JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Perspective

Progress, Challenges and Opportunities of NMR and XL-MS for Cellular Structural Biology

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Cite This: *JACS Au* 2024, 4, 369−383
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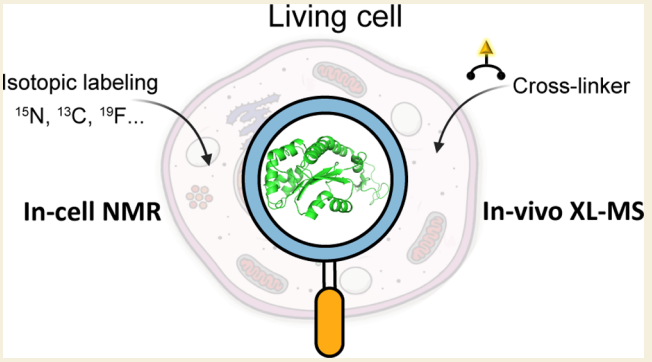
ABSTRACT: The validity of protein structures and interactions, whether determined under ideal laboratory conditions or predicted by AI tools such as Alphafold2, to precisely reflect those found in living cells remains to be examined. Moreover, understanding the changes in protein structures and interactions in response to stimuli within living cells, under both normal and disease conditions, is key to grasping proteins' functionality and cellular processes. Nevertheless, achieving high-resolution identification of these protein structures and interactions within living cells presents a technical challenge. In this Perspective, we summarize the recent advancements in in-cell nuclear magnetic resonance (NMR) and in vivo cross-linking mass spectrometry (XL-MS) for studying protein structures and interactions within a cellular context. Additionally, we discuss the challenges, opportunities, and potential benefits of integrating in-cell NMR and in vivo XL-MS in future research to offer an exhaustive approach to studying proteins in their natural habitat.



KEYWORDS: *cellular structural biology, protein structure, protein interaction, in-cell NMR, in vivo XL-MS*

1. INTRODUCTION

In living cells, proteins perform essential biological functions through conformational changes and interactions. Investigating the structure, interaction, and dynamics of proteins is crucial for understanding their functions and the inner workings of the cell.[1] Most of the current studies on proteins are typically carried out in vitro on purified samples with optimized experimental conditions, which differ greatly from the physiologically natural environment inside cells. The cellular interior is a crowded environment containing up to 400 g/L macromolecules, which produce excluded volume effect and weak, soft chemical interactions of proteins with the surrounding cellular components.[2−4] Additionally, the distinctive subcellular compartments intricately complicate the dynamics of protein conformation and interactions within live cells, setting them significantly apart from those observed in whole cells. However, the relatively scarce in situ structural analysis techniques impede our access to information about protein complexes within their native functional microenvironment, thereby limiting our understanding of disease pathways and impeding the development of new therapies. Whether and how the cellular environment modulates the behaviors of proteins remains an open question. However, it is difficult to fully reproduce the cellular environment under in vitro conditions, while studying proteins in living cells is much more technically challenging than studying proteins in a dilute solution.

Over the past few decades, numerous efforts have been made on the development of methods for directly visualizing proteins in living cells, including nuclear magnetic resonance (NMR) spectroscopy, in vivo chemical cross-linking mass spectrometry (XL-MS), cryo-electron tomography (cryo-ET), light microscopy, and various computational approaches, enabling researchers to derive insights into intracellular protein structures, protein folding, protein dynamics, and protein interactions in their native environment. Cryo-ET can visualize the 3D structures of macromolecules, viruses, and cellular components, as well as directly study protein−protein interactions, the assembly and operation of protein machinery, and the phase separation of biomacromolecules in near-nanometer resolution.[5] Besides, the development of fluorescent proteins and advanced imaging techniques has significantly expanded the capabilities of light microscopy for

Received: November 14, 2023
Revised: January 5, 2024
Accepted: January 16, 2024
Published: February 5, 2024

ACS Publications
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https://doi.org/10.1021/jacsau.3c00712
JACS Au 2024, 4, 369−383

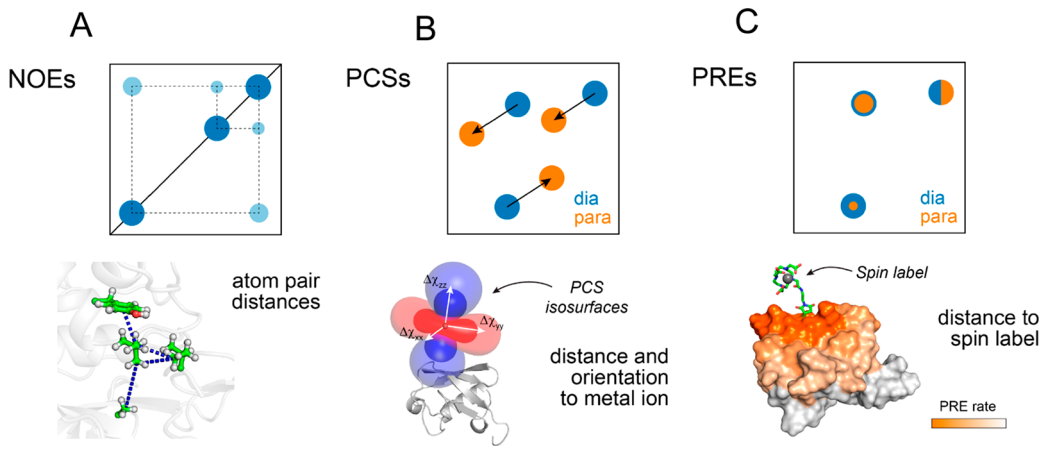

Figure 1. Experiments for obtaining distance restraints in living cells by solution NMR, including NOEs (A), PCSs (B), and PREs (C).[17] Reproduced with permission from ref 17. Copyright 2023 MDPI.

studying protein structure within cells.[6] By expressing fluorescent proteins in fusion, these methods not only allow for precise localization of proteins but also provide distance information that characterizes structural features, although such information is relatively sparse and low-resolution. In addition to the aforementioned experimental techniques, various computational methods have also been applied in the study of protein structures. With the optimization of sampling algorithms, the application of coarse-grained models, and the development of computer hardware, such as the widespread use of GPUs, MD simulations can now achieve the study of protein molecular structures in different cellular environments.[7]

Among the existing structural biology techniques, nuclear magnetic resonance (NMR) spectroscopy is the only one that allows high-resolution structure determination of macromolecules in solution. It can also provide information on macromolecules in a nondestructive manner under "near-to biologically relevant" conditions or even in living cells, because of the low energy of the electromagnetic radiations employed in NMR spectroscopy. The advantage of NMR to selectively "visualize" a chosen type of isotope as well as the noninvasive nature make NMR spectroscopy ideally suited for study of protein dynamic behaviors in living cells. Since Dötsch and coworkers acquired the first two-dimensional (2D) high-resolution NMR spectrum of a small protein (NmerA) in living *Escherichia coli* cells in 2001,[8] in-cell NMR has emerged as a powerful technique for monitoring proteins directly inside living cells with atomic resolution. In-cell NMR has been introduced comprehensively in many reviews.[9−12] Here, we focus on some recent progress in methodology development and applications for probing protein structure and interactions in living cells.

In-cell NMR generally focuses on a single target protein at a time by specific isotope labeling; on the contrary, in vivo chemical cross-linking mass spectrometry (XL-MS) enables analysis of protein structure and interaction at the cellular proteomic level. In vivo XL-MS method is crucial for unraveling the native conformation of protein complexes directly within the complex and dynamic microenvironments of cells and tissues. By employing chemical cross-linking techniques, researchers can probe the intricate spatial organization and interactions of proteins, providing a comprehensive understanding of the structural dynamics of protein complexes in their physiological context. This approach not only sheds light on the molecular details of cellular and tissue processes but also contributes to advancing our understanding of the functional roles played by these complexes in vivo.

