conformational substates, and structural rearrangements in conditions ranging from dilute solutions to crowded cellular milieus and biomolecular condensates [6]. Technical advances in site-specific fluorophore labeling, alternating/pulsed excitation schemes, and multiparameter detection have improved quantitative analysis, while integration with polymer physics models and molecular simulations has refined the extraction of full inter-dye distance distributions and dynamic reconfiguration times [7] (Figure 2a).

SAS, including both X-ray (SAXS) and neutron (SANS) scattering, provides global information on molecular size, shape, and compactness with minimal perturbation to the system in solution. For IDPs, SAS delivers key global descriptors such as the radius of gyration (Rg), scaling exponent (ν), and pairwise distance distribution P(r), complementing the residue-level detail of NMR and smFRET [8]. Recent advances include enhanced beamline sensitivity, rapid data acquisition enabling kinetic studies from microseconds to hours, and improved analysis frameworks capable of extracting polymer scaling behavior directly from scattering profiles [9] (Figure 2c).

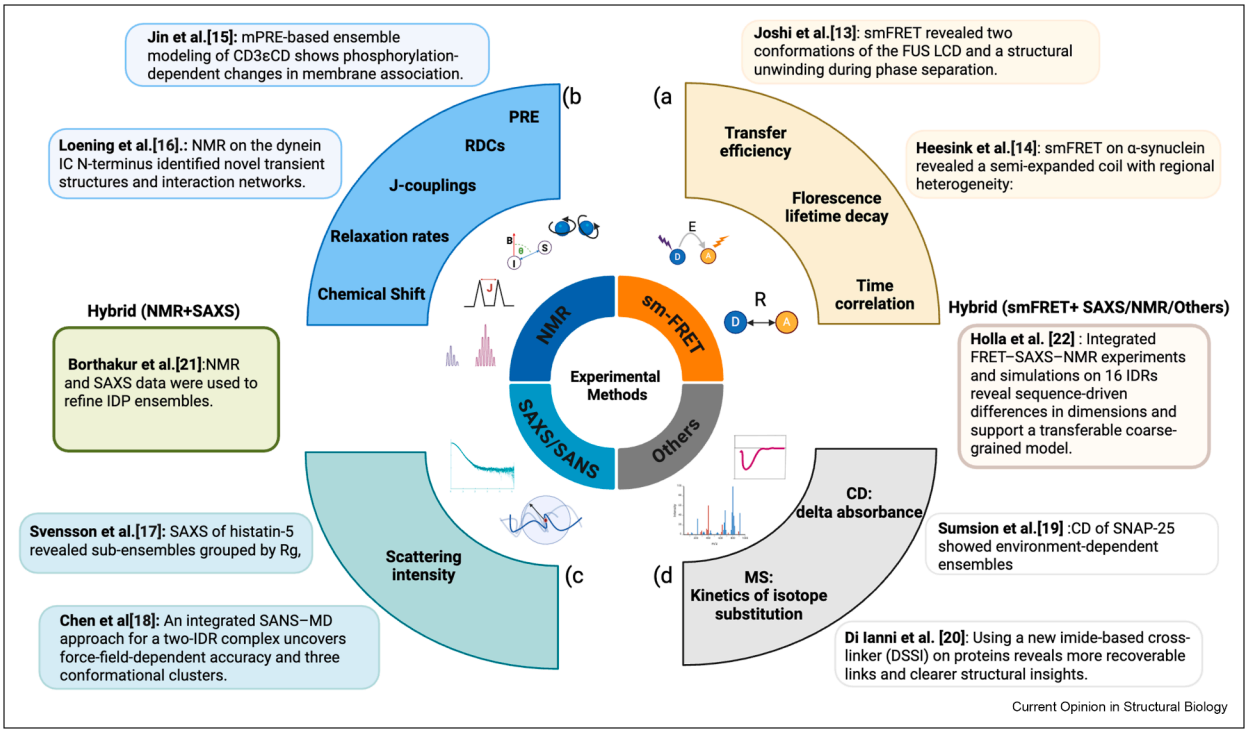
Complementary techniques have gained traction in probing additional facets of IDP structure and dynamics. Hydrogen–deuterium exchange, particularly coupled to mass spectrometry, maps transient secondary structure and conformational flexibility, while covalent labeling and hydroxyl radical footprinting capture solvent exposure at side chain resolution [10] (Figure 2d).

