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Recent advances in quantifying protein conformational ensembles with dipolar EPR spectroscopy

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Abstract

This perspective highlights recent applications and technological progress in dipolar electron paramagnetic resonance (EPR) spectroscopy, including double electron–electron resonance (DEER) spectroscopy. These methods provide nanoscale distance distributions between site-specific spin labels in biomacromolecules. The resulting data are particularly well suited for quantifying the structure and energetics of conformational ensembles of multi-state and flexible proteins. Recent applications span a wide range of systems and are accompanied by innovations in spin labeling, deuteration, in-cell measurements, integrative multi-technique approaches, and novel computational modeling methods combined with structure prediction tools.

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Introduction

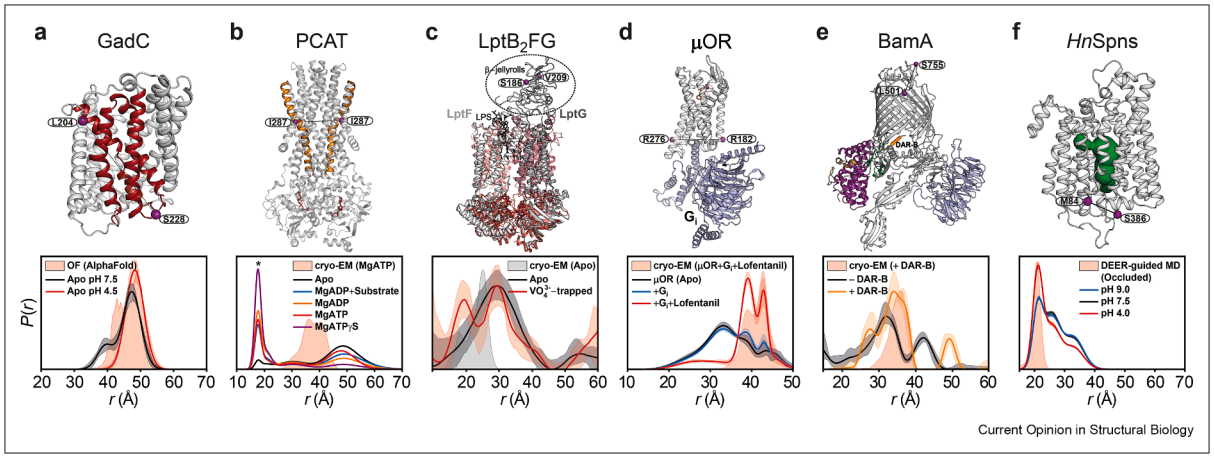
Pulse dipolar electron paramagnetic resonance (EPR) spectroscopy techniques, particularly double electron–electron resonance (DEER), serve as powerful tools for exploring the structural dynamics of small-to medium-sized and highly dynamic biological macromolecules in diverse physiologically relevant environments [1–3]. These techniques measure the distribution of distances between site-specific spin labels in an ensemble of biomacromolecules, with an accessible range of about 15–80 Å. They provide unique structural and energetic insights into disordered, dynamic, multi-conformational proteins, low-symmetry large complexes, and transient protein–protein interactions. Dipolar EPR is increasingly integrated with other structural techniques, such as cryo-electron microscopy, Förster resonance energy transfer (FRET), nuclear magnetic resonance (NMR), and small-angle scattering, and combined with computational structure prediction tools to characterize mechanistically relevant conformational states. This perspective highlights recent applications of dipolar EPR to study protein conformational transitions and ensembles (Fig. 1), summarizes experimental and computational advances (Fig. 2), and discusses future opportunities and challenges in the field.

Recent applications

Dipolar EPR spectroscopy continues to reveal detailed mechanistic insights into protein conformational dynamics across diverse systems. For example, the functional dynamics of the APC transporter GadC was studied by DEER combined with computational modeling, revealing proton-induced transitions between inward- and outward-facing conformations (Fig. 1a) [4]. In an ABC transporter, DEER spectroscopy was recently combined with extensive cryo-EM studies to elucidate its coupled cargo protein export mechanism, capturing a snapshot of a high-energy, outward-facing conformation essential for substrate release (Fig. 1b) [5]. Similarly, studies on the *E. coli* lipopolysaccharide (LPS) transport protein complex $LptB_2FGC$ demonstrated, through DEER distance measurements in detergent and liposomes and continuous-wave (CW) EPR spin label mobility analysis, how ATP binding and hydrolysis regulate the dynamics of lateral gates and structurally unresolved periplasmic domains (Fig. 1c), underscoring the importance of LptC in regulating directional LPS transport across the periplasm [6,7].