2. PROTEIN STRUCTURE DETERMINATION USING IN-CELL NMR

In vivo determination of protein 3D structures is essential for explicit understanding of the structural basis of their functions inside cells. Protein structure determination by NMR relies on the collection of structural restraints, typically including the distance restraints, hydrogen bonds, and dihedral angle restraints, which are critical to the precision of the calculated protein structure.

2.1. Protein Structure Determination Using Solution NMR in Cellular Environment

In solution NMR experiments, distance restraints are traditionally obtained via the Nuclear Overhauser Effect (NOE), which is one of the most important phenomena in NMR spectroscopy. NOE arises from dipole−dipole relaxation between a dipolar-coupled nuclear spin pair, and the NOE cross-relaxation rate between spins is proportional to r^{-6} (with r being the distance between two nuclei). NOE measurements depend on the NOE spectroscopy (NOESY), where the volume of the corresponding cross-peak is correlated to r^{-6} and can reveal a close distance in space between nuclei (up to ∼5−6 Å in most cases) (Figure 1A).[13] Three dimensional (3D) NMR experiments for resonance assignment and obtaining NOE-based structure restraints normally require long acquisition times. Therefore, although NMR is powerful in protein structure determination, its low sensitivity, severe line broadening in the cellular environment, and the short lifetime of cells challenge the acquisition of sufficient structural information on proteins in living cells. The first de novo protein structure in *E. coli* cells was determined by the Ito group in 2009, via high protein expression (3−4 mM in concentration) and rapid measurement of the 3D NMR spectra by nonlinear sampling scheme for the indirectly acquired dimensions.[14] In combination with maximum-entropy processing, the duration of each 3D experiment was reduced to 2−3 h to make assignments and obtain NOE distance restraints for structure calculation. NOE-based structure determination in eukaryotic cells is hampered mainly

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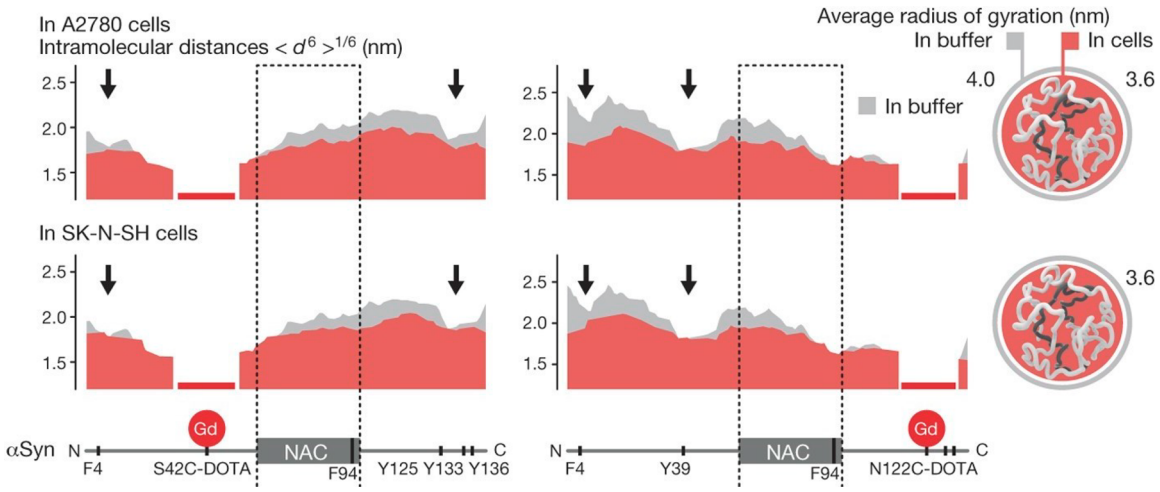

Figure 2. Compact α-syn structures in cells. Intramolecular PRE-derived distance profiles of N-terminally acetylated α-syn in buffer (gray) and in A2780 and SK-N-SH cells (red).[18] Reproduced with permission from ref 18. Copyright 2016 Springer Nature.

due to the inherent poor sensitivity of NMR and the fact that the achievable protein concentration in eukaryotic cells was too low to obtain a sufficient number of NOE-derived distance restraints, since 3D spectra for side-chain resonance assignment and 3D NOESY spectra suffered from the short lifetime of the cells. Ito and colleagues further improved their method for de novo structural determination from lower levels of expressed proteins.[15,16] In 2019, they reported a strategy for protein structure determination in sf9 cells by introducing a bioreactor system that supplies fresh medium into the NMR tube continuously to prolong the lifetime (at least 24 h) of the cells in the NMR tube. The 3D NMR data were sparsely sampled and reconstructed by quantitative maximum entropy, thus producing improved sensitivity of the in-cell spectra.[16]

Conventional NOE-based structure determination is advantageous to provide information on side-chain atoms as well as global structures of proteins. However, its widespread application is still hampered by the difficulty in obtaining well-resolved 3D spectra in living cells. NMR methods that provide structure restraints based on paramagnetic effects, which can be measured by heteronuclear 2D correlation or even one-dimensional (1D) NMR spectra, provide an outstanding advantage for structural studies in living cells. Pseudocontat shift (PCS) is present in molecules chelating a paramagnetic moiety with anisotropic unpaired electrons. It induces chemical shift perturbations of the surrounding nuclei depending on both the distance (r^{-3}) and the orientation between the paramagnetic center and the observed nuclei, thus providing both distance and angular information (Figure 1B). Paramagnetic relaxation enhancement (PRE) is the acceleration of magnetization relaxation. It is caused by the dipolar interaction between a nucleus and isotropic unpaired electrons and is usually measured with a paramagnetic probe covalently linked to a specific site of an otherwise diamagnetic protein. PRE is proportional to r^{-6}, thus providing distance information between a nucleus and the paramagnetic center (Figure 1C). Due to the larger gyromagnetic ratio of electrons, both PRE and PCS experiments can provide long-range distance restraints (up to 35 Å) for structural calculation. In 2016, Theillet et al. used PRE restraints derived from 2D ^{1}H−^{15}N spectra to get insights into the structure of the intrinsically disordered protein α-synuclein (α-syn) in mammalian cells. They show that α-syn adopts a more compact conformation in mammalian cells than it does in buffer (Figure 2).[18] In addition, Pan et al. and Müntener et al. independently proposed the method to determine the structure of Streptococcal protein G B1 domain (GB1) from simple 2D ^{1}H−^{15}N heteronuclear single quantum coherence (HSQC) spectra using PCS restraints in *Xenopus laevis* oocytes,[15] providing an effective approach for globular protein structure determination in living eukaryotic cells.[19,20]

The nonspecific interactions of proteins with large cellular components increase their transverse relaxation, resulting in severe line broadening in the conventional ^{1}H-detected heteronuclear in-cell NMR spectra, which severely limits the widespread application of in-cell NMR in studies on globular proteins. ^{19}F is a spin-1/2 nucleus with a high gyromagnetic ratio, making it a highly sensitive probe (83% of ^{1}H) for NMR studies. The resonances of ^{19}F nuclei are extremely sensitive to the surrounding chemical environment, which produces a wide chemical shift range. Labeling with ^{19}F is a powerful approach for protein in-cell NMR studies,[21] since it is naturally absent from biological systems and can be detected by 1D NMR spectra. ^{19}F PRE was successfully used to characterize the globular protein structure in mammalian cells.[22] By comparing the ^{19}F PRE-derived distance restraints measured in cells with in a buffer, it was found that the cellular environment does not influence the structure of GB1. The use of ^{19}F labeling expands the applicability of NMR for atomic-level characterization of protein structure in mammalian cells.[22] In general, in-cell NMR signals of membrane proteins normally suffer from the restricted rotational motion of the protein and are difficult to detect by traditional 2D NMR spectra in cells. More recently, a direct observation of membrane-associated H-Ras in HeLa cells using ^{19}F in-cell NMR was reported by Ikari and colleagues.[23] P-trifluoromethoxyphenylalanine (OCF_3Phe) was site-specifically incorporated into H-Ras, thus producing a relatively narrow ^{19}F resonance due to the rapid rotation of the trifluoromethyl (CF_3) group. It was found that H-Ras adopts conformational multiplicity on the plasma membrane, suggesting that ^{19}F provides an effective approach for getting insight into membrane-associated states of proteins at atomic resolution in living cells.