Collectively, these methodological advances have reshaped the experimental landscape of IDP research, fostering increasingly integrative approaches to decipher sequence-encoded interactions, conformational dynamics, and the resulting structural transitions and phase separation. Recent entries in the Protein Ensemble Database (PED) [11] often combine SAXS and NMR data and/or smFRET measurements [12], frequently complemented by other orthogonal techniques such as electron paramagnetic resonance and circular dichroism (CD), to generate ensembles constrained at both global and local levels.

Recent advances in computational methods

Computational modeling of IDPs as conformational ensembles is a widely used approach for interpreting experimental data and gaining insight into their conformational heterogeneity [23]. There are three main strategies for generating conformational ensembles of IDPs (Table 1): (a) physics-based methods, primarily using molecular dynamics (MD) simulations; (b) knowledge-based techniques, which rely on heuristics or statistical patterns derived from known protein structures; and (c) ML–based approaches, particularly

Figure 2



Experimental methods for the determination of conformational ensembles of IDPs. For each technique we highlight the most important observables and recent studies to showcase the potential of each method for the determination of conformational ensembles. **(a)** smFRET provides intramolecular distance distributions at the nanometer scale [13,14], **(b)** NMR yields residue-specific information on local structure and dynamics through chemical shifts, couplings, relaxation parameters [15,16], **(c)** SAXS/SANS reports on overall chain dimensions and global conformational properties [17,18], d) other techniques such as CD and MS-based techniques probe secondary structure content and long-range residue–residue distance restraints [19,20]. Moreover, **hybrid techniques** integrate complementary observables from different experimental methods to generate ensembles consistent with multiple structural restraints [21,22]. CD, circular dichroism; IDP, intrinsically disordered protein; MS, mass spectrometry; NMR, nuclear magnetic resonance; SANS, small-angle neutron scattering; SAXS, small-angle X-ray scattering; smFRET, single-molecule Förster resonance energy transfer.

generative models, which infer structural features from trained models without relying on explicit physical principles. Another category of computational techniques for determining IDP ensembles involves integrating experimental data into computational models to obtain ensembles that more accurately reflect experimental observations. These methods are broadly divided into two classes, maximum entropy and maximum parsimony [24], explained later in this section.

Ensemble generation techniques

Knowledge-based methods

Knowledge-based methods offer the fastest way to generate conformational ensembles. They construct conformers by sampling residue fragments from nonredundant, high-resolution experimental structures, using the observed torsion angle distributions in these datasets as empirical descriptors of allowable backbone geometries rather than as explicit statistical potentials or derived force fields. Early examples include TraDES [25], which used probabilistic sampling with secondary structure biases, and flexible-meccano [26], which added IDP-specific conformational propensities and back-calculation of experimental observables. Recent tools, such as IDPConformerGenerator [27], extend earlier approaches by incorporating fragment-based $\phi/\psi/\omega$ sampling, all-atom modeling, integration of experimental restraints, and pipelines for ensemble analysis and reweighting. The main limitation of such methods is their neglect of time-dependent physical dynamics, which prevents them from capturing kinetically relevant processes and may lead to ensembles that do not fully reflect the underlying energy landscape.

Physics-based methods

In physics-based approaches, MD simulations use force fields to model protein motions over time, offering detailed insights into the dynamic behavior of IDPs.