In the human μ-opioid receptor system, a combination of DEER and single-molecule FRET identified multiple conformational states, including a pre-activated conformation primed for G-protein engagement and a fully active state that modulates GDP affinity within the ternary complex (Fig. 1d) [8]. A novel approach

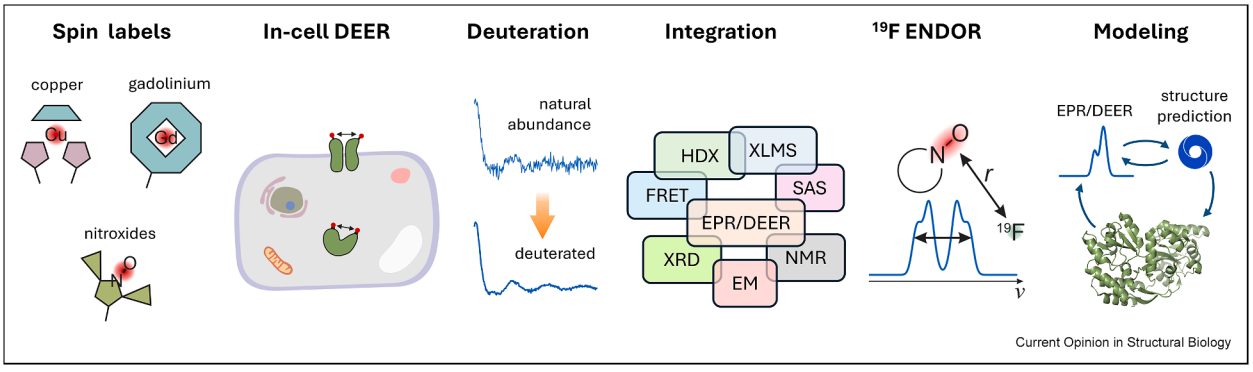
Figure 1



Capturing conformational intermediates using pulsed dipolar EPR spectroscopy. (**a**) Distance distributions $P(r)$ at low and neutral pH of the bundle domain of the APC transporter GadC [4]. The spin label pair used for the DEER distance measurement (bundle domain, across the membrane) is represented by purple spheres. Confidence bands (2σ) are shown around the best-fit lines. These bands, which depict the estimated uncertainty in $P(r)$, reflect errors associated with the fitting of the primary DEER trace. The protonated GadC adopts an outward-facing (OF) conformation, as predicted by the AlphaFold model. In all panels, predicted distributions from models and structures are shaded in light colors.
(**b**) A distinct conformation (population indicated by a star) of the core protease-containing ABC transporter (PCAT) in the nucleotide-bound state, compared to its cryo-EM structure (PDB: 8VP1) in lipid nanodiscs [5]. (**c**) DEER spectroscopy on the vanadate-trapped intermediate of the structurally unresolved periplasmic domains (PDB: 6MHZ, apo state) of the *E. coli* $LptB_2FG$ LPS transporter [6]. (**d**) Capturing the ternary complex of the human μ-opioid receptor (μOR) bound to its ligand lofentanil and G protein G_i, consistent with the cryo-EM structure of this state (PDB: 7T2H) [8]. (**e**) *E. coli* BAM complex bound to darobactin B (DAR-B) in its native environment, compared to its compatible cryo-EM structure (PDB: 8BVQ) [10]. (**f**) Proton-dependent conformational changes of the gating helices 2 and 11 on the intracellular side of a bacterial Spns transporter [11]. Modeling of the protonated conformation using DEER spectroscopy distance constraints indicated an occluded state. Tunnels (green) were calculated using MOLEonline 2.5 software.

combining DEER spectroscopy between spin-labeled non-canonical amino acids and semi-reduced flavin and molecular dynamics (MD) resolved conformational heterogeneity in the one-electron-reduced state of cytochrome P450 reductase, revealing open-state ensembles [9]. Studies on the β-barrel assembly machinery (BAM complex) in native *E. coli* cells demonstrated the power of in-cell DEER combined with cryo-EM to capture substrate-bound conformations (Fig. 1e) [10]. Using an integrated approach in lipid membranes,

Figure 2



Areas of recent methodology advances in dipolar EPR spectroscopy. From left to right: novel copper, gadolinium and nitroxide spin labels; innovative methods for dipolar EPR of spin-labeled proteins inside cells; deuteration of proteins to improve sensitivity; integrative use of EPR with other experimental biostructural approaches including FRET, hydrogen–deuterium exchange and crosslinking mass spectrometry (HDX, XLMS), small angle X-ray and neutron scattering (SAS), X-ray diffraction (XRD), cryo-electron microscopy (EM), and NMR; short-distance measurements using fluorine-19 ENDOR; structural modeling combining EPR/DEER data with biomolecular structure prediction tools.

extensive DEER spectroscopy was combined with enhanced-sampling MD simulations to identify a previously unknown, mechanistically relevant occluded conformation of a bacterial membrane transporter (Fig. 1f) [11]. Despite numerous structures reported in the past decade, DEER spectroscopy uniquely captures the functionally relevant asymmetric conformation of nucleotide-binding sites in the mammalian multidrug transporter P-glycoprotein, offering a mechanism to distinguish substrates from inhibitors [12]. DEER has also been instrumental in understanding how membrane potential affects conformational and functional dynamics in the sodium-dependent glucose transporter (SGLT1), providing quantitative distance constraints that define functionally coupled states [13]. Multi-state de novo designed hinge-like proteins that switch between distinct conformations upon ligand binding have been characterized by combining DEER to capture conformational equilibria with measurements of binding kinetics and structural determination [14]. The application of dipolar EPR extends beyond proteins. EPR studies on riboswitch RNAs captured conformational flexibility that remains challenging for other methods [15]. Finally, integrative approaches combining DEER with mass spectrometry, small-angle scattering, and ensemble modeling have proven especially effective for resolving complex conformational landscapes [16–22].