AlphaFold2 has been developed to predict the 3D structure of proteins from amino acid sequences with atomic-level accuracy.[24] It is still an open question whether the structures in

https://doi.org/10.1021/jacsau.3c00712
JACS Au 2024, 4, 369−383

a cellular environment can be truthfully reproduced by AlphaFold2 prediction or in in vitro experiments. De novo protein structure determination in living cells is hampered by the long acquisition time and the poor resolution of 3D NMR spectra obtained in a cellular environment. To date, it is still challenging for structure determination on most proteins exclusively on the basis of NOE restraints obtained in living cells, particularly in human cells. Benefit from the development of protein labeling strategies and paramagnetic probes in recent years, protein structure can be studied by performing 2D or 1D NMR experiments, which greatly expands the applications of NMR in protein structure determination in living cells. Although studies on GB1 showed that the cellular environment does not affect the protein structure, the structure of intrinsically disordered protein, α-syn, is shown to be more compact than that in solution. A study on a multidomain protein, Calmodulin (CaM), in reverse micelles demonstrated that confinement dramatically altered CaM structure,[25] suggesting that proteins with large conformational plasticity may be more likely to be affected by the cellular environment. Therefore, multidomain proteins or proteins with conformational multiplicity may attract more attention in the study of in situ protein structure in the future. The interdomain motion and conformational distribution are often closely related to multifunctionality and are very sensitive to the environment. Rapidly obtaining structure restraints based on 2D or 1D in-cell NMR experiments to validate the structure determined in solution would be an effective way to get insight into the protein structure in a functional environment. Further development in methods for protein labeling, and NMR techniques for improving detection limits and resolution should further empower NMR in in situ protein structure studies.

2.2. Protein Structure Determination Using Solid-State NMR in Cellular Environment

Solution NMR has emerged as a powerful technique for studying protein structures and interactions in living cells. However, this technique is limited by molecular tumbling times, which depend on molecular size and solvent viscosity. Interactions of proteins with large cellular components often broaden the resonance lines beyond the detection limit of solution NMR. Therefore, solution NMR can usually only be used for small proteins that are intrinsically disordered or tumbling rapidly in cells. Magic angle spinning (MAS) solid-state NMR (ssNMR) is particularly suitable to study large assemblies such as protein complexes, amyloid fibrils, and membrane-embedded proteins, as it is not limited by molecular correlation times and uses spinning to minimize anisotropic interactions.[26]

In 2012, the Dötsch group reported an in-cell ssNMR study on two well-folded cytosolic proteins, *E. coli* thioredoxin (Trx) and human FK506-binding protein (FKBP) in *E. coli* cells, both of which are involved in high molecular weight complexes inside the cell and thus cannot be detected by solution NMR. By employing ^{13}C-depleted glucose as the sole carbon source combined with protein selective-labeling schemes, these two proteins were successfully measured by recording a series of 1D ^{13}C and 2D ^{15}N,^{13}C correlation experiments on a frozen solid-state in-cell sample under low temperature at which cross-polarization (CP) transfer efficiency is enhanced.[27] Therefore, solid-state NMR provides an alternative technique to investigate cytosolic proteins that are involved in interactions in the cellular milieu and tumble too slowly for detection by solution NMR.

Membrane proteins reside in a highly heterogeneous and complex lipid environment. The majority of in vitro membrane protein structural analyses rely on detergents to extract these proteins from their native environment, which may alter the structure and function of the membrane proteins, and structure determination is commonly carried out in membrane mimicking environments, such as detergent micelles, bicelles, and synthetic lipid bilayers.[28,29] Cellular solid-state NMR allows for the investigation of membrane proteins in native cellular membranes without the need for protein purification using detergents. In the past decade, substantial progress has been made in sample preparations and detection techniques.[30−34] ssNMR has been applied to study membrane proteins in native cellular membranes.[33,35−37] However, the high-resolution 3D structure determination of membrane proteins in cellular membranes is still a challenge mainly due to the low concentration of the membrane protein and the high complexity of native cellular membranes, resulting in low sensitivity of the spectra as well as severe interference from background signals.

More recently, the Yang group reported approaches for structure determination of membrane proteins in *E. coli* cellular inner membranes.[38,39] A "dual-media" expression approach and antibiotic treatment were employed to suppress the interference of background proteins, while signal sensitivity was effectively enhanced upon a high level of overexpression of aquaporin Z (AqpZ) and the removal of outer membrane components. Based on the high sensitivity and good resolution of the ssNMR multidimensional spectra, almost all residues in AqpZ were successfully assigned. More than 1000 ^{13}C−^{13}C distance restraints were then obtained from ^{13}C−^{13}C combined R2$^{\nu}_{n}$-driven (CORD) spectra for structure calculation, and the 1.7-Å ssNMR structure of AqpZ in *E. coli* cellular inner membranes was determined based on the experimental distance restraints.[39] This work provides a strategy for determining the structure of highly expressed membrane proteins in the cellular environment, while structure determination of proteins with lower expression levels is still hampered by the inherent low sensitivity of ssNMR.

Dynamic nuclear polarization (DNP), in which the high polarization of an unpaired electron is transferred to the observed nuclei, can greatly enhance NMR sensitivity.[40] In recent years, DNP-based ssNMR (DNP-ssNMR) has been used for the study of proteins in cell lysate, bacteria, mammalian cells, as well as 3D cell culture.[41−45] Frederick et al. applied DNP-ssNMR to investigate the structure of yeast prion protein Sup35 in yeast cell lysates by adding the exogenously prepared isotopically labeled protein to deuterated lysates. Using the combination strategy of DNP with an isotopic labeling scheme, protein structure is measured at endogenous levels in biological contexts, and the results show that the native context has a dramatic influence on protein structure.[41] Moreover, the Baldus group developed an in situ DNP-ssNMR approach for studying the protein structure inside human cells. Isotope-labeled protein was introduced into human cells by electroporation, followed by functional treatment on the cells and introduction of DNP-radicals for ssNMR. This in situ DNP-ssNMR scheme enabled the detection of ubiquitin at endogenous levels in human cells.[42] More recently, they utilized SNAPol-1, a novel biradical, to conduct DNP-ssNMR at high magnetic fields (800 MHz/527

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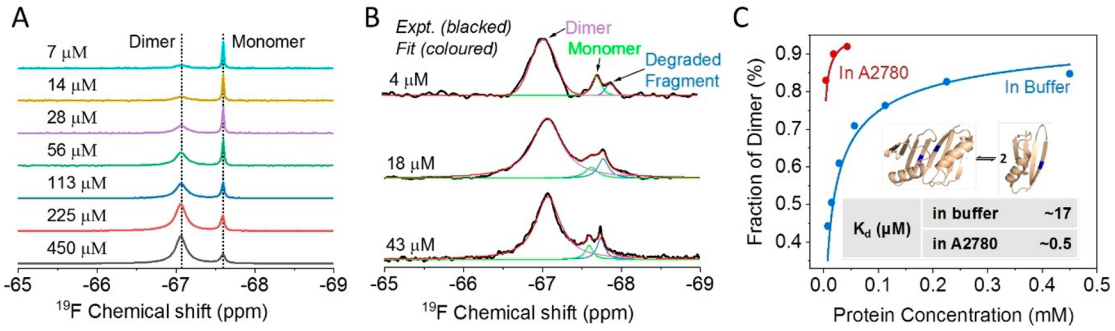

Figure 3. ^{19}F NMR spectra of tagged GB1 variant at different concentrations in PBS buffer (A) and in A2780 cells (B); and corresponding fitting curves of the dissociation constant (C). Inset shows the dissociation of the homodimer. PDB ID: 2rmm. The mutant of T16C was marked with blue.[53] Reproduced with permission from ref 53. Copyright 2023 John Wiley and Sons.