The choice of method depends strongly on the biological question and the available computational resources. In particular, all-atom MD simulations with force fields

Table 1

Recent ensemble generation techniques, grouped into knowledge-based, physics-based, and machine learning approaches, with applications, strengths, and limitations.

Ensemble Generation Strategy	Technique	Underlying concept	Application context	Advantages	Drawbacks
Knowledge-based	MoMA [74]	Generates conformers through motion-planning algorithms that apply steric constraints and may integrate energy models.	When extensive sampling with realistic geometry is required	Incorporates all-atom representations with physically realistic structures.	Physical dynamics are not accounted for
	IDPConformer Generator [27]	Draws torsion angles from fragment libraries, potentially refined through restraints	When incorporating both sequence data and experimental constraints	Modular framework that allows reweighting and incorporation of SAXS/NMR constraints	Relies on the quality of fragments and the effectiveness of scoring functions.
	DIPEND [75]	Generates ensembles by sampling statistical dihedral angles conditioned on neighboring residues and applying structural filters	For rapid backbone ensemble generation directly from sequence with minimal requirements	Flexible and efficient, incorporating experimental priors with rapid clash resolution and refinement.	Relies on heuristics and rotamer libraries without modeling physical dynamics, offering only backbone-level sampling.
Physics-based	Amber ff99SB ws/ff03ws [76]	All-atom modeling framework refined to better capture protein−solvent interactions	For simulating IDPs where protein−water interactions are accurately balanced.	Enhanced accuracy of global dimensions and protein−protein interactions	Computationally intensive
	DES-amber [29]	All-atom force field optimized to capture protein−protein interactions	Suitable for simulating IDPs, folded proteins, and protein−protein complexes	Enhanced accuracy in global dimensions, transient helix formation, and protein−protein interactions	Limited to amber ecosystem
	MARTINI-3 [77]	Coarse-grained approach for studying complex biomolecular structures	Suitable for studying large biomolecular or membrane-associated systems	Scales efficiently to simulate large assemblies	Simplified interactions
	CALVADOS [40,41]	Implicit-solvent coarse-grained force field for IDPs and multi-domain proteins, parameterized using a top-down approach.	Efficient modeling approach that accounts for sequence effects in IDPs and multi-domain proteins	Efficient; accurate predictions of IDP compaction	Provides residue-level resolution without accounting for short-lived secondary structure or hydrogen bonds
Machine-Learning	idpGAN [42]	GAN trained on CG-MD ensembles to produce new conformers that reproduce the statistical properties of the training data	When rapid ensemble generation is needed	Reproduces realistic ensemble variability and is highly scalable	Constrained by training data patterns from CG simulations; generates Cα-only ensemble representations.
	idpSAM [78]	Latent diffusion approach (transformer autoencoder plus diffusion) trained on ABSINTH implicit-solvent ensembles to generate new conformers	Designed to generate ensembles of short IDRs (<60 residues) and generalize to unseen sequences.	Strong ability to generalize to new sequences with robust training stability.	Depends on large amounts of simulation-derived training data.
	BioEmu [79]	Diffusion-based model trained on extensive datasets combining simulations and experimental measurements	Fast production of equilibrium ensembles directly from the sequence.	Rapid and sequence-aware, generating diverse ensembles characteristic of disordered proteins.	Restricted to backbone sampling without side chains; tends to overestimate Rg for longer IDPs (>100 residues).
Hybrid	AlphaFold-Metainference [57]	Integrates AlphaFold-derived constraints with Bayesian ensemble	Aimed at integrating AI-based insights into the	Incorporates prior knowledge	Depends on AlphaFold-derived priors and confidence metrics.