Collectively, these studies demonstrate how dipolar EPR, particularly when integrated with complementary techniques, enables the characterization of elusive, functionally relevant intermediate states in biological macromolecules.

Novel spin labels

While cysteine-ligated nitroxide spin labels such as R1 remain widely used, they are limited by long side chains, labile ligation chemistry, and reduction sensitivity in cellular environments. In cell lysates, nitroxides are prone to enzymatic reduction by NADPH/NADH-dependent enzymes. This reduction can be mitigated through protective maleimide derivatization, improving nitroxide stability in *E. coli*, HeLa, and HEK293T lysates [23]. Nitroxide stability has also been improved through spirocyclic modifications, enhancing resistance to cellular reducing agents and allowing measurements at elevated temperatures (up to 120 K) [24]. Thioester-based cysteine-reactive rigid nitroxide radicals offer improved distance precision and chemical stability [25]. Gd(III)-based labels are gaining popularity due to their exceptional stability in intracellular environments [2]. New nucleic acid labeling strategies are also emerging [26].

Non-covalent Cu(II)-based spin labels, particularly the one coordinated by two histidines (dHis) and capped with nitrilotriacetate (NTA), offer distinct advantages by preserving native cysteines and minimizing label flexibility, resulting in narrower distance distributions that more accurately reflect protein backbone conformations (Fig. 2). Notably, removal of native histidines is unnecessary [27]. In silico labeling tools now support labeling with dHis-Cu(II)-NTA and other bifunctional labels [28,29]. Additionally, these Cu(II) labels enable room-temperature measurements of side chain dynamics [30]. Another Cu(II) labeling strategy based on the genetically encoded noncanonical amino acid 2,2′-bipyridin-5-yl alanine has also emerged [31].

Bioorthogonal labeling strategies offer crucial advantages for intracellular labeling, particularly by preserving native cysteines [32]. For instance, aldehyde-functionalized pyrrolidine and 3-pyrroline-1-oxyl radicals can react with an added N-terminal cysteine, enabling in-cell measurements without perturbing native disulfides [33]. This method was used to show that the trigger factor protein predominantly exists in the monomeric state in *E. coli* lysate. Genetic encoding of tetrazine-functionalized amino acids followed by reaction with strained trans-cyclooctene (sTCO)-modified nitroxides enables direct in-cell spin labeling in mammalian cells [34]. Cyclooctyne-based spin labeling using non-canonical amino acid p-azidophenylalanine has been used for distance measurements to native flavin radicals in human cytochrome P450 reductase [35].

These diverse spin-labeling chemistries collectively expand the versatility of dipolar EPR, allowing tailored labeling approaches for different biological contexts.

In-cell measurements

Expanding EPR into native intracellular environments remains a major frontier [2,36,37]. While cell-surface proteins are readily labeled, intracellular targets typically require *ex situ* labeling followed by delivery via microinjection, electroporation, osmotic or heat shock. However, strategies for direct in-cell labeling are rapidly advancing (Fig. 2). For example, Cu(II)-NTA complexes can be delivered directly to intracellular overexpressed proteins containing genetically encoded dHis motifs to form dHis-Cu(II)-NTA spin labels [38–40]. As mentioned, tetrazine-sTCO labeling allows site-specific spin labeling during intracellular protein expression in mammalian systems [34]. Nanodiscs-mediated delivery has enabled insertion of spin-labeled membrane proteins into intact bacterial membranes [41]. Metal-based spin labels, particularly Gd(III), provide robust intracellular stability, while newer nitroxide derivatives (e.g., ethyl or spirohexyl-substituted) offer partial resistance to reduction [24,34]. Continued progress in intracellular labeling will be critical for fully realizing EPR's potential to study biomolecular conformations in native cellular environments.

Deuteration

Deuteration of the spin label environment extends spin phase memory time (T_m), improving sensitivity and accessible distance range (Fig. 2). Solvent deuteration is now routine, while protein deuteration—though powerful—remains underutilized [42]. Deuteration has enabled DEER measurements at distances up to 160 Å in proteins and 100 Å in nucleic acids [43,44]. Recent advances include selective methyl protonation in otherwise deuterated proteins to resolve multimodal distributions [45], perdeuterated spin-labelled affinity proteins for non-covalent labeling of undeuterated targets [46], and complete deuteration in cells to extend intracellular T_m [47]. Lipid deuteration remains challenging to extend phase memory time for spin-labelled membrane proteins in liposomes or nanodiscs. Deuteration will likely be a key tool for maximizing the sensitivity and accuracy of future dipolar EPR studies.