GHz) inside human cells and cell nuclei electroporated with isotopically labeled ubiquitin, enabling high-field DNP-ssNMR with superior sensitivity, enhanced spectral resolution, and subcellular specificity.[43]

Traditional in-cell NMR experiments commonly depend on the overexpression or introduction of exogenous protein into the cells with concentration exceeding endogenous levels for sufficient NMR sensitivity, which may alter the interaction and physiological relevance of target proteins in the cellular interior. Further development of polarization agents, methods for DNP-ssNMR sample preparation, and NMR detection strategies will enable NMR for investigating protein structure and interaction with superior sensitivity in a native cellular environment and extend the study to biomacromolecules that cannot be enriched at high concentrations.

3. PROTEIN INTERACTIONS STUDIED BY IN-CELL NMR

3.1. Protein−Protein Interactions

In the cellular environment, the transient contacts between macromolecules that organize the cellular interior are known as quinary interactions. It has been found in recent years that the quinary interaction can affect protein folding, protein electrostatic interaction, and protein dynamics in the cellular environment,[46−49] indicating a great significance of studying proteins in living cells. NMR is powerful for probing protein interactions since it is sensitive to the environmental changes of atoms. Studying protein interactions in living cells is essential for understanding the mechanisms by which proteins function. In 2020, Burmann et al. reported the interaction of α-syn with chaperones and membranes in mammalian cells by using in-cell NMR.[50] [U-^{15}N]-labeled recombinant α-syn was delivered into HEK293 cells, yielding ^{1}H−^{15}N HSQC spectra with intensity patterns that are canonical chaperone-interaction signatures. The N-terminal interactions of α-syn in cells are abrogated by inhibition of two major chaperones and result in transient interactions of α-syn with cellular membranes and relocalization to mitochondria. Using in-cell NMR, the interaction of α-syn with endogenuous chaperones and membranes are monitored in a residue-resolved manner, thus providing the molecular details for understanding protein functions in the cellular environment.

Although in-cell NMR is proven to be effective on some proteins in cells, the interactions of many globular proteins with cellular components often broaden the resonances beyond detection in traditional ^{1}H−^{15}N HSQC-based NMR spectra. Using ^{19}F NMR, direct observation of globular proteins in mammalian cells was achieved.[22] Human Cyclophilin A (CypA), whose in-cell SOFAST ^{1}H−^{15}N HMQC and methyl ^{1}H−^{13}C HMQC spectra are completely invisible, was observed in the ^{19}F in-cell NMR spectrum. The signal of CypA, enabling further detection of its interaction with Cyclosporin A. In addition to delivering protein into cells via electroporation, Luchinat, Banci, and colleagues reported a medium switch strategy, by which fluorinated amino acids can be incorporated into proteins within human cells, thus enabling ^{19}F direct detection in living cells without the need for protein delivery.[51] By coexpression of two proteins incorporating 3FY, 4FF, and 6FW, the interaction between superoxide dismutase 1 (SOD1) and its structural homologue, the second domain of the copper chaperone for SOD (CCS-D2), was observed in living HEK293T cells.

Protein−protein interactions are essential for cellular function, but nearly all equilibrium thermodynamic and kinetic studies of protein−protein interactions were performed in a dilute buffer. In 2020, Li, Pielak, and colleagues thermodynamically quantified protein−protein interactions in living cells.[52] They employed simple 1D ^{19}F NMR to detect A34F GB1 dimerization in a prokaryote (*E. coli*) and a eukaryote (*Xenopus laevis* oocytes). It was shown that the dimer is more stable in cells than in the buffer and more stable in oocytes than *E. coli*. They further test the role of electrostatic interactions between GB1 and the cellular milieu and found that increasing the negative charge on the protein surface enhances a specific protein−protein interaction. The findings have important implications for understanding how widespread nonspecific and specific interactions between proteins in cells coherently evolve for protein function in crowded cellular environments. However, quantifying protein−protein interactions in mammalian cells is challenging due to the low achievable protein concentration and the inherently low sensitivity of NMR. More recently, the Li group developed a water-soluble tag of wPSP-6F with high reaction selectivity and appreciable environmental sensitivity, which can be incorporated into proteins via cysteine residue under mild conditions.[53] Due to six equivalent fluorine atoms and fast trifluoromethyl rotation, it significantly enhances the signal-to-noise ratio and therefore enables detection of globular proteins in human cells at concentrations as low as 1.0 μM. Using this ^{19}F tag, protein dimerization of a GB1 variant (A34F, K10N) was quantified in human cells (Figure 3). The big difference of the equilibrium dissociation constant in the buffer and in

https://doi.org/10.1021/jacsau.3c00712
JACS Au 2024, 4, 369−383

A2780 cells indicated the profound effect of chemical interactions in human cells.

3.2. Protein−Ligand Interactions

In-cell NMR has been shown to be an effective method for the direct observation of protein−ligand interactions at atomic resolution within the cellular environment. Most of the studies probe the interaction by selective observation of a ^{15}N-labeled protein target.[54,55] Recently, Luchinat et al. reported an approach to perform protein-observed ligand screening directly in living human cells by fast, inexpensive ^{1}H NMR experiments.[56] Signals from slow-exchanging histidine side chain amide protons located in the active site of CA2 (the second isoform of human carbonic anhydrase) were detected in a background-free region of the 1D ^{1}H in-cell NMR spectrum between 11 and 16 ppm, allowing protein−ligand interactions to be monitored in living cells without the need for isotopic labeling. They further improved their method to allow real-time quantification of protein−ligand binding by introducing bioreactors to maintain human cell viability for up to 72 h.[57] The binding of the two inhibitors, AAZ and MAZ, to human CA2 overexpressed in the cytosol was monitored by 1D ^{1}H NMR spectra. They propose that these approaches can be applied to other proteins that give rise to ^{1}H signals in any background-free spectral region for investigating kinetic behaviors of proteins in living cells.

Ligand-observing NMR methods, including saturation transfer difference (STD) and transfer NOESY (Tr-NOESY), are powerful for probing ligand binding to large receptors.[58] STD NMR can provide information on the binding epitope of the ligand and reveal the closest bound moieties to the receptor, whereas Tr-NOESY NMR determines the conformational changes of the ligand on binding to the receptor. Primikyri et al. reported research on probing the interaction of a quercetin bioconjugate with the nonlabeled antiapoptotic protein Bcl-2 located on the outer membrane of mitochondria in living human cancer cells.[59] STD and Tr-NOESY in-cell NMR spectroscopy were employed to investigate the direct binding via a ligand-observed strategy. The STD signals indicate that all of the protons of 3′ quercetin-alanine were involved in the interactions with intracellular receptors. Moreover, new Tr-NOE cross-peaks of the ligand within the cells were detected, suggesting the adaptation of a new conformation of the bioconjugate upon binding to Bcl-2. G protein-coupled receptors (GPCRs) family are well-established drug targets within pharmaceutical intervention. Ligand-based NMR methods were used to study the interaction of peptide heptamers and the C-X-C Chemokine Receptor Type4 (CXCR4) on membranes of human T-Leukemia cells.[60] The Tr-NOESY results demonstrate the presence of a well-defined side chain cluster involved in the binding to the receptor on the cell surface.

Interactions of transmembrane receptors with their ligands are essential for cellular signaling and are, therefore, a major focus for drug development. It is known that the failures of drug design are often associated with poor cell membrane permeability or low binding specificity within the physiological environment.[61] NMR studies of living cells enable direct observation of protein−ligand interactions at atomic resolution within the cell. However, due to the limited rotation motion of receptors on cell membranes, it is extremely difficult to characterize the receptor−ligand interaction by observing proteins. STD and Tr-NOESY allow the monitoring of ligand−receptor binding by detecting signals from the ligand, taking the advantage of being labeling-free and not being limited by the molecular weight of the receptor. Information about ligand binding sites, binding thermodynamics, and receptor-bound ligand conformation can be obtained from these experiments. Therefore, a ligand-observing strategy seems to be an ideal choice for the study of ligand−protein interactions in living cells, as well as discovery of new therapeutic agents targeting large membrane receptors.