Table 1 (*continued*)

Ensemble Generation Strategy	Technique	Underlying concept	Application context	Advantages	Drawbacks
		modeling, blending ML and physics-based methods.	refinement of ensembles.		
	DynamICE [50]	Employs a Generative recurrent neural network to create conformers, refined with experimental data using a Bayesian reward function (X-EISD).	For situations where fixed conformer pools are inadequate and diverse, data-driven IDP ensembles are required.	Allows *de novo* generation of conformers consistent with data, integrating physical and experimental constraints with iterative improvement.	Local torsion-based sampling limits accuracy in meeting distance restraints such as NOEs and PREs.
	bAles [56]	Incorporates AlphaFold2 distograms as distance restraints into a random coil prior using Bayesian integration.	Designed to efficiently generate atomistic IDP ensembles, particularly under limited experimental data.	Rapid, atomistic-level modeling that is resilient to AF2 errors and captures both local and long-range features.	Relies on AF2 accuracy and is less effective for environment-dependent or transient interactions.
	AFflecto [80]	Constructs IDP ensembles through fragment-based sampling guided by AlphaFold models, incorporating secondary strucutre elements (SSEs) and steric constraints.	For efficient conformer generation directed by AlphaFold predictions with optional control over SSEs.	Rapid and adjustable, integrates partial structural information without relying on MD or force fields.	Lacks dynamic sampling; side chains are represented by pseudo-atoms.

AF2, AlphaFold2; CD, circular dichroism; IDP, intrinsically disordered protein; IDR, intrinsically disordered proteins and region; MD, molecular dynamics; NMR, nuclear magnetic resonance; Rg, radius of gyration; SAXS, small-angle X-ray scattering; smFRET, single-molecule Förster resonance energy transfer. GAN, generative adverserial network; X-EISD, extended experimental inferential structure determination; NOEs, nuclear overhauser effects; PRE, paramagnetic relaxation enhancement.

adapted for IDPs [28–30] have proven valuable for unraveling binding mechanisms with protein partners [31], clarifying sequence–ensemble–function relationships [32], assessing the impact of small-molecule drugs [33], and elucidating the molecular processes underlying liquid–liquid phase separation [34]. Although computationally demanding, recent advances in GPU-accelerated MD simulations have greatly increased the accessibility of this approach [35]. In addition, enhanced sampling techniques now allow a more efficient exploration of the complex free energy landscapes of IDPs, yielding improved convergence and more reliable ensemble descriptions [36]. Altogether, ongoing research is addressing force field inaccuracies and expanding conformational sampling through AI and ML techniques [37,38]. For further details on atomistic MD simulations applied to IDP systems, we refer readers to this review paper [39].

On the other hand, coarse-grained (CG) MD simulations provide an efficient approach to study global parameters of IDPs across larger timescales and system sizes. Recent CG models, particularly those employing data-driven force fields optimized with SAXS and NMR data [40,41], have proven highly effective in capturing realistic global ensemble features such as radius of gyration (Rg), end-to-end distance (Ree), and the apparent Flory scaling exponent ($\boldsymbol{\nu}$). However, their reduced resolution limits the accuracy of local structural features and specific residue–residue interactions. Taken together, physics-based methods remain constrained by limited accessible timescales and the inherent approximations of current force fields which make it challenging to achieve convergence in IDP simulations.

Machine learning methods

Machine learning–based methods have almost resolved the challenge of predicting stable folded protein structures, whose conformational ensembles exhibit only limited heterogeneity. Thanks to AlphaFold [2] and other state-of-the-art models, it is now possible to predict the 3D structures of folded proteins with near-experimental accuracy using only the primary sequence. The next major challenge for AI-based approaches, however, lies in predicting protein conformational dynamics, where each sequence maps to a highly heterogeneous distribution of structural ensembles. A

growing number of ML models have recently been developed to overcome the limitations of conventional experimental and computational approaches in determining IDP ensembles. Broadly, these models can be classified into three main categories:

a. **Sampling techniques** – it includes models that generate structural ensembles either de *novo* or by enhancing molecular simulations. These range from generative models that directly sample conformational space [42–44] to ML approaches that accelerate or guide MD trajectories by learning effective energy landscapes or proposing long timescale transitions [45,46].
b. **Feature predictors** – it comprises models that learn to link sequence features to ensemble-averaged properties. Rather than generating full ensembles, these models predict global or local descriptors of conformational behavior, such as chain compaction, phase separation propensity, or transient secondary structure content [47–49].
c. **Experimentally-aware models** – it includes models that integrate experimental restraints into the ML framework, either during training (e.g. incorporating NMR, SAXS, or spectroscopy data) or at the inference time by biasing the sampling process. Such approaches allow generated ensembles to be directly tuned toward agreement with experimental observables [50,51].