Integration with FRET and other methods

FRET and dipolar EPR offer complementary strengths for probing site-specific distances in biomacromolecules (Fig. 2). FRET enables room temperature, single-molecule sensitivity, whereas dipolar EPR provides a broader distance range and more accurate distance distributions due to its significantly smaller labels and highly localized unpaired electrons. Also, the distance axis in dipolar EPR is absolute and unlike FRET does not need calibration. Comparative studies reveal excellent overall agreement between FRET and EPR, though differences of ~5 Å often arise due to label size and protein interactions [48]. Integrative EPR/FRET approaches have recently illuminated conformational dynamics in GPCRs, such as μ-opioid (Fig. 1d) [8] and β_2-adrenergic receptors [49]. Beyond FRET, dipolar EPR has been combined with techniques such as anomalous X-ray scattering [50]. EPR has been combined with NMR, mass spectrometry and small angle scattering to map conformational ensembles of complex RNA-binding proteins (Fig. 2) [16]. Integration with emerging computational structure prediction methods (e.g., AlphaFold) further enhances structural interpretation [51]. These integrated multipronged approaches offer powerful new avenues for dissecting conformational landscapes inaccessible to individual methods alone.

^{19}F ENDOR

At short distances (<15 Å), conventional dipolar EPR is limited by excitation bandwidth. Another EPR method, ^{19}F electron–nuclear double resonance (ENDOR) addresses this regime (Fig. 2) [52–54]. ^{19}F ENDOR measures distances between unpaired electrons (nitroxide, trityl, Gd(III), Cu(II), etc.) and nearby ^{19}F nuclei introduced via fluorinated amino acids such as p-trifluoromethyl-phenylalanine or nucleotides such as 2′-fluororibose [55]. Operating at high fields (ca. 94 GHz, 3.4 T), ^{19}F ENDOR has enabled in-cell measurements [54] and structural studies on flexible fluoride-binding riboswitches [15]. Recent work has clarified the influence of spin label identity and spectral broadening on the accessible distance range [56,57]. Selective ^{19}F labeling also enhances conformational studies by NMR [58,59]. The growing adoption of ^{19}F ENDOR expands the dynamic range of distance measurements, complementing conventional DEER spectroscopy.

Data analysis and modeling

Extracting distance distributions from dipolar EPR data relies on approaches ranging from neural networks trained on simulated data [60] to regularized least-squares fitting [61]. The results can be ambiguous if the dipolar EPR data are truncated and noisy, but they can be stabilized by introducing additional distribution constraints (e.g., smoothness, number of Gaussians, compactness) or fitting globally across a variety of biochemical conditions. In all cases, it is essential to quantify the uncertainty in the obtained distance distributions, as this directly impacts the reliability of structural inferences. Bayesian methods are increasingly used to quantify uncertainty in distance estimates [62].

DEER-derived constraints are now routinely integrated into protein conformational modeling pipelines [63,64]. Software platforms such as MMMx, a platform for modeling biomacromolecular ensembles [65], and chiLife, a Python library for modeling non-canonical amino acids including spin labels [66] support spin-label modeling and ensemble refinement [67,68]. Advances in combining MD simulations with DEER data for membrane proteins have improved accuracy [69]. Several promising approaches have emerged that combine DEER constraints with AlphaFold modeling [4,70] or directly embed DEER constraints into AlphaFold training [51]. Efforts are ongoing to assess how well AlphaFold-predicted contact maps reflect conformational distributions [71].

These advances in data analysis and modeling continue to improve the resolution and reliability of distance constraints obtained by dipolar EPR spectroscopy, ultimately enabling more accurate structural and energetic characterizations of biomolecular ensembles.

Outlook

Dipolar EPR spectroscopy has emerged as a versatile tool to study the conformational landscapes of dynamic proteins and nucleic acids under physiologically relevant conditions. Continued innovations in spin-label chemistry, in-cell methodologies, deuteration, advanced pulse sequences, computational modeling, and integration with other techniques will further broaden its impact. As the field evolves, the combination of EPR with complementary biophysical, computational, and cellular

approaches will enable increasingly sophisticated dissection of protein conformational energy landscapes and the elucidation of allosteric mechanisms in complex biomolecular systems.

Declaration of competing interest

There are no competing interests to disclose.

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Data availability

No data was used for the research described in the article.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Galazzo L, Bordignon E: **Electron paramagnetic resonance spectroscopy in structural-dynamic studies of large protein complexes**. *Prog Nucl Magn Reson Spectrosc* 2023, **134–135**: 1–19.

2. Goldfarb D: **Exploring protein conformations in vitro and in cell with EPR distance measurements**. *Curr Opin Struct Biol* 2022, **75**, 102398.

3. Schiemann O, Heubach CA, Abdullin D, Ackermann K, Azarkh M, Bagryanskaya EG, Drescher M, Endeward B, Freed JH, Galazzo L, *et al.*: **Benchmark test and guidelines for DEER/PELDOR experiments on nitroxide-labeled biomolecules**. *J Am Chem Soc* 2021, **143**:17875–17890.