4. CHALLENGES AND PROSPECTS OF IN-CELL NMR

Most of the early in-cell NMR studies focused on proteins overexpressed in *E. coli* cells, in which high protein levels could be obtained to facilitate NMR detection. In the past decade, efforts toward protein labeling, protein delivery and expression, and NMR sampling strategies have been made, enabling study of proteins in eukaryotic cells and even human cells. More importantly, globular proteins at concentration as low as 1.0 μM were successfully visualized in human cells,[53] which indicates considerable potential of in-cell NMR for deriving insight into biological events in physiological conditions, as well as pathogenic mechanisms on a disease-cell model[62] or patient primary cells. In addition to the use of highly sensitive tags, DNP can greatly enhance NMR sensitivity.[40] Currently, DNP is mainly applied to solid-state NMR experiments on frozen samples,[42,63] and it is still a challenge to enhance the signal of biological samples in solution. Methodological development on chemically induced DNP and novel pulse schemes suitable for large proteins is expected to improve the detection limits of NMR. Besides, taking advantage of noninvasive, in-cell NMR makes it possible to monitor dynamic patterns of protein behaviors by recording time series of successive spectra on a single sample at the atomic level, which potentially expands the application of NMR for monitoring the changes of protein conformation, interaction, stability, and post-translational modifications within living cells in response to small molecule inhibitors or external stimuli on the temporal resolution.

5. DETERMINING IN-CELL PROTEIN COMPLEX CONFORMATIONS WITH IN VIVO XL-MS TECHNOLOGY

NMR obtains structural information about proteins through spatial details, such as distances and angles between atoms. In contrast, XL-MS utilizes cross-linkers that react with specific amino acids, and structural information about proteins can be derived from the arm length of the cross-linker. Compared with NMR, XL-MS features advantages such as increased sensitivity, higher throughput, and lower sample requirements, making it particularly suitable for conducting comprehensive conformational analyses of protein complexes at the scale of proteome studies in the cellular microenvironment.

5.1. Conformational Analysis of Protein Complexes within Cells

In the realm of cell biology, the quest to unravel intricate protein structures and interactions within cells has propelled the continuous development and refinement of in vivo chemical cross-linking methods. The corresponding distances between the cross-linked residues were compared with those of the known structures. The so-called "over-length" cross-links, meaning the distances between corresponding cross-linking sites exceed the maximum arm length of the corss-linker,

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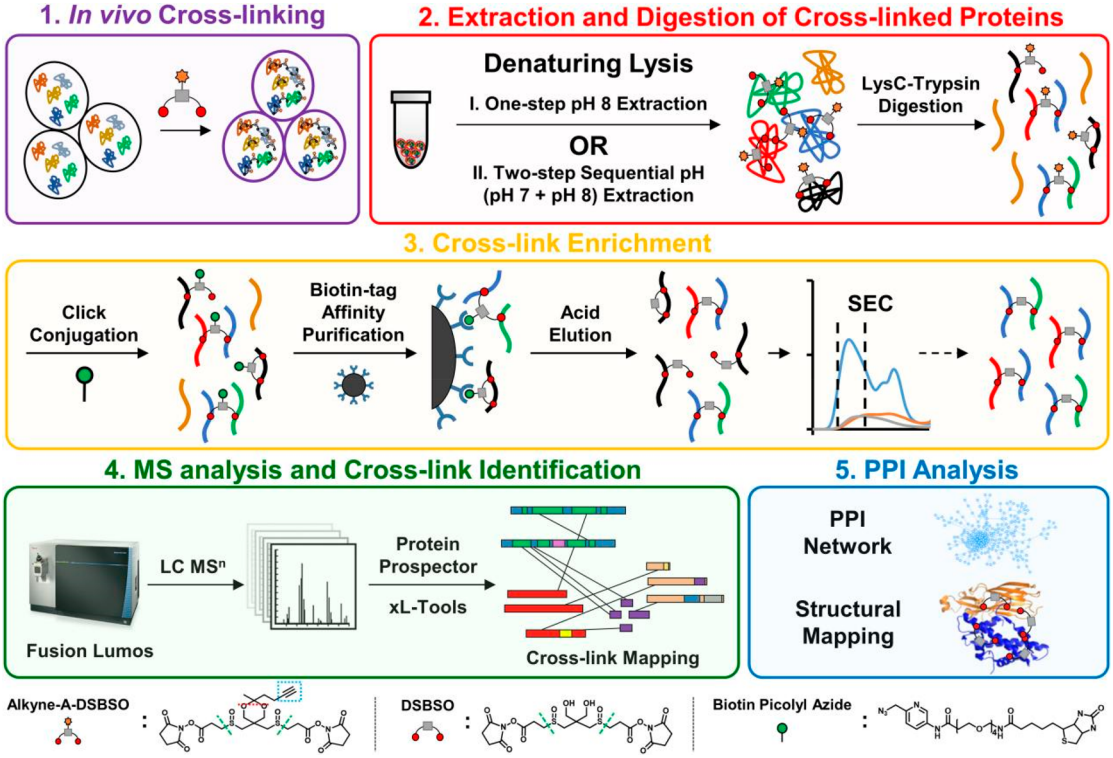

Figure 4. A robust in vivo XL-MS platform to enhance in-depth protein complex analysis.[66] Reproduced with permission from ref 66. Copyright 2021 National Academy of Sciences.

suggest that the protein undergoes conformational dynamics. In such cases, the structure refinements were performed with the distance restraints from cross-linked residues. By employing these techniques, researchers can probe the complex spatial organization and interactions of proteins, providing a comprehensive understanding of the structural dynamics of protein complexes in their physiological context.

In 2008, the use of the cross-linker of PIR (MRN) on Shewanella facilitated the analysis of the extracellular protein complex OmcA-MtrC.[64] Subsequently, the membrane permeability of the cross-linker was continuously regulated, enhancing the cross-linking efficiency of the protein complexes within various cellular organelles. In 2013, in vivo cross-linking of human HeLa cells was achieved, identifying 368 cross-linking sites within the cell membrane, cytoplasm, and cell nucleus, effectively facilitating the cross-linking of intracellular proteins.[65] Subsequent breakthroughs in the field include the development of novel cross-linkers with enhanced membrane permeability,[64−67] efficient enrichment methods for cross-linking peptides,[68−71] mass spectrometry analytical methods for cleavable cross-linkers,[72] and retrieval software, such as pLink[73,74] and XlinkX,[75] which are actively being developed to enhance the efficiency of identifying cross-linked sites within intracellular protein complexes. As a pivotal milestone in 2021, Wheat et al. developed a robust in vivo XL-MS platform to enhance in-depth protein complex analysis.[66] This achievement involved the integration of a multifunctional MS-cleavable cross-linker with sample preparation strategies, including a two-step sequential pH extraction procedure to minimize histone interference, cross-link enrichment through click chemistry and acid cleavage, peptide separation via SEC, and identification using high-resolution MS, revealing 6,439 interactions among 2,484 proteins and offering a detailed yet panoramic view of cellular PPIs in their native environments (Figure 4).

Building upon the achievement of achieving depth in intracellular cross-linking, the new challenge lies in enhancing the dynamic capture capabilities and biocompatibility of cross-linking methods to obtain the in situ conformation of protein complexes within living cells. Jiang et al. introduced the tBu-PhoX cross-linker in 2021, combining membrane permeability and IMAC enrichment to enable in vivo cross-linking within just 30 min, minimizing cellular interference and ensuring comparable cross-linking results.[75] Pushing the boundaries further in 2022, Gao et al. introduced the BSP cross-linker, reducing in vivo cross-linking time to a mere 5 min without significant cellular disruption.[76] Moreover, in 2023, the TDS cross-linker was developed, utilizing a trehalose disaccharide scaffold known for its excellent amphiphilicity and biocompatibility.[77] This novel approach enabled in vivo cross-linking at the cellular level without auxiliary solvents, preserving cell viability and proteome integrity, while capturing a more authentic dynamic conformation of proteins.