Detailed descriptions about these methods are available in these two reviews [52,53].

Despite these advances, ML-based approaches still struggle to fully capture IDP conformational heterogeneity as they depend heavily on training data quality and may miss physically realistic dynamics or rare yet functionally important states.

Experimental data integration methods

After generating conformational ensembles with the aforementioned methods, a common practice is to assess their agreement with experimental data (Figure 3). This process involves computing experimental observables from each conformer using *forward models* (Figure 3a) and comparing the ensemble-averaged predictions with experimental measurements (Figure 3b). In practice, however, the calculated ensembles often show poor agreement with experimental data. This mismatch typically arises from three main factors: insufficient sampling of the protein energy landscape, inaccuracies and approximations in force fields, and uncertainties or sparsity in both forward models and experimental measurements [54]. To overcome these limitations, experimental data integration techniques are employed to reweight ensembles or guide their generation, thereby ensuring that the resulting conformers more accurately reflect experimental observations (Figure 3c). Notably, two main approaches exist for integrating experimental data with ensemble generation: maximum entropy (ME) and maximum parsimony (MP). ME methods reweight ensembles to remain as close as possible to the original prior distribution while enforcing agreement with experimental observations. This strategy preserves the inherent heterogeneity of IDPs and is particularly powerful in maintaining ensemble diversity in high-entropy systems. In contrast, MP methods aim to identify the smallest subset of conformers that can explain the data, thereby simplifying the representation of conformational space. While ME emphasizes unbiased refinement, MP favors minimal models and is best suited for systems dominated by a few major conformations. Further details on these approaches can be found in a recent review [24]. Suppl. Table 1 summarizes the recent integration methods and their respective use cases.