4. del Alamo D, DeSousa L, Nair RM, Rahman S, Meiler J, Mchaourab HS: **Integrated AlphaFold2 and DEER investigation of the conformational dynamics of a pH-dependent APC antiporter**. *Proc Natl Acad Sci* 2022, **119**, e2206129119.

5. * Zhang R, Jagessar KL, Brownd M, Polasa A, Stein RA, Moradi M, Karakas E, Mchaourab HS: **Conformational cycle of a protease-containing ABC transporter in lipid nanodiscs reveals the mechanism of cargo-protein coupling**. *Nat Commun* 2024, **15**:9055.
This study combines DEER spectroscopy with cryo-EM to capture a high-energy outward-facing conformation of an ABC transporter, revealing how it forms an exit pathway for the cargo protein, shedding light on the mechanism of coupled protein export.

6. Dajka M, Rath T, Morgner N, Joseph B: **Dynamic basis of lipopolysaccharide export by LptB2FGC**. *eLife* 2024, **13**, RP99338.

7. Cina NP, Klug CS: **The LptC transmembrane helix undergoes a rigid body movement upon LptB2FG cavity collapse**. *Protein Sci* 2025, **34**, e70133.

8. * Zhao J, Elgeti M, O'Brien ES, Sár CP, Ei Daibani A, Heng J, Sun X, White E, Che T, Hubbell WL, *et al.*: **Ligand efficacy modulates conformational dynamics of the μ-opioid receptor**. *Nature* 2024, **629**:474–480.
This work utilizes DEER and single-molecule FRET to identify multiple active and inactive conformations of the μ-opioid receptor, which interconvert on different timescales, offering insights into the functional dynamics of an important drug target.

9. Bizet M, Byrne D, Biaso F, Gerbaud G, Etienne E, Briola G, Guigliarelli B, Urban P, Dorlet P, Kalai T, *et al.*: **Structural insights into the semiquinone form of human Cytochrome P450 reductase by DEER distance measurements between a native flavin and a spin labelled non-canonical amino acid**. *Chem Eur J* 2024, **30**, e202304307.

10. Haysom SF, Machin J, Whitehouse JM, Horne JE, Fenn K, Ma Y, El Mkami H, Böhringer N, Schäberle TF, Ranson NA, *et al.*: **Darobactin B stabilises a lateral-closed conformation of the BAM complex in E. coli cells**. *Angew Chem* 2023, **135**, e202218783.

11. Dastvan R, Rasouli A, Dehghani-Ghahnaviyeh S, Gies S, Tajkhorshid E: **Proton-driven alternating access in a spinster lipid transporter**. *Nat Commun* 2022, **13**:5161.

12. Dastvan R, Mishra S, Peskova YB, Nakamoto RK, Mchaourab HS: **Mechanism of allosteric modulation of P-glycoprotein by transport substrates and inhibitors**. *Science* 2019, **364**:689–692.

13. Khan F, Elgeti M, Grandfield S, Paz A, Naughton FB, Marcoline FV, Althoff T, Ermolova N, Wright EM, Hubbell WL, *et al.*: **Membrane potential accelerates sugar uptake by stabilizing the outward facing conformation of the Na/glucose symporter vSGLT**. *Nat Commun* 2023, **14**:7511.

14. Praetorius F, Leung PJY, Tessmer MH, Broerman A, Demakis C, Dishman AF, Pillai A, Idris A, Juergens D, Dauparas J, *et al.*: **Design of stimulus-responsive two-state hinge proteins**. *Science* 2023, **381**:754–760.

15. Remmel L, Meyer A, Ackermann K, Hagelueken G, Bennati M, Bode BE: **Pulse EPR methods in the angstrom to nanometre scale shed light on the conformational flexibility of a fluoride riboswitch**. *Angew Chem Int Ed* 2024, e202411241. **n/a**.

16. ** Dorn G, Gmeiner C, de Vries T, Dedic E, Novakovic M, Damberger FF, Maris C, Finol E, Sarnowski CP, Kohlbrecher J, *et al.*: **Integrative solution structure of PTBP1-IRES complex reveals strong compaction and ordering with residual conformational flexibility**. *Nat Commun* 2023, **14**:6429.
This integrative study combines EPR, NMR, DEER, mass spectrometry, small-angle scattering, and extensive ensemble modeling to map the full dynamic conformational landscape of an RNA-binding protein.

17. Chubarov AS, Endeward B, Kanarskaya MA, Polienko YF, Prisner TF, Lomzov AA: **Pulsed dipolar EPR for self-limited complexes of oligonucleotides studies**. *Biomolecules* 2024, **14**:887.

18. Baker ZD, Rasmussen DM, Levinson NM: **Exploring the conformational landscapes of protein kinases: perspectives from FRET and DEER**. *Biochem Soc Trans* 2024, **52**: 1071–1083.

19. Lane BJ, Ma Y, Yan N, Wang B, Ackermann K, Karamanos TK, Bode BE, Pliotas C: **Monitoring the conformational ensemble and lipid environment of a mechanosensitive channel under cyclodextrin-induced membrane tension**. *Structure* 2024, **32**: 739–750.e4.