With the progress of XL-MS techniques, efforts have been made to enhance the precision of understanding intracellular protein dynamics. This involves using cross-linking data to set up distance restraints, not only for well-characterized proteins but also for those whose structures have eluded traditional methods in studying protein functional dynamics.[78,79] Moreover, the incorporation of cross-linking data into advanced algorithms has enabled the evolution of hybrid experimental and deep learning strategies, adding a new dimension to our comprehension of protein dynamics.[80]

By leveraging cross-linking data that surpass the maximum linker length, researchers have successfully unraveled the dynamics of protein complexes. This is particularly applicable

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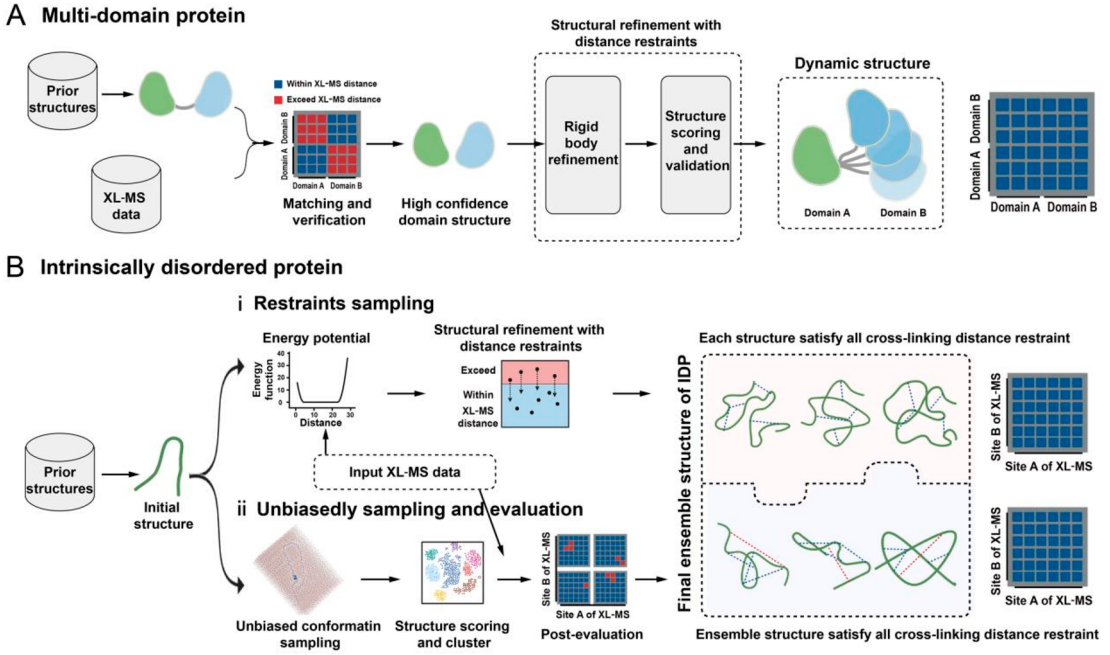

Figure 5. Algorithm workflow for hierarchical decoding of protein dynamics using XL-MS. (A) Workflow for decoding the ensemble conformation of multidomain proteins. (B) Strategies for decoding the ensemble conformation of IDPs. Structure calculations were performed using distance restraints from XL-MS. (i) Restraints sampling and (ii) unbiased sampling combined with postevaluation using XL-MS. The final ensemble structure of IDP from both strategies complement each other.[81] Reproduced with permission from ref 81. Copyright 2023 John Wiley and Sons.

to intrinsically disordered proteins (IDPs), known for their high dynamism.[82,83] Zhang et al. employs two strategies to characterize IDPs.[81] One method utilizes XL-MS data to calculate IDP structures by converting it into distance restraints, which is also applicable to obtaining the dynamic structure of multidomain proteins (Figure 5a). The other involves unbiased sampling through all-atom molecular dynamics simulations, followed by evaluation and selection of structures based on XL-MS data (Figure 5b). These approaches successfully decode dynamic ensembles of highly mobile proteins, such as HMG-I/Y and HMG-17, within cellular contexts. This technical support enhances our understanding of IDP functionality in the cellular microenvironment.

Despite these accomplishments, challenges persist in the field. Researchers are actively pursuing faster cross-linking reactions, improved methods to distinguish intramolecular from intermolecular cross-links in oligomeric proteins, and techniques for quantitatively determining dynamic conformational states. Nevertheless, these advancements create new opportunities for investigating structural plasticity, an essential and dynamic characteristic of many proteins and their complexes, presenting challenges to traditional structural biology techniques. Furthermore, XL-MS provides a unique perspective on the functionality of IDPs, known for their flexible structures crucial in processes such as liquid−liquid phase separation (LLPS).[84]

5.2. Conformational Analysis of Protein Complexes within Specific Cellular Organelles

Within living cells, the interior is intricately partitioned into subcellular compartments, each with its own unique biological functions, resulting in specific microenvironments.[85] These microenvironments exert a profound influence on the conformation and interactions of proteins, serving as the foundation for their functional roles within these subcellular compartments.[85,86] Expanding upon in vivo cross-linking and incorporating techniques for organelle isolation and enrichment, a diverse array of subcellular fractionation-assisted chemical cross-linking methods has emerged in recent years. Schweppe et al. applied cross-linking to intact organelles, providing insights into the interactions between complex I and III in the electron transport chain (ETC) in functional mitochondria.[87] Liu et al. explored intact mouse heart mitochondria, confirming interactions within the ETC supercomplex I−III−IV.[88] Additionally, Fasci et al. utilized the DSSO cross-linker to investigate the proteome interacting with the isolated intact human cell nucleus, resulting in structural models of protein interactions.[89] Despite these advancements, challenges related to the purity of subcellular fractionation and the potential disruption of subcellular structures persist, necessitating rigorous data quality control.[90,91]

To address these limitations, it is imperative to devise methods capable of selectively targeting specific subcellular compartments and facilitating cross-linking within live cells. Although enzyme-mediated proximity labeling strategies, such as BioID[92,93] and APEX,[94,95] have been designed for various subcellular compartments, they often lack the ability to resolve protein conformations. In response to this challenge, An et al. introduced the SubPiXL (suborganelle proteome labeling-assisted in vivo cross-linking) strategy, enabling the identification of protein conformations within the mitochondrial matrix in live cells.[96] Additionally, Chen et al. developed the targeted cross-linker delivery-mass spectrometry (CD-MS) method, leveraging nanoparticles for cross-linker delivery, thus facilitating the analysis of mitochondrial protein conformations and interactions.[97] These innovative approaches hold the potential to revolutionize our understanding of dynamic subcellular protein interactions and structures.