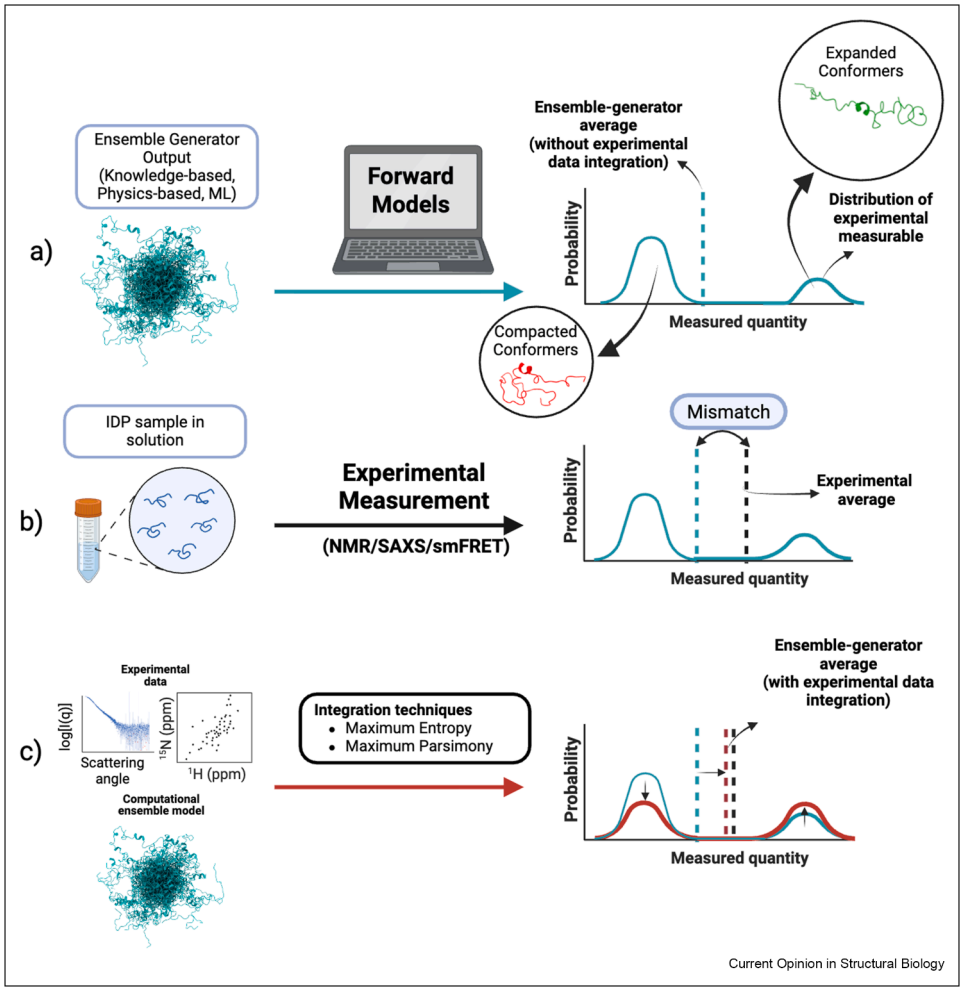
Hybrid techniques

Some computational strategies for modeling IDPs adopt hybrid approaches that combine complementary methods for ensemble generation and data integration. One direction integrates physics-based and knowledge-based sampling to balance computational efficiency with conformational diversity. A common example is fragment-based protocols refined with energy functions, which leverage both empirical knowledge and physical accuracy [55]. Another strategy merges ML with physics-based modeling, where deep learning predictions are refined within Bayesian or simulation frameworks [56,57]. A further class of approaches directly couples ensemble generation with experimental restraints, iteratively adjusting conformers to fit NMR or other measurements [50]. An additional hybrid framework combines hierarchical chain growth with MD simulations, enabling statistically rigorous and computationally efficient sampling of IDP ensembles that can be refined through integrative modeling or experimental reweighting [58]. Such flexible pipelines exploit the complementary strengths of different paradigms, enabling more realistic ensembles in cases where a single approach proves insufficient.

Comparison and validation techniques

As discussed earlier, conformational ensembles of IDPs can be derived from a variety of experimental and computational approaches. However, resolving these ensembles is inherently an ill-posed problem [3] as sparse experimental data often permit multiple solutions. This highlights the need for robust methods to compare ensembles and quantify differences between those obtained from distinct data sources. In the context of IDPs, an effective comparison metric should capture both global ensemble properties and local structural variations by reflecting differences in the underlying

Figure 3



Schematic representation of experimental data integration strategies. **(a)** Forward models calculate observables from the coordinates of each conformer in the ensemble and provide the distribution of experimental measurable in the ensemble. In this figure, we have shown Rg as an example **(b)** Ensemble-averaged value is compared to experimental measurement. In this step, we normally observe mismatches between them. The reasons are mainly insufficient sampling, uncertainties in experimental measurements and forward models, and approximations in force fields **(c)** Integration methods reweight or refine ensembles to fit the computational ensemble model to experimental data. Two main approaches for integration of experimental data are maximum entropy and maximum parsimony.

distributions rather than solely ensemble-averaged information. Such approaches are essential not only for validating ensembles derived from diverse experimental inputs but also for extracting mechanistic insights, for example, identifying binding regions or dynamic linkers, comparing homologous IDPs, and evaluating the effects of solution conditions. Next, we outline recent advances in IDP ensemble comparison techniques and introduce complementary approaches for validating conformational ensembles.

