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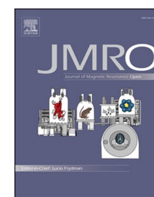

Intrinsically disordered proteins studied by NMR spectroscopy

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ABSTRACT

Intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) of complex multi-domain proteins are now identified as a trend topic by the scientific community. NMR constitutes a unique investigation tool to access atom resolved information on their structural and dynamic properties, in isolation or upon interaction with potential partners (metal ions, small molecules, proteins, nucleic acids, membrane mimetics etc.). Their high flexibility and disorder, in contrast to more compact structures of globular protein domains, has a strong impact on NMR observables and NMR experiments should be tailored for their investigation. In this context, ^{13}C direct detection NMR has become a very useful tool to contribute to IDPs/IDRs characterization at atomic resolution. 2D CON spectra can now be collected in parallel to 2D HN ones, and reveal information, which in some cases is not accessible through 2D HN spectra only, particularly when studying proteins in experimental conditions approaching physiological pH and temperature. The 2D HN/CON spectra are thus becoming a sort of identity card of an IDP/IDR in solution. Their simultaneous acquisition through multiple receiver NMR experiments is particularly useful to investigate the properties of highly flexible intrinsically disordered regions within complex multi-domain proteins, rather than in isolation as often performed to reduce the complexity of the system, an interesting perspective in the field.

1. Introduction

Our understanding of protein function has been, until recently, largely based on the concept of structural order. A vast number of atomic coordinates of three-dimensional protein structures accumulated in the protein data bank (PDB - https://www.rcsb.org/) providing information on how molecular architecture can encode specific functions. Beautiful images of proteins can be found in textbooks to show how they assemble, interact with other proteins through complementary surfaces and bind with high affinities with small molecules through well-defined binding pockets. Drug discovery largely relies on the knowledge of three-dimensional protein structures to identify small molecules that perfectly fit into protein cavities and design novel drugs with improved affinity following a lock-and-key drug design approach. Recent progress in machine learning/artificial intelligence (AI) methods enabled the expansion of the structure coverage (https://alphafold.ebi.ac.uk/), learning from the information deposited in the PDB [1]. Still, many entire proteins or fragments of complex proteins that do not adopt a stable structure and cannot be modeled remain [2].

Neglected for a long time as outliers in the mainstream structure-function paradigm, proteins that function also in the absence of a stable structure, even when identified were reported in the early literature in very naive ways (malleable, vulnerable, chameleon, protein clouds, dancing proteins…) [3,4] and seldom studied in detail. On the other hand, it is obvious that a high extent of disorder and flexibility confers to proteins several properties that are highly complementary to the ones deriving from a well-defined conformation. Highly flexible disordered protein fragments can carry out complementary functional roles with respect to folded ones [5–13]. For example, while enzyme catalysis is often optimally carried out by stable folds, recognition, signaling and regulatory processes are strongly linked to protein flexibility [14–16]. The possibility to adopt different conformations enables proteins to bind to different partners, an important feature for proteins that act in nodal points of interaction pathways [12,14,17,18]. The exposed proteins' backbone is often site of post translational modifications, an extra layer of complexity encoded in protein primary sequences [19–21]. Alternative splicing is also generally linked to exposed parts of polypeptide chains [22,23]. Short sequences with specific aminoacidic composition (the so called short linear motives—SLiMs - or Eukaryotic Linear Motifs—ELMs) [24–26] can engage in interactions with minimal use of

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information introducing novel ways to communicate inside a cell. Short disordered regions, constituted by repeated amino acids of the same type, called low-complexity regions, might be involved in liquid-liquid phase separation, another process important for cells' life [27,28]. Recent work is revealing a number of novel functional modules that can be encoded in the primary sequence of highly flexible protein regions by specific patterns of amino acids. The image that emerges from this initial collection of data is even more fascinating than originally expected!

2. NMR: a unique tool to access atom-resolved information on IDPs/IDRs

NMR constitutes a strategic tool, if not the unique one, to access atomic resolution information on intrinsically disordered proteins (IDPs) or on highly flexible regions of complex multi-domain proteins (IDRs). The high extent of disorder and flexibility has a profound impact on NMR observables that should be carefully considered before the selection or the design of the most appropriate NMR experiments for the investigation of IDPs/IDRs. Indeed, while some aspects are advantageous, such as the long coherence lifetimes linked to the high flexibility which enable the use of sophisticated multidimensional pulse sequences [29–33], some others introduce critical points such as extensive chemical shift clustering and efficient solvent exchange of amide protons [33–35]. These are discussed in detail in the following paragraphs. However just a quick look at a 2D HN correlation spectrum, the first one usually acquired on a protein, provides interesting insights on the different structural and dynamic properties of an IDP with respect to a globular protein.

Let's start with a paradigmatic IDP, α-synuclein. This protein attracted the attention of the scientific community because of its link with Parkinson's disease [36]. Constituted by 140 amino acids it features different regions with specific properties: a positively charged N-terminal region, that tends to adopt helical conformations when in contact with membranes [37–41], a central NAC region as well as a negatively charged C-terminal region also hosting three out of four tyrosine and all the proline residues [42,43]. The chameleonic structural and dynamic properties of α-synuclein, ranging from the highly flexible and disordered monomer, to various helical forms when membrane-bound, to oligomeric forms, all the way to fibrillary aggregates, make it one of the most eclectic proteins in terms of structural dynamics [44], probably a key aspect at the basis of its physiological role, an aspect that is still a matter of debate. The highly flexible, disordered monomeric form is taken here as an example to illustrate how the increased disorder and flexibility, with respect to globular proteins, impact NMR spectra.