20. Jaiswal M, Tran TT, Li Q, Yan X, Zhou M, Kundu K, Fanucci GE, Guo Z: **A metabolically engineered spin-labeling approach for studying glycans on cells**. *Chem Sci* 2020, **11**: 12522–12532.

21. Bogetti X, Bogetti A, Casto J, Rule G, Chong L, Saxena S: **Direct observation of negative cooperativity in a detoxification enzyme at the atomic level by Electron Paramagnetic Resonance spectroscopy and simulation**. *Protein Sci* 2023, **32**, e4770.

22. Jash C, Feintuch A, Nudelman S, Manukovsky N, Abdelkader EH, Bhattacharya S, Jeschke G, Otting G, Goldfarb D: **DEER experiments reveal fundamental differences between calmodulin complexes with IQ and MARCKS peptides in solution**. *Structure* 2022, **30**:813–827.e5.

23. Wang X-W, Zhang X, Cui C-Y, Li B, Goldfarb D, Yang Y, Su X-C: **Stabilizing nitroxide spin labels for structural and conformational studies of biomolecules by maleimide treatment**. *Chem Eur J* 2023, **29**, e202301350.

24. Sowiński MP, Mocanu EM, Ruskin-Dodd H, McKay AP, Cordes DB, Lovett JE, Haugland-Grange M: **Sigmatropic rearrangement enables access to a highly stable spirocyclic nitroxide for protein spin labelling**. *Chem Commun* 2025, **61**: 6755–6758.

25. Chen Y-T, Zhang X, Chen J-L, Pan B-B, Goldfarb D, Yang Y, Su X-C: **Rigid and stable nitroxide spin label for high-resolution distance measurements on proteins by DEER experiments**. *Magn Reson Lett* 2025, https://doi.org/10.1016/j.mrl.2025.200194.

26. Hirsch M, Hofmann L, Yakobov I, Kahremany S, Sameach H, Shenberger Y, Gevorkyan-Airapetov L, Ruthstein S: **An efficient EPR spin-labeling method enables insights into conformational changes in DNA**. *Biophys Rep* 2024, **4**, 100168.

27. * Heubach CA, Hasanbasri Z, Abdullin D, Reuter A, Korzekwa B, Saxena S, Schiemann O: **Differentiating between label and protein conformers in pulsed dipolar EPR spectroscopy with the dHis-Cu^{2+} (NTA) motif**. *Chem Eur J* 2023, **29**, e202302541.
This work uses di-histidine Cu(II) labels to reveal a single backbone conformation of the Yersinia outer protein O, demonstrating that Cu(II) labels more directly report backbone conformations. Also shows that native histidines do not bind Cu(II) label in the presence of introduced di-His sites.

28. Tessmer MH, Stoll S: **A rotamer library approach to modeling side chain ensembles of the bifunctional spin label RX**. *Appl Magn Reson* 2024, **55**:127–140.

29. Hasanbasri Z, Tessmer MH, Stoll S, Saxena S: **Modeling of Cu(II)-based protein spin labels using rotamer libraries**. *Phys Chem Chem Phys* 2024, **26**:6806–6816.

30. Singewald K, Hunter H, Cunningham TF, Ruthstein S, Saxena S: **Measurement of protein dynamics from site directed Cu(II) labeling**. *Anal Sens* 2023, **3**, e202200053.

31. Tabares LC, Daniel DT, Vázquez-Ibar JL, Kouklovsky C, Alezra V, Un S: **Using the noncanonical metallo-amino acid [Cu(II)(2,2′-Bipyridin-5-yl)]-alanine to study the structures of proteins**. *J Phys Chem Lett* 2023, **14**:3368–3375.

32. Feng R, Wang M, Zhang W, Gai F: **Unnatural amino acids for biological spectroscopy and microscopy**. *Chem Rev* 2024, **124**:6501–6542.

33. Meng W-H, Zhang X, Pan B-B, Tan X, Zhao J-L, Liu Y, Yang Y, Goldfarb D, Su X-C: **Efficient orthogonal spin labeling of proteins via aldehyde cyclization for pulsed dipolar EPR distance measurements**. *J Am Chem Soc* 2025, **147**:234–246.

34. ** Jana S, Evans EGB, Jang HS, Zhang S, Zhang H, Rajca A, Gordon SE, Zagotta WN, Stoll S, Mehl RA: **Ultrafast bioorthogonal spin-labeling and distance measurements in mammalian cells using small, genetically encoded tetrazine amino acids**. *J Am Chem Soc* 2023, **145**:14608–14620.
This work demonstrates that proteins can be directly overexpressed and spin labeled inside mammalian cells using bioorthogonal tetrazine-based amino acids.

35. Bizet M, Balázsi Á, Biaso F, Byrne D, Etienne E, Guigliarelli B, Urban P, Dorlet P, Truan G, Gerbaud G, *et al.*: **Expanding the diversity of nitroxide-based paramagnetic probes conjugated to non-canonical amino acids for SDSL-EPR applications**. *Chembiochem* 2025, **26**, e202500064.