Analyzing protein complexes with subcellular spatial resolution is crucial for exploring the conformational and

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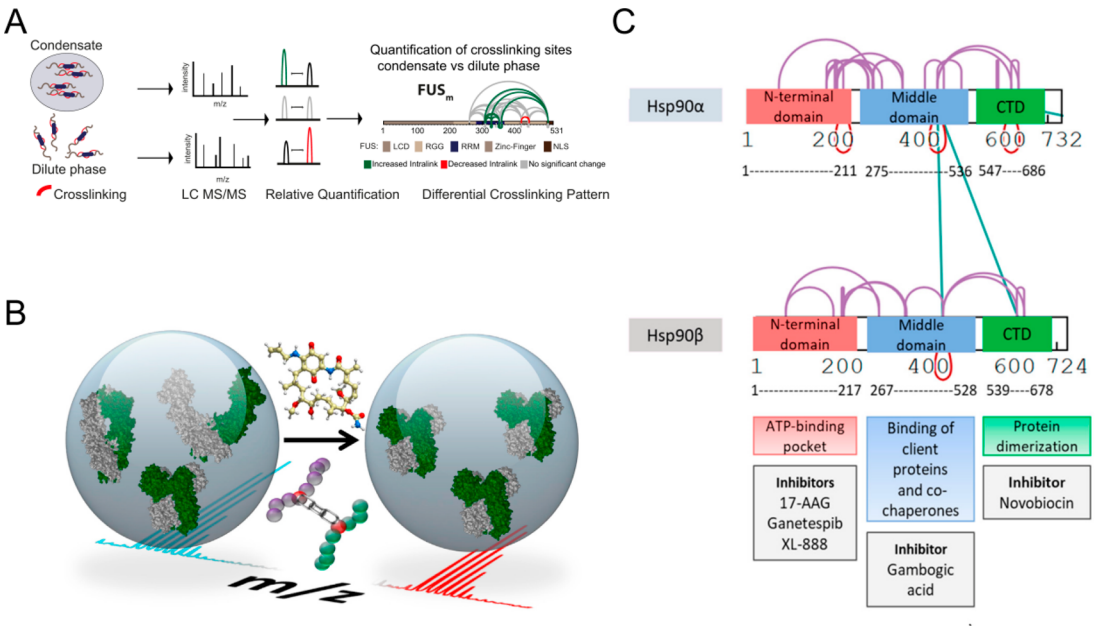

Figure 6. Conformational changes in response to corresponding stress or drug treatment. (A) Quantitative and time-resolved XL-MS analysis of the domain-specific changes during the FUSm condensates formation. (B) Identification of in vivo conformational changes of Hsp90 upon the inhibitors treatment using XL-MS. (C) Conformational and interactive changes investigation of Hsp90 isoforms specific to the domain-bias inhibitors upon systems-level quantitative XL-MS.[101−103] Reproduced with permission from ref 101. Copyright 2021 eLife Sciences Publications, Ltd. Reproduced with permission from ref 102. Copyright 2016 Cell Press. Reproduced with permission from ref 103. Copyright 2022 American Chemical Society.

functional heterogeneity and dynamics of proteins in diverse cellular localizations. Lysosomes, critical for macromolecule degradation, have emerged as central regulators of cellular metabolism, influencing homeostasis and various cellular functions. Singh et al. conducted XL-MS analysis on lysosome-enriched fractions in both intact (IT) and disrupted (DR) states, identifying significant cross-links associated with flotillin proteins FLOT1 and FLOT2, and their cross-linking experiments on early endosome-enriched fractions revealed a consistent structural assembly of FLOT1 and FLOT2 across lysosomes and early endosomes.[98] An et al. introduced SubPiXL, enabling the exploration of regional protein conformations and interactions within living cells. When applied to the mitochondrial matrix, SubPiXL not only enhances our understanding of unique protein conformations and interactions specific to this subcellular region crucial for cellular energy production but also enables a sophisticated analysis of protein complexes in subcellular regions characterized by low abundance and dynamic structures. Notably, SubPiXL refines the ensemble structure of mitochondrial protein complexes, such as PHB and HSP90AA1, revealing distinctions from the structures observed in the whole cell.[99]

However, it is crucial to emphasize that although subcellular chemical cross-linking has showcased its potential in advancing our comprehension of region-specific conformations within protein complexes, there remains a necessity to offer avenues for cross-validation through alternative techniques, including NMR and Cryo-EM. This is imperative for the validation and elucidation of finer details in structural alterations. Moreover, it is crucial to advance the development of organelle-specific and reaction-controlled cross-linking strategies to enable targeted in vivo analysis of protein complexes within different organelles, as well as development enabling the exploration of dynamics at the subminute level in the future. This is essential for accurately analyzing conformational changes in protein complexes within specific organelles and characterizing the spatiotemporal dynamics of protein complexes among organelles.

5.3. Conformational Changes in Physiological and Pathological Processes

In pathological states, protein misfolding or aberrant conformational changes can occur, leading to diseases. XL-MS represents a robust tool for probing conformational changes in proteins in their native state, contributing significantly to our understanding of protein functions and modifications in both health and disease.[100]

In the context of biological processes, such as protein aggregation, the development of time-resolved XL-MS is crucial for analyzing the conformational dynamics of proteins at different time points. Boczek et al. employed integrated time-resolved XL-MS and label-free quantitation to reveal domain-specific alterations and binding modalities of molecular chaperones during the formation and aging of FUS condensates. Their findings highlighted shifts in the interactions of RRM domains, showcasing the pivotal role of the molecular chaperone HspB8 in preventing a disease-associated aberrant phase transition mediated by FUS, attributed to the high interaction between HspB8-αCD and FUS-RRM (Figure 6a).[101]

Moreover, Chavez et al. developed a quantitative XL-MS approach by combining protein interaction reporter (PIR) technology with SILAC to investigate additional protein conformation and interaction changes induced by various stimuli in vivo, such as changes in cellular Hsp90 conformations and interactions upon treatment with Hsp90 inhibitors (Figure 6b).[102]

To advance quantitative XL-MS toward systems-level studies, multiplexing capability is desirable to increase

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throughput and reduce missing values between samples. Wippel et al. presented a 6-plex isobaric quantitative protein interaction reporter (iqPIR) technology to perform a large-scale screening of protein conformational and interaction changes specific to the molecular class of Hsp90 inhibitors in MCF-7 breast cancer cells. Comparison of the 44 cross-link pairs, all quantitated for Hsp90α/β in 6 channels, elucidated that NTD inhibitors and MD inhibitors induced an upregulation of Hsp90β/α heterodimer, disrupted by CTD inhibitor (Figure 6c).[103] The iqPIR strategy illustrates a significant advancement for in vivo protein conformation characterization using XL-MS, enabling monitoring of changes at the global proteome level with high throughput and allowing for the comparison of multiple biological samples from a single LC-MS acquisition.

Furthermore, the invaluable insights into complex diseases and functions that are challenging to replicate in simpler cell models. The application of XL-MS was expanded to tissue research. However, the presence of intercellular substances and collagen in tissues poses challenges for analysis. Chavez et al. have shown the feasibility of implementing XL-MS in mammalian tissues, furnishing structural insights into critical protein systems within the heart, including the sarcomere and OXPHOS complexes.[104] Additionally, Wittig et al. unveiled protein interactions and functional assemblies, including both active and inactive conformations of the vesicular ATPase complex in synaptic vesicle membranes purified from the rat brain.[105]

Moreover, building upon the aforementioned foundation, there is a critical need to enhance precision in characterizing conformational alterations. The widespread use of slower-reacting homobifunctional cross-linkers, especially NHS ester derivatives, poses the risk of introducing bias. Despite the current availability of photoactivated cross-linking, its application for conformation identification in complex systems is hindered by unpredictable fragmentation and the presence of multiple reactive moieties.[106] Significant opportunities exist to optimize cross-linking agent reactions and refine site identification, thereby advancing our understanding of in vivo protein conformational changes. As the accuracy of protein conformational analysis in physiological and pathological processes improves, comprehending these changes becomes crucial for drug development.

6. PROSPECTS AND CHALLENGES OF XL-MS IN IN SITU CONFORMATIONAL ANALYSIS

Currently, XL-MS has significantly advanced the fields of protein interaction and structural biology, providing crucial insights into spatial proximity, organizational structure, and the dynamic nature of protein assemblies under native cellular conditions. It excels at detecting transient and low-affinity interactions often overlooked by other methods, offering a comprehensive view of the interactome landscape. Through cross-linking to stabilize these interactions, XL-MS reveals the dynamics of protein complexes, capturing various subcomplex states and providing 3D coordinates of structural ensembles, rather than a single averaged structure. Integration with computational modeling further expands its applications, potentially transforming our understanding of protein interactions and their functional implications.