Ensemble comparison methods

A variety of approaches have been developed to compare conformational ensembles of IDPs, each capturing different aspects of their structural variability. Scalar descriptors provide a simple measure of conformational heterogeneity, allowing ensembles to be compared via single values, though at the cost of detail [59]. Distribution-based methods extend this by analyzing differences in probability distributions of structural features, such as inter-residue distances, using similarity measures like Kullback–Leibler or Jensen–Shannon divergence, Hellinger distance, or Wasserstein distance, which also account for geometric relationships but at higher computational cost [60]. Descriptor-based approaches rely on specific structural parameters: atomic coordinates with root-mean-square deviation (RMSD), torsion angles, or other local properties, sometimes combining local and global descriptors for a more integrated view [61]. Finally, alternative

frameworks employ concepts such as topology or the detection of local energy traps to highlight unique structural features [62]. Together, these strategies enable ensemble comparison across multiple levels of resolution and complexity.

Conformational ensemble validation

Ensemble validation techniques focus on testing the reliability of IDP ensembles by combining structural checks, cross-validation against experimental data, and assessments of biological relevance. Structural validation normally begins with stereochemical checks such as bond lengths, torsion angles, Ramachandran distributions, and clash avoidance, criteria not always satisfied in PED ensembles [11]. It then extends to global parameters like radius of gyration, end-to-end distance, asphericity, and scaling exponents, derived from SAXS, smFRET, and NMR measurements [63]. Local descriptors, including inter-residue contact probabilities [64], secondary structure content (CD and NMR), and residue-specific torsion preferences (NMR: CSs, RDCs, and NOEs), provide finer evaluation. Cross-validation with experimental data can validate IDP ensembles by testing robustness to data removal [65] and by verifying the ability to predict independent observables [66]. Applications to α-synuclein, tau, and viral IDPs using NMR, SAXS, and smFRET highlight this strategy [67]. Finally, functional validation examines biological relevance, such as binding interfaces [68], conserved linker dimensions [69], or disease mechanisms revealed through small-molecule binding and structure-activity relationship (SAR) analysis [70]. Emerging in-cell approaches [71] and sequence conservation analyses [72] provide ultimate physiological benchmarks.

Future perspectives

Despite recent progress, several challenges remain before conformational ensembles of IDPs can be routinely determined and applied across biology. A first priority is the establishment of robust community-wide critical assessment (e.g. IDP-bench [3]), which will allow systematic evaluation of generation methods, forward models, and integration strategies. Notably, a recent study proposed a benchmarking protocol based on a curated set of experimental data, primarily SAXS and NMR, to assess the performance of several ensemble generation approaches including machine learning and physics-based techniques [73]. Equally important is the development of data standards for reporting and exchanging experimental information, including rigorous annotation of uncertainties and error estimates. Without explicit error modeling, ensemble refinement risks overfitting and limited reproducibility. Finally, the field must expand beyond monomeric systems to include the structural heterogeneity of protein−protein, protein−RNA, and protein−DNA assemblies, which are central to cellular signaling and phase separation. Addressing these challenges will require coordinated efforts across experimentalists, biocurators and computational scientists, ultimately enabling more predictive, transferable, and biologically relevant ensemble descriptions.

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.

Acknowledgements

This work has been supported by: ELIXIR, the research infrastructure for life-science data; COST Action ML4NGP [CA21160], supported by the COST (European Cooperation in Science and Technology); European Union through NextGenerationEU; PNRR project ELIXIRxNextGenIT [IR0000010]; the National Center for Gene Therapy and Drugs based on RNA Technology [CN00000041]; co-funded by the European Union under grant agreement no. 101182949 (HORIZON-MSCA-SE project IDPfun2); views and opinions expressed are, however, those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Executive Agency. Neither the European Union nor the granting authority can be held responsible for them. Funding for open access charge: University of Padua.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbi.2025.103198.

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