36. Kucher S, Elsner C, Safonova M, Maffini S, Bordignon E: **In-cell double electron–electron resonance at nanomolar protein concentrations**. *J Phys Chem Lett* 2021, **12**:3679–3684.

37. Pierro A, Drescher M: **Dance with spins: site-directed spin labeling coupled to electron paramagnetic resonance spectroscopy directly inside cells**. *Chem Commun* 2023, **59**:1274–1284.

38. Shenberger Y, Gevorkyan-Airapetov L, Hirsch M, Hofmann L, Ruthstein S: **An *in-cell* spin-labelling methodology provides structural information on cytoplasmic proteins in bacteria**. *Chem Commun* 2023, **59**:10524–10527.

39. Hunter HR, Kankati S, Hasanbasri Z, Saxena S: **Endogenous Cu(II) labeling for distance measurements on proteins by EPR**. *Chem Eur J* 2024, e202403160. n/a.

40. Meron S, Peleg S, Shenberger Y, Hofmann L, Gevorkyan-Airapetov L, Ruthstein S: **Tracking disordered extracellular domains of membrane proteins in the cell with Cu(II)-Based spin labels**. *J Phys Chem B* 2024, **128**:8908–8914.

41. * Cheng C-C, Tsai R-F, Lin C-K, Tan K-T, Kalendra V, Simenas M, Lin C-W, Chiang Y-W: **In-cell DEER spectroscopy of nanodisc-delivered membrane proteins in living cell membranes**. *JACS Au* 2024, **4**:3766–3770.
This work introduces a novel method for delivering spin-labelled membrane proteins into the outer membranes of both Gram-negative and Gram-positive bacteria using nanodiscs.

42. Ward R, Bowman A, Sozudogru E, El-Mkami H, Owen-Hughes T, Norman DG: **EPR distance measurements in deuterated proteins**. *J Magn Reson* 2010, **207**:164–167.

43. Schmidt T, Wälti MA, Baber JL, Hustedt EJ, Clore GM: **Long distance measurements up to 160 Å in the GroEL tetradecamer using Q-band DEER EPR spectroscopy**. *Angew Chem Int Ed* 2016, **55**:15905–15909.

44. Endeward B, Hu Y, Bai G, Liu G, Prisner TF, Fang X: **Long-range distance determination in fully deuterated RNA with pulsed EPR spectroscopy**. *Biophys J* 2022, **121**:37–43.

45. Schmidt T, Stadnytskyi V: **Temperature-dependent rotation of protonated methyl groups in otherwise deuterated proteins modulates DEER distance distributions**. *Appl Magn Reson* 2025, **56**:91–102.

46. Szalai VA, Bergonzo C, Lyon RB, Kelman Z, Schmidt T, Grishaev A: **Structure and dynamics of monoclonal antibody domains using spins, scattering, and simulations**. *ChemMedChem* 2025, e202400917. n/a.

47. Torricella F, Vitali V, Banci L: **A systematic study on the effect of protonation and deuteration on electron spin Tm/T2 in a cellular context**. *Phys Chem Chem Phys* 2024, **26**:20246–20250.

48. Peter MF, Gebhardt C, Mächtel R, Muñoz GGM, Glaenzer J, Narducci A, Thomas GH, Cordes T, Hagelueken G: **Cross-validation of distance measurements in proteins by PELDOR/DEER and single-molecule FRET**. *Nat Commun* 2022, **13**:4396.

49. Casiraghi M, Wang H, Brennan PC, Habrian C, Hübner H, Schmidt MF, Maul L, Pani B, Bahriz SMFM, Xu B, *et al.*: **Structure and dynamics determine G protein coupling specificity at a class A GPCR**. *Sci Adv* 2025, **11**, eadq3971.

50. Stubhan S, Baptist AV, Körösy C, Narducci A, Muñoz GGM, Wendler N, Lak A, Sztucki M, Cordes T, Lipfert J: **Determination of absolute intramolecular distances in proteins using anomalous X-ray scattering interferometry**. *Nanoscale* 2025, **17**:3322–3330.

51. ** Wu T, Stein RA, Kao T-Y, Brown B, Mchaourab HS: **Modeling protein conformational ensembles by guiding AlphaFold2 with Double Electron Electron Resonance (DEER) distance distributions**. *Nat Commun* 2025, **16**:7107.
This pioneering work shows how DEER distance constraints can be integrated into AlphaFold2 structure prediction to guide them toward alternative conformational states.

52. Judd M, Abdelkader EH, Qi M, Harmer JR, Huber T, Godt A, Savitsky A, Otting G, Cox N: **Short-range ENDOR distance measurements between Gd(III) and trifluoromethyl labels in proteins**. *Phys Chem Chem Phys* 2022, **24**:25214–25226.