Despite significant progress, further developments are necessary to enhance the depth and coverage of XL-MS. This entails advancements in cross-linking reagents, sample preparation, and data analysis. The growing importance of quantitative XL-MS applications in visualizing protein structural and interaction dynamics demands new informatics strategies for data processing, visualization, and interpretation. Integration with other structural biology techniques can complement information, advancing our understanding of protein complexes. Continued advancements in XL-MS hold great promise for unraveling the complexities of the interactome and advancing our knowledge of protein structure and function.

Moreover, although XL-MS has made remarkable strides in revealing protein structural dynamics, its limitations in temporal resolution hinder the definition of dynamic structures at precise time points. Future developments should focus on capturing protein structures on a shorter time scale, exploring dynamics at the subminute level rather than providing ensemble structures. This advancement promises a more profound exploration of protein behavior within the cellular milieu, offering invaluable insights into biological processes and protein functionality.

In parallel, advances in chemical cross-linking techniques not only explore dynamic conformational changes but also broaden our understanding of in situ protein−protein interactions. Utilizing cross-linkers with enhanced properties, such as improved membrane permeability and rapid reaction times, enables scientists to gain insights into subtle, time-sensitive structural transformations within the cellular milieu. The development of cross-linkers that minimize cellular interference and preserve cell viability, coupled with improved cellular spatial in situ resolution, enhances the accurate representation of protein conformations. Challenges persist, including the need for faster cross-linking reactions, distinguishing intramolecular and intermolecular cross-links in oligomeric proteins, and quantitatively determining dynamic protein conformations. As technology advances, the future of in vivo chemical cross-linking promises to unveil intricate details of protein structures and interactions within the dynamic, crowded environment of living cells.

7. CONVERGENCE OF NMR AND XL-MS FOR IN SITU CHARACTERIZATION OF PROTEIN STRUCTURES AND INTERACTIONS

Both NMR and XL-MS primarily characterize protein structures through distance information[107] and can be utilized for studying dynamic protein structures and protein−protein interactions.[108] Therefore, these two methods can be effectively combined to study the protein structure and dynamics in the cell (Figure 7).

First, NMR and XL-MS can be combined for the dynamic characterization of large and complex protein systems within cells. NMR typically obtains distance information through NOE experiments, with a detection range usually limited to within 6 Å. Although paramagnetic NMR methods can measure the distances up to 40 Å,[109] these techniques often necessitate mutations and labeling of the protein system with paramagnetic probes. In contrast, XL-MS does not require premodification or labeling of proteins, making it directly applicable for the detection of protein structures within cells.[110] The distance range obtained by XL-MS depends on the length of the cross-linker (usually more than 20 Å) and can be flexibly designed according to the specific requirements.[111] This characteristic of chemical cross-linking makes it suitable for investigating protein molecules or complexes in large

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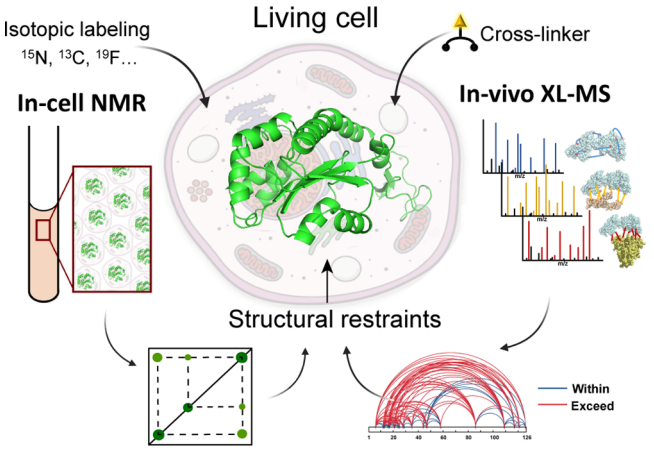

Figure 7. Convergence of NMR and XL-MS for in situ characterization of protein structure in the cellular environment.

systems. In contrast, NMR is constrained by spectral resolution and is generally more applicable to studying smaller protein systems up to about 40 kDa.[112] Therefore, in terms of the spatial scale of detection, the long-range distance information obtained by cross-linking complements the short-range distance information obtained by NMR.[113] For the study of protein structures within cells, especially in larger and complex protein molecules, leveraging the advantages of both NMR and chemical cross-linking is advantageous. Initially, high-resolution structures of smaller structural units can be resolved using NMR. Subsequently, long-range distance information between these structural units can be obtained through XL-MS, allowing for the integration of both sets of information to characterize the whole protein structure.[114]

Second, the integration of NMR and XL-MS offers a more comprehensive insight into protein dynamics. NMR usually can detect thousands of NOEs for a modest-sized protein.[115] In contrast, XL-MS typically occurs only at amino acids with reactive groups (such as Lys, Glu, Asp, Cys), and the success of cross-linking reactions depends on the environment and exposure of these amino acids.[116] Consequently, the distances obtained through chemical cross-linking are relatively sparse. On the other hand, NMR experiments typically require high sample concentrations up to hundreds of micromolar. However, within cells, the functional concentrations of proteins are generally much lower, making it challenging to reach the detection limits of NMR. Therefore, NMR experiments within cells often involve the use of probe labeling with multiple fluorine atoms to enhance sensitivity. In contrast, XL-MS offers significantly lower sensitivity limits, requiring only a few micromolar concentrations. Consequently, in different protein systems and within various intracellular microenvironments, NMR and XL-MS complement each other in terms of concentration detection.

Moreover, the intrinsically disordered proteins (IDP) play a crucial role in cellular functions.[117,118] However, due to the highly dynamic nature of their structure, NMR is currently the only experimental method capable of studying the atomic-resolution structures of intrinsically disordered proteins.[119] Recently, XL-MS has also been applied to characterize the structures of intrinsically disordered proteins within cells.[81] Hence, combining NMR with XL-MS for studying the structure, dynamic changes, and interactions of intrinsically disordered proteins within cells holds significant value. Besides, NMR can detect dynamic changes in protein structures ranging from picoseconds to seconds. However, chemical cross-linking is constrained by the time scale of chemical reactions, limiting the detection of dynamic changes. Therefore, the development of faster-reacting cross-linking agents, such as photo-cross-linking agents, can enable the capture of faster time scales in protein dynamic changes. Within cells, crucial dynamic structures and processes exist in proteins, such as the excited state of enzymes and encounter complexes. These structures are often challenging to detect and analyze using conventional methods due to their short-lived existence and rapid exchange rates. The combination of NMR and XL-MS proves to be effective in studying these vital life processes. This integrated approach can provide valuable insights, aiding in the design of interventions and regulatory strategies.

8. CONCLUSION

In conclusion, the ongoing examination and understanding of the in situ structure and interactions of cellular proteins remain paramount in deciphering the complexities of cellular functions and biological processes. Substantial progress has been achieved through the use of NMR and XL-MS techniques, but there is an urgent need for further advancements and the inception of innovative methodologies to accommodate the growing demand for a profound understanding of intracellular protein structures and interactions.

The continuous development of more sensitive NMR techniques and specific isotopic labeling is critical, with the main goal being to enhance detection limits and resolution within the complex cellular environment. Parallel to this, improvements in XL-MS techniques are vital to reaching improved selectivity, cross-linking efficiency, and spatiotemporal precision, all done while minimizing the effects on the delicate cellular conditions. Additionally, the integration of various multiscale characterization techniques and functional information offers a promising perspective in delivering a comprehensive and holistic view of cellular processes. Through joint efforts and innovation, the research community can persist in breaking the boundaries of knowledge, ultimately contributing to progress in medicine and biotechnology and our overall understanding of life at the cellular level.

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https://doi.org/10.1021/jacsau.3c00712
JACS Au 2024, 4, 369−383

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by grants from the National Natural Science Foundation of China (21991080, 21991083, 21925406, 32088101, 22074139), the National Key R&D Program of China (2021YFA1301501, 2021YFA1302602, 2018YFE0202300), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB37040105, XDB0540000).

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https://doi.org/10.1021/jacsau.3c00712
JACS Au 2024, 4, 369−383