The 2D HN HSQC spectrum of α-synuclein shown in Fig. 1(left panel), clearly illustrates the profound effect of its structural and dynamic properties on NMR spectra. The first major consequence that emerges consists of a pronounced reduction in the chemical shift dispersion of the resonances, responsible for a high extent of cross-peak overlap in the spectrum. This effect however is more evident in the ^{1}H dimension with respect to the ^{15}N one, in agreement with the notion that heteronuclear spins are very important for the study of proteins in general but especially for the study of IDPs/IDRs. While in globular proteins the local environment differentiates the spins and causes large chemical shift dispersion, in IDPs/IDRs these contributions are largely averaged out and only contributions to chemical shifts arising from the covalent structure remain [45,46]. This is particularly evident when looking at the spectrum of ubiquitin, a small globular protein often used as a model system for the set-up of NMR experiments, reported in Fig. 1 (right panel).

The increased local mobility, when passing from globular proteins to highly flexible ones, is reflected in smaller effective local rotational correlations times, a crude approximation of the complex motions in IDPs/IDRs that however describes the major effect of the lack of a globular structure on motional properties [47,48]. This effect is responsible for narrower NMR lines in IDPs with respect to folded proteins, which partly alleviates the problem of severe cross-peak overlap.

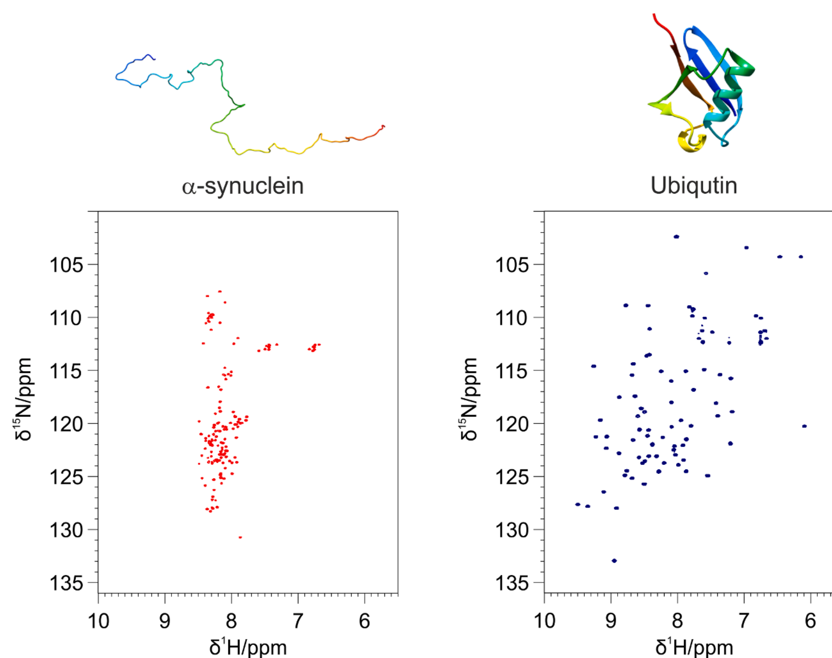

Fig. 1. Impact of protein disorder and flexibility on NMR spectra.
The figure shows the comparison between the 2D HN (^{1}H-^{15}N HSQC) spectra of two proteins with different structural and dynamic properties: (left panel) an intrinsically disordered protein (α-synuclein 0.6 mM in 20 mM potassium phosphate buffer, 100 mM NaCl, 50 μM EDTA at pH 6.5 and 298 K); (right panel) a globular protein (ubiquitin 1.0 mM in 50 mM HEPES at pH 7.0 and 298 K) both acquired at 16.4 T. The left panel reports one of the possible models of α-synuclein, the right panel reports the ribbon representation of ubiquitin (PDB 1UBQ). The ^{1}H-^{15}N HSQC spectrum of α-synuclein is characterized by a low cross-peak dispersion and presents many peaks in overlap one to the other, a common feature of intrinsically disordered protein spectra. The ^{1}H-^{15}N HSQC spectrum of ubiquitin shows the large cross-peak dispersion typical of folded proteins.

The opening of protein backbones to the solvent introduces many more degrees of freedom for the chemical exchange of amide protons with the solvent. This effect is modulated by the samples' conditions, and especially by pH, temperature (T) and ionic strength. Fine-tuning of these factors can be used to minimize exchange effects and enables the measurement of informative 2D HN spectra [49]. However, it is important to be able to study the protein of interest in a broad range of informative conditions (pH, T, ionic strength), that approach the physiological ones. Very sensitive 2D spectra, like 2D HC HSQCs, often have poor resolution when acquired on IDPs due to the limited chemical shift dispersion of aliphatic ^{1}H and ^{13}C nuclear spins as well as to the presence of many ^{1}H-^{1}H scalar couplings. Worth noting, experimental interesting alternatives have recently been proposed [50].

2.1. The contribution of ^{13}C direct detection NMR for the study of IDPs/IDRs

Needless to say, additional tools are important to render the investigation of IDPs more accurate and straightforward. As discussed above, cross peaks in ^{1}H detected NMR spectra of an IDP tend to cluster in regions foreseen for the different types of amino acids, especially moving away from the backbone. Backbone resonances, in particular those of the peptide bond involving two different amino acids, are the ones that retain a certain chemical shift dispersion also in the absence of a 3D structure. From this, the idea is to exploit as much as possible heteronuclear spins, such as ^{13}C and ^{15}N, to obtain informative NMR spectra [51].

The increase in instrumental sensitivity experienced in recent years allowed us to reintroduce heteronuclear direct detection also in biomolecular NMR investigations [52–54]. Heteronuclear spins have many valuable properties for the study of IDPs: they are characterized by large intrinsic chemical shift dispersion, do not suffer from exchange processes with the solvent and reveal information about proline residues [55,56], often abundant in IDPs, in a straightforward and clean way. The intrinsic lower sensitivity of heteronuclei compared to protons is the major drawback which however has been partly compensated by the development of tailored probeheads and by the increasing of magnetic field strength [54,57].

A suite of NMR experiments based on ^{13}C detection has been developed throughout the years [58] and is now widely used for the investigation of IDPs in parallel to the set of experiments based on ^{1}H detection. A key experiment is the 2D CON [35,59], which correlates two nuclear spins involved in the peptide bond, a feature that contributes to the excellent chemical shift dispersion of the cross peaks in these spectra also in the case of IDPs [60].

Thanks to these properties the use of 2D CON spectra in parallel to 2D HN ones has become widely employed to ease IDPs investigations, as shown in Fig. 2A and B through the example of CBP-ID4, a 207 amino acids long linker connecting two structured domains of the CREB-binding protein [61]. The availability of 2D CON spectra in parallel to 2D HN ones enriches the information content thanks to the excellent chemical shift dispersion of heteronuclei. This allows for example to count the number of signals and compare them with the expected ones, a key aspect to understand in the initial phases of an NMR investigation whether one will manage to fully characterize the protein of interest. The 2D HN and 2D CON spectra can thus be considered as an initial identity card (ID) of a protein. Inspection of Fig. 2 clearly shows that the combined use of the two spectra allows focusing on proteins displaying different structural and dynamic properties. However, it's worth noting that the relaxation properties, exemplified at this stage in the cross-peak linewidths, are not markedly influenced by the protein size, as can be noted comparing the 2D CON spectra reported in the various Figures of this paper, at variance to what is observed for globular proteins of similar size. The possibility of detecting signals of proline ^{15}N nitrogen spins in a very clean region of the spectra enables also the design of experiments tailored for this residue, which allow the acquisition of

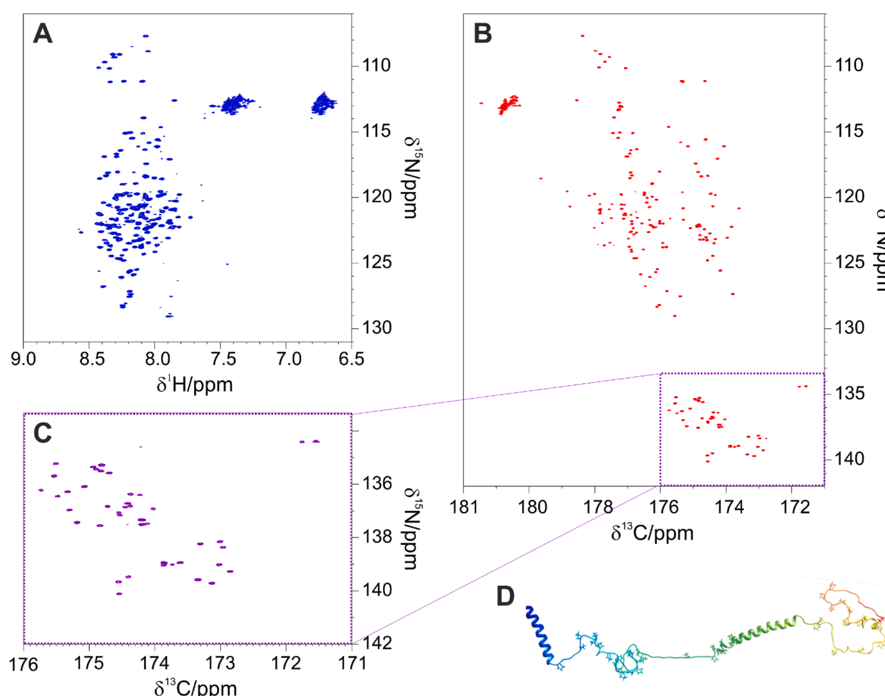

Fig. 2. Complementary information on IDPs/IDRs from 2D HN, 2D CON, 2D CON^{PRO} NMR spectra.
Panels A and B show the 2D HN (^{1}H-^{15}N HSQC) and the 2D CON spectra recorded at 16.4 T on a 0.2 mM CBP-ID4 sample in 20 mM potassium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA at pH 6.5 and 283 K. The ^{13}C dimension in the CON spectrum features a higher chemical shift dispersion if compared to the ^{1}H dimension of the HSQC spectrum, letting to count signals and helping in the initial analysis of the protein.
The content of information in the 2D HN is limited also by the absence of the proline residues correlations due to the lack of the amide proton. As shown in panel C, the CON experiment can be designed to zoom on the proline region, particularly informative in CBP-ID4. Proline residues, displayed in the conformer of CBP-ID4 reported in panel D, constitute 21.9 % of the protein's content.

spectra that can be considered a "proline fingerprint" for all the IDPs that count a high number of residues of this type (Fig. 2C) [55].

Proline-rich regions are often found in IDRs and indeed proline residues were initially classified as disorder promoters. The disorder is generally associated with high flexibility. However, prolines feature the most constrained side chain (a closed ring, lacking the amide proton) and these residues are even used as "rigid spacers" in some experimental techniques [62–64]. Recent work indeed showed how dipeptides constituted by a proline and an aromatic residue have a key role in stabilizing compact states of disordered protein regions in which long-range contacts are present in the absence of short-range ones [56, 65]. Cis-trans isomers of amide bonds involving proline in disordered regions were recently investigated and correlated to functional features [66–69]. It is thus important to investigate in detail the properties that prolines confer to intrinsically disordered protein regions as they are likely to cover unexplored functional niches. If sensitivity allows, minor isomers of peptide bonds can also be detected and easily identified in the ^{15}N proline region of 2D CON spectra [56,68,69], as demonstrated for osteopontin (OPN, Fig. 3) which features 12 proline residues grouped in key regions of the polypeptide.

Increasing the protein size also means obtaining spectra with several peaks clustering in crowded regions with an overall increased difficulty in the spectral analysis. For this reason, the possibility of obtaining as much resolution as possible becomes a fundamental argument in the study of IDPs through NMR. The application of NMR experiments at very high fields provides exquisite resolution thanks to the larger dispersion of the NMR signals in the frequency domain (in Hz) moving from lower to higher fields [57,70].

A striking example is provided by proteins containing low complexity "polyQ" regions, an acronym that is used to indicate protein tracts constituted by several consecutive glutamine residues. These are linked to the so-called polyQ diseases, which include different kinds of neurodegenerative diseases that have, as the only common feature, a polyQ fragment as part of different proteins involved in the formation of aggregates at the origin of the disease [71,72]. These proteins do not crystallize, as most IDPs, and are not easy to study through NMR. Conflicting evidence was reported in the literature about their structural and dynamic properties achieved by studying small constructs. Recent work

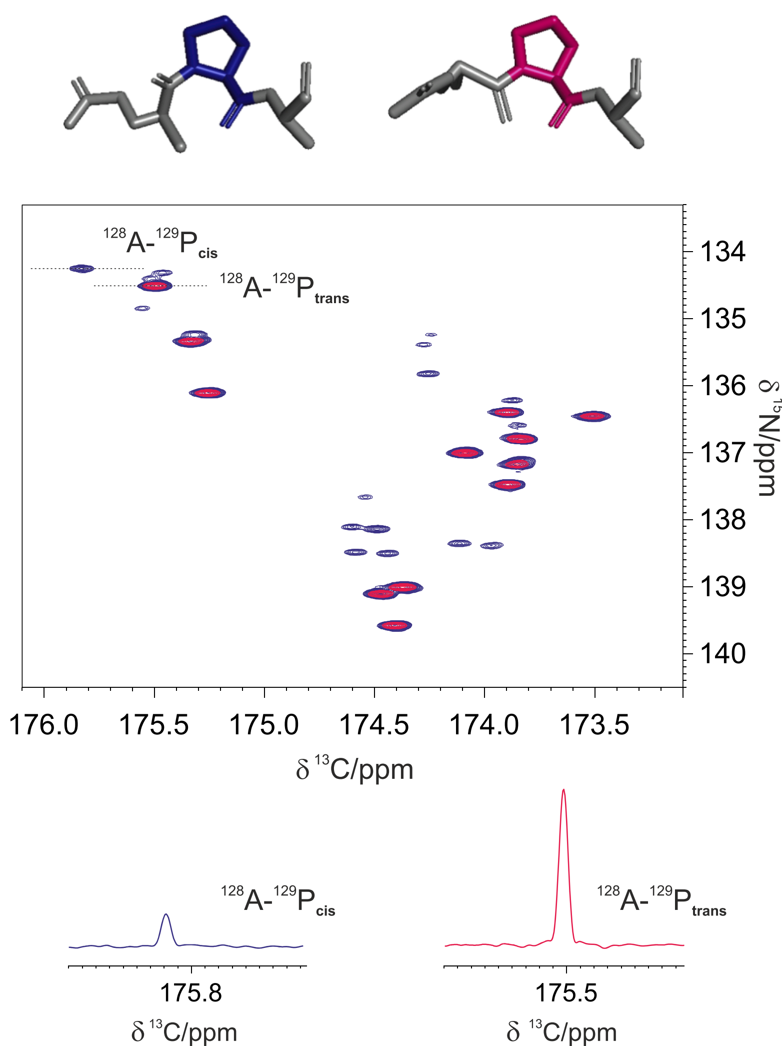

Fig. 3. Identification of cis/trans X-Pro conformations from 2D CON spectra.
The figure reports the 16.4 T 2D CON spectrum acquired on 2.0 mM osteopontin protein in PBS buffer at pH 6.5 and 310 K (only the region containing C′ X - N Pro cross-peaks is shown).
The spectrum is shown using different base levels, to highlight the presence of minor forms deriving from cis/trans isomers of X-Pro peptide bonds. The magenta contours are optimized to visualize only the major trans conformation while the blue ones also show the minor cis conformation. Traces of the peaks arising from the resonances of C′ Ala 128 - N Pro 129 are reported in the bottom of the figure to compare the intensity of the major trans (magenta) and minor cis (blue) forms. A model of a tripeptide with the X-Pro peptide bond in cis (blue) and trans (magenta) conformation is also shown on the top of the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

on the N-terminal fragment of the androgen receptor, involved in the onset of spinal bulbar muscular atrophy, highlighted the role of the sequence context in modulating the structural and dynamic properties of polyQ regions (Fig. 4) [73,74]. In particular, a leucine-rich region preceding a polyQ tract was shown to induce a helical conformation in the subsequent glutamine residues engaging backbones in intramolecular interactions (rather than intermolecular ones); in other words, a transient secondary structural element (a “polyQ helix”) exists and protects the protein from aggregation as experimentally verified in vitro [73,74]. Surprisingly this module was then found in other naturally occurring proteins, revealing that this module deserves further investigation [75–77].

Low complexity regions are not limited to polyQ. Another interesting case is provided by protein tracts that are very rich in amino acids with a defined charge, such as aspartates and glutamates conferring a negative charge or arginine and lysine conferring a positive charge at neutral pH to the protein stretch. An obvious role for these highly charged regions would be to mediate intermolecular recognition contributing to targeting, signaling and regulatory functions. Indeed, electrostatic interactions are among the strongest ones and can be established at long distances. Although a few nice examples were recently described in the literature [78–80], very little high-resolution information is available so far. These examples are probably just a glimpse of novel ways in which flexible protein regions modulate functions that are not yet described in the PDB.

2.2. *Sequence specific assignments are necessary: a quick overview*

The possibility to access atom-resolved information derives from associating each cross peak in 2D NMR spectra to a specific pair of nuclear spins within the protein under investigation. This process is commonly known as “sequence-specific” assignment of the resonances. Many different strategies have been proposed throughout the years. Among them, the most successful ones rely on exploiting the backbone scalar couplings within ^{13}C and ^{15}N enriched proteins. Triple resonance experiments, based on amide proton detection have been widely applied to obtain the backbone assignment of proteins [81–83]. More recently ^{13}C′ detected 3D experiments have been proposed for the assignment of IDPs [51,84–86]. Variants that exploit ^{1}H$^{\alpha}$ or ^{15}N direct detection were also proposed [87–89].

To understand how 3D spectra are used for the residue-specific assignment of proteins, the 3D CBCACON [59,90] spectrum of α-synuclein is reported as an example in Fig. 5. This is a ^{13}C detected experiment in which the backbone carbonyl carbon (C′) is the directly observed nuclear spin. Each signal of the spectrum encodes the information about the chemical shifts of the ^{15}N nucleus of the residue *i*, the ^{13}C′ and the ^{13}C^{ali} (both ^{13}C$^{\alpha}$ and ^{13}C$^{\beta}$) of the residue $i-1$. In this way, it is possible to correlate two adjacent residues in the primary sequence. The 3D spectrum is usually sliced along one of the three axes (^{15}N in this example) to obtain a series of planes that can be analyzed like 2D spectra. As shown in Fig. 5, each plane obtained by slicing the 3D CBCACON spectrum at a given ^{15}N chemical shift value contains the resonances of the ^{13}C$^{\alpha/\beta}$ of the previous residue in the sequence. This experiment can be analyzed in parallel to the 3D CCCON which also provides information about the remaining ^{13}C nuclear spins of the side chain (the aliphatic part). Once most expected resonances are identified in 3D CBCACON/3D CCCON spectra, it is important to place them in the primary sequence by correlating them one with another exploiting the information of the primary sequence of the protein. To this end the 3D CBCACON experiment is analyzed together with the 3D CBCANCO (not shown), in which two sets of cross peaks involving ^{13}C$^{\alpha/\beta}$ are present: one from residue $i-1$ and one from residue *i*, providing the information required for sequence specific assignment [59,90]. An additional experiment that is very useful towards this objective is the 3D COCON experiment [51,56,91,92] which provides the correlations between carbonyls of neighboring amino acids ($i-1$, i, $i+1$), contributing to more accurate and robust results.

The set of ^{13}C detected NMR experiments is thus particularly well

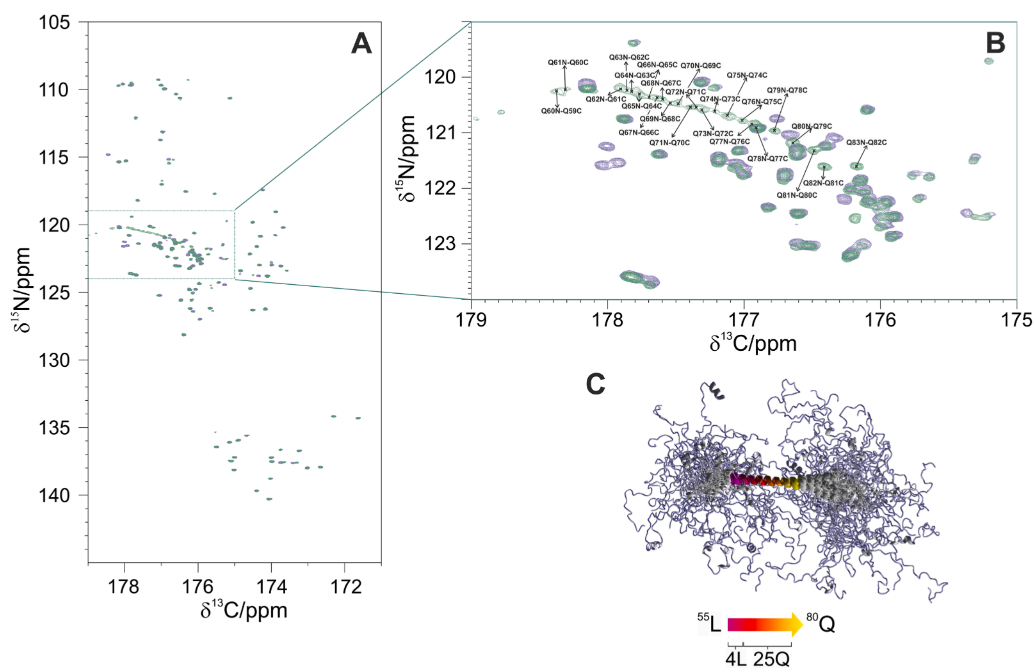

Fig. 4. Atomic resolution information on polyQ tracts from 2D CON spectra.
Panel A shows the superimposition of 16.4 T 2D CON spectra acquired on 0.4 mM 4Q (lilac) and 25Q (green) constructs of the N-terminal region of the androgen receptor (20 mM sodium phosphate buffer, 1 mM TCEP at pH 7.4 and 278 K) [73]. Panel B is an enlargement of the region of the spectrum where the polyQ tract peaks are observed. The specific sequence assignment of the resonances is shown. An ensemble of possible conformers of 25Q is reported in panel C, highlighting in colors the ^{55}L-^{80}Q fragment (structures are superimposed considering residues 55–80). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

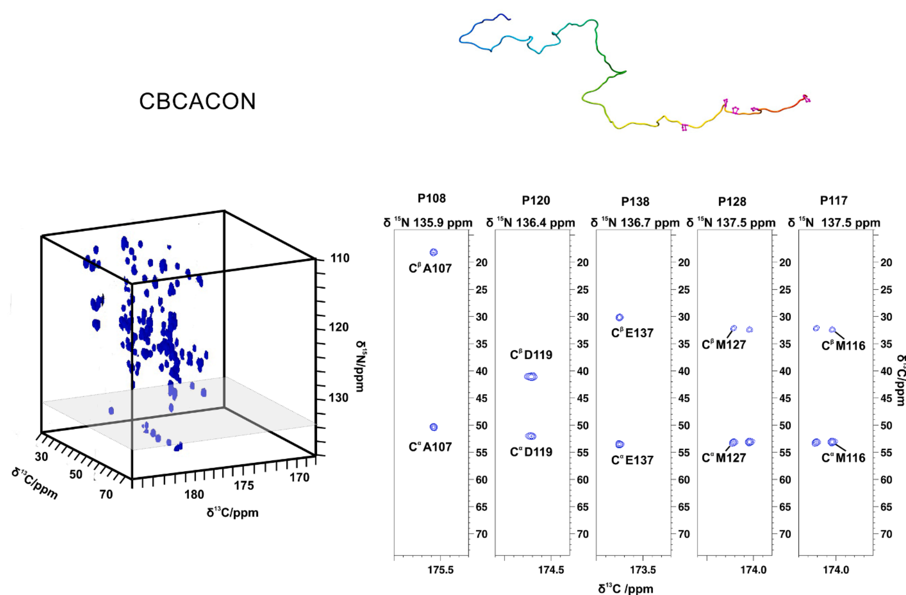

Fig. 5. Sequence specific resonance assignment through ^{13}C-detected 3D NMR experiments.
The left panel shows the 3D CBCACON spectrum of 0.4 mM α-synuclein in 20 mM TRIS buffer, 50 mM NaCl, 0.5 mM EDTA at pH 7.4 and 298 K recorded at 28.2 T. This experiment correlates the amide nitrogen of residue *i* with the C′, C$^{\alpha}$ and C$^{\beta}$ of the residue $i-1$. Usually, the 3D spectra are sliced along one axis, as shown by the light grey plane inside the cube. By slicing the spectrum along the ^{15}N axis, the ^{13}C′-^{13}C$^{\alpha/\beta}$ planes are obtained. The slices corresponding to the ^{15}N chemical shift of the five proline residues of α-synuclein, highlighted in magenta in the model, are reported on the right. In each plane, at the C′ chemical shift of the $i-1$ residue, the cross-peaks of the C$^{\alpha}$ and C$^{\beta}$ of the $i-1$ residue are detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

suited to focus on highly flexible IDPs/IDRs as it provides extended information about the amino acids in the protein primary sequence, including proline residues as well as amino acids whose amide proton is affected by line broadening beyond detection. Of course, the acquisition of a complete set of 3D NMR experiments combining ^{1}H and ^{13}C detected ones, is always welcome when focusing on complex systems. Experimental conditions may be optimized to improve the detectability of amide proton resonances by reducing the temperature and/or the pH to minimize exchange broadening with solvent protons. In these conditions, in particular when protein solubility is limited, it may be useful to focus through ^{13}C direct detection on the information that is completely lacking in ^{1}H^{N} detected experiments, that is correlations involving proline ^{15}N resonances [55]. The latter ^{15}N resonances fall in a very particular spectral region in IDPs, quite isolated from the one where ^{15}N resonances of amide nitrogen nuclei fall. This is a property that can be used to design NMR experiments that focus on this region by utilizing band-selective ^{15}N radio-frequency pulses [93]. Along these lines, a suite of 3D experiments tailored for ^{15}N proline resonances was proposed and offers a useful tool to complement information that is completely lacking in ^{1}H^{N} detected triple resonance experiments, even if in optimized pH and T conditions [93]. These experiments can be imagined as a small region of the complete 3D experiments and can be acquired with excellent resolution in a reduced amount of time with respect to the ones acquired on the full ^{15}N spectral region.

The set of ^{13}C detected NMR experiments for sequence-specific assignment, as well as the ^{1}H detected variants, can also be extended to include additional dimensions and acquire higher dimensionality NMR experiments (nD, with $n > 3$) [30,92,94]. These NMR experiments are very useful to focus on complex systems. Indeed, the increased number of frequencies associated with each cross peak detected in these spectra provides an increase in resolution reducing the chances of accidental cross peak overlap, one of the major problems encountered when focusing on IDPs/IDRs. It should also be mentioned that the success of these experiments is strongly linked to the sample properties, in terms of protein concentration as well as high flexibility/disorder. Indeed, long coherence lifetimes typical of IDPs allow for complex experiments with many coherence transfer pathways. Acquisition of these experiments with the needed resolution to be useful for the investigation of IDPs/IDRs through conventional uniform sampling strategies would require excessively long times (several days) and large disk spaces. Many non-uniform sampling (NUS) approaches have been proposed in recent years to overcome this limitation and render these spectra very useful in challenging cases [95–101].

As a result of the sequence specific resonance assignment procedure chemical shifts are available for most of the nuclear spins within each amino acid in the protein primary sequence. These are crucial to achieve information with atomic resolution through NMR. However, they also provide initial information about the structural and dynamic properties of the protein. This can be evinced by comparing the observed chemical shifts with those predicted for the protein primary sequence assuming a fully disordered state (random coil chemical shifts). The approach allows to easily identify regions of the primary sequence that transiently adopt secondary structural elements, generally indicated as secondary structural propensities [102–104].

2.3. ^{15}N relaxation provides useful information about differential local dynamics

The ^{15}N NMR relaxation measurements are one of the first observables to be determined when starting the investigation of a protein. They allow to have an initial idea of the overall relaxation properties of the protein and, in the second stage of the investigation, to determine local differences in relaxation rates between different parts of the protein itself to link ^{15}N NMR relaxation rates to differential local mobility along the polypeptide chain.

The ^{15}N R_1, ^{15}N R_2 and ^{1}H-^{15}N NOE values measured for α-synuclein are reported in Fig. 6. ^{15}N R_1 values are generally not very informative at high fields, while ^{15}N R_2 and ^{1}H-^{15}N NOE values immediately give us some indications about overall and local flexibility of the protein along its primary sequence. Indeed, both values are low with respect to the expected values for a well-folded protein of similar size (schematically indicated by a dashed line in Fig. 6). The large reduction in transverse

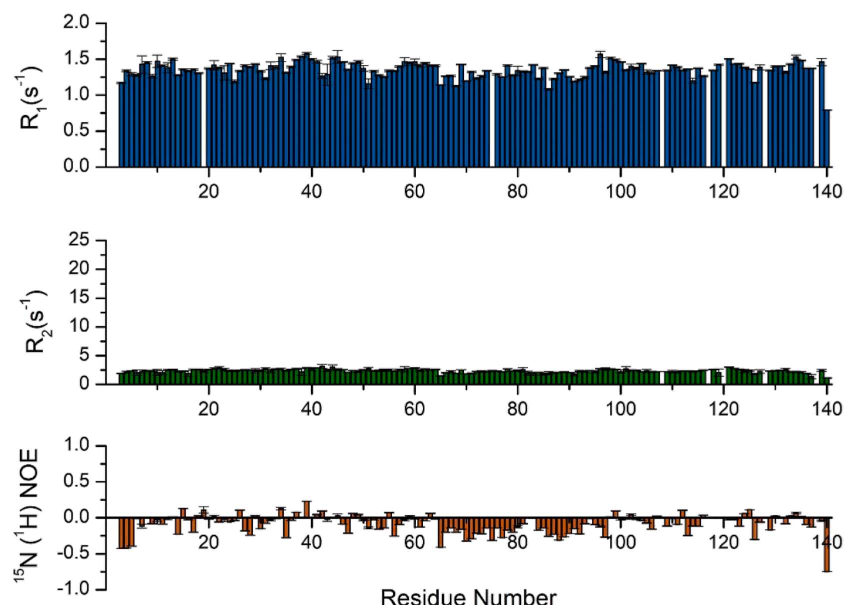

Fig. 6. Relaxation properties of ^{15}N in IDPs: the example of α-synuclein.
The ^{15}N R_1, ^{15}N R_2 and ^{15}N {^{1}H} NOE values for 1.0 mM α-synuclein in 20 mM potassium phosphate buffer, 200 mM NaCl at pH 6.3 and 298 K, measured at 16.4 T, are shown. The extensive disorder and high flexibility of α-synuclein are responsible for the low values of ^{15}N R_2 and ^{15}N {^{1}H}NOE. For comparison, a globular protein constituted by a similar number of amino acids has a very different behavior such as ^{15}N R_1, ^{15}N R_2 and ^{15}N {^{1}H} NOE values of about 1.5 s^{-1}, 15 s^{-1}, 0.8 respectively [105].

relaxation rates upon loss of a stable 3D structure that characterizes highly flexible IDPs/IDRs has a general impact on the characteristics of NMR spectra of IDPs/IDRs. Indeed, the low transverse relaxation rates are linked to narrow linewidths, an aspect that allows for mitigating the problem of resonance overlap deriving from the low chemical shift dispersion. The low transverse relaxation rates permit long evolution times in multidimensional experiments, a key aspect for achieving good resolution. Finally, experiments which feature many coherence transfer steps, such as the nD ones, can be collected thanks to the long coherence lifetimes.

A comment is due on ^{13}C detected experiments to determine ^{15}N relaxation rates [106]. While several variants are available, probably the most interesting and useful ones are those devoted to the determination of ^{15}N relaxation rates for proline residues [107] that cannot be achieved through other methods. The ability to selectively focus on the ^{15}N region of proline residues allows researchers to narrow down the spectral region of interest, resulting in excellent resolution with minimal increments, and thus saving in the overall experimental time, compared to studying the entire amide region in proteins. This feature is particularly attractive for investigating complex proline-rich proteins. It is worth noting that the absence of a directly bound proton contributes to a reduction in the ^{15}N nuclear relaxation rates, leading to sharp NMR lines.

At higher pH and temperature, the interpretation of ^{15}N relaxation rates for non-proline amino acids, obtained from ^{1}H detected experiments, becomes challenging due to efficient solvent exchange. In such cases, heteronuclear relaxation rates involving nonexchangeable nuclear spins like ^{13}C can be beneficial and offer complementary information to that obtained from ^{15}N relaxation. Experimental variants to determine ^{13}C' R_1, ^{13}C' $R_{1\rho}$, ^{13}C' R_2 and ^{1}H-^{13}C NOE data have been proposed [108–110].

2.4. IDPs/IDRs fingerprints

The complementarity between 2D HN and 2D CON spectra, and the inclusion of 2D CACO and 2D CBCACO spectra, that reveal information about two additional nuclear spins ($^{13}C^{\alpha}$ and $^{13}C^{\beta}$) as well as about side chain resonances of aspartate, glutamate, asparagine, and glutamine residues, provide a set of experiments that can be exploited to obtain atom resolved information on the properties of a protein in different environments (buffer, pH, etc.) as well as upon addition of potential partners. The possibility of studying the system through different experiments and exploring different points of view can help to increase the quality and the quantity of the information that can be achieved, expanding the panorama of sample conditions accessible.

As an example, Fig. 7 recaps the complementarity of 2D HN (A), 2D CON (B), and 2D CACO (C) on α-synuclein. The 2D HN spectrum shows extensive overlap of the peaks due to the absence of a stable three-dimensional structure of the protein. Moreover, only a subset of the expected cross peaks is observed, in agreement with the notion that amide proton signals are very sensitive to solvent exchange processes, which broaden the peaks beyond detection. ^{13}C detection helps to study the system in temperatures and buffer conditions in which the exchange processes are effective ($T = 308$ K and pH = 7.4 in this case). Indeed, a quick count of the observed cross peaks in the CON spectrum shows that the vast majority of the expected cross peaks can be detected, revealing information lost in 2D HN spectra in these experimental conditions (note as an example the region of the spectra where ^{15}N resonances of glycine residues are detected). Moreover, 2D CON allows monitoring the five proline residues present in the final part of the α-synuclein primary sequence. The CACO clearly shows two regions of the spectrum reporting information about the backbone and the side chains of amino acids with carbonyl or carboxylate functional groups in their side chains. The latter are expected to be the primary actors involved in interactions with potential partners but are seldom investigated due to extensive cross peak overlap, in particular in IDPs. ^{13}C detection offers the possibility to monitor these resonances in detail through simple 2D NMR spectra as illustrated here through the example of the interaction of α-synuclein with calcium ions. Indeed α-synuclein is exposed to Ca^{2+} concentration bursts associated with neurotransmitter release [111] and the interaction with calcium ions might also trigger easier aggregation and be linked to the onset of Parkinson's disease. The possibility of monitoring resonances of side chain carboxylate functional groups present in aspartate and glutamate residues is very helpful to reveal the initial steps of the interaction with Ca^{2+} [79]. Interestingly, only a subset of glutamate and aspartate side chains is found to be affected by