53. Meyer A, Kehl A, Cui C, Reichardt FAK, Hecker F, Funk L-M, Ghosh MK, Pan K-T, Urlaub H, Tittmann K, *et al.*: **19F electron-nuclear double resonance reveals interaction between redox-active tyrosines across the α/β interface of E. coli ribonucleotide reductase**. *J Am Chem Soc* 2022, **144**:11270–11282.

54. Seal M, Zhu W, Dalaloyan A, Feintuch A, Bogdanov A, Frydman V, Su X-C, Gronenborn AM, Goldfarb D: **Gd^{III}-^{19}F distance measurements for proteins in cells by electron-nuclear double resonance**. *Angew Chem Int Ed* 2023, **62**, e202218780.

55. Asanbaeva NB, Novopashina DS, Rogozhnikova OYu, Tormyshev VM, Kehl A, Sukhanov AA, Shernyukov AV, Genaev AM, Lomzov AA, Bennati M, *et al.*: **^{19}F electron nuclear double resonance (ENDOR) spectroscopy for distance measurements using trityl spin labels in DNA duplexes**. *Phys Chem Chem Phys* 2023, **25**:23454–23466.

56. Bogdanov A, Gao L, Dalaloyan A, Zhu W, Seal M, Su X-C, Frydman V, Liu Y, Gronenborn AM, Goldfarb D: **Spin labels for ^{19}F ENDOR distance determination: resolution, sensitivity and distance predictability**. *Phys Chem Chem Phys* 2024, **26**:26921–26932.

57. Kehl A, Sielaff L, Remmel L, L. Rämisch M, Bennati M, Meyer A: **Frequency and time domain ^{19}F ENDOR spectroscopy: role of nuclear dipolar couplings to determine distance distributions**. *Phys Chem Chem Phys* 2025, https://doi.org/10.1039/D4CP04443F.

58. Huang Y, Reddy KD, Bracken C, Qiu B, Zhan W, Eliezer D, Boudker O: **Environmentally ultrasensitive fluorine probe to resolve protein conformational ensembles by ^{19}F NMR and cryo-EM**. *J Am Chem Soc* 2023, **145**:8583–8592.

59. Qianzhu H, Abdelkader EH, Otting G, Huber T: **Genetic encoding of fluoro-L-tryptophans for site-specific detection of conformational heterogeneity in proteins by NMR spectroscopy**. *J Am Chem Soc* 2024, **146**:13641–13650.

60. Keeley J, Choudhury T, Galazzo L, Bordignon E, Feintuch A, Goldfarb D, Russell H, Taylor MJ, Lovett JE, Eggeling A, *et al.*: **Neural networks in pulsed dipolar spectroscopy: a practical guide**. *J Magn Reson* 2022, **338**, 107186.

61. Fábregas Ibáñez L, Jeschke G, Stoll S: **DeerLab: a comprehensive software package for analyzing dipolar electron paramagnetic resonance spectroscopy data**. *Magnes Res* 2020, **1**:209–224.

62. Sweger SR, Cheung JC, Zha L, Pribitzer S, Stoll S: **Bayesian probabilistic inference of nonparametric distance distributions in DEER spectroscopy**. *J Phys Chem A* 2024, **128**:9071–9081.

63. Belyaeva J, Elgeti M: **Exploring protein structural ensembles: integration of sparse experimental data from electron paramagnetic resonance spectroscopy with molecular modeling methods**. *eLife* 2024, **13**, e99770.

64. Tessmer MH, Stoll S: **Protein modeling with DEER spectroscopy**. *Annu Rev Biophys* 2025, **54**:35–57.

65. Jeschke G: **Protein ensemble modeling and analysis with MMMx**. *Protein Sci* 2024, **33**, e4906.

66. Tessmer MH, Stoll S: **chiLife: an open-source Python package for in silico spin labeling and integrative protein modeling**. *PLoS Comput Biol* 2023, **19**, e1010834.

67. Kao T-Y, Chiang Y-W: **DEERefiner-assisted structural refinement using pulsed dipolar spectroscopy: a study on multidrug transporter LmrP**. *Phys Chem Chem Phys* 2023, **25**:24508–24517.

68. Kazemi S, Lopata A, Kniss A, Pluska L, Güntert P, Sommer T, Prisner TF, Collauto A, Dötsch V: **Efficient determination of the accessible conformation space of multi-domain complexes based on EPR PELDOR data**. *J Biomol NMR* 2023, **77**:261–269.

69. Mittal S, Dutta S, Shukla D: **Reconciling membrane protein simulations with experimental DEER spectroscopy data**. *Phys Chem Chem Phys* 2023, **25**:6253–6262.

70. Schwartz AC, Stein RA, Gil-Iturbe E, Quick M, Mchaourab HS: **Alternating access of a bacterial homolog of neurotransmitter: sodium symporters determined from AlphaFold2 ensembles and DEER spectroscopy**. *Proc Natl Acad Sci* 2024, **121**, e2406063121.

71. Brown BP, Stein RA, Meiler J, Mchaourab HS: **Approximating projections of conformational Boltzmann distributions with AlphaFold2 predictions: opportunities and limitations**. *J Chem Theor Comput* 2024, **20**:1434–1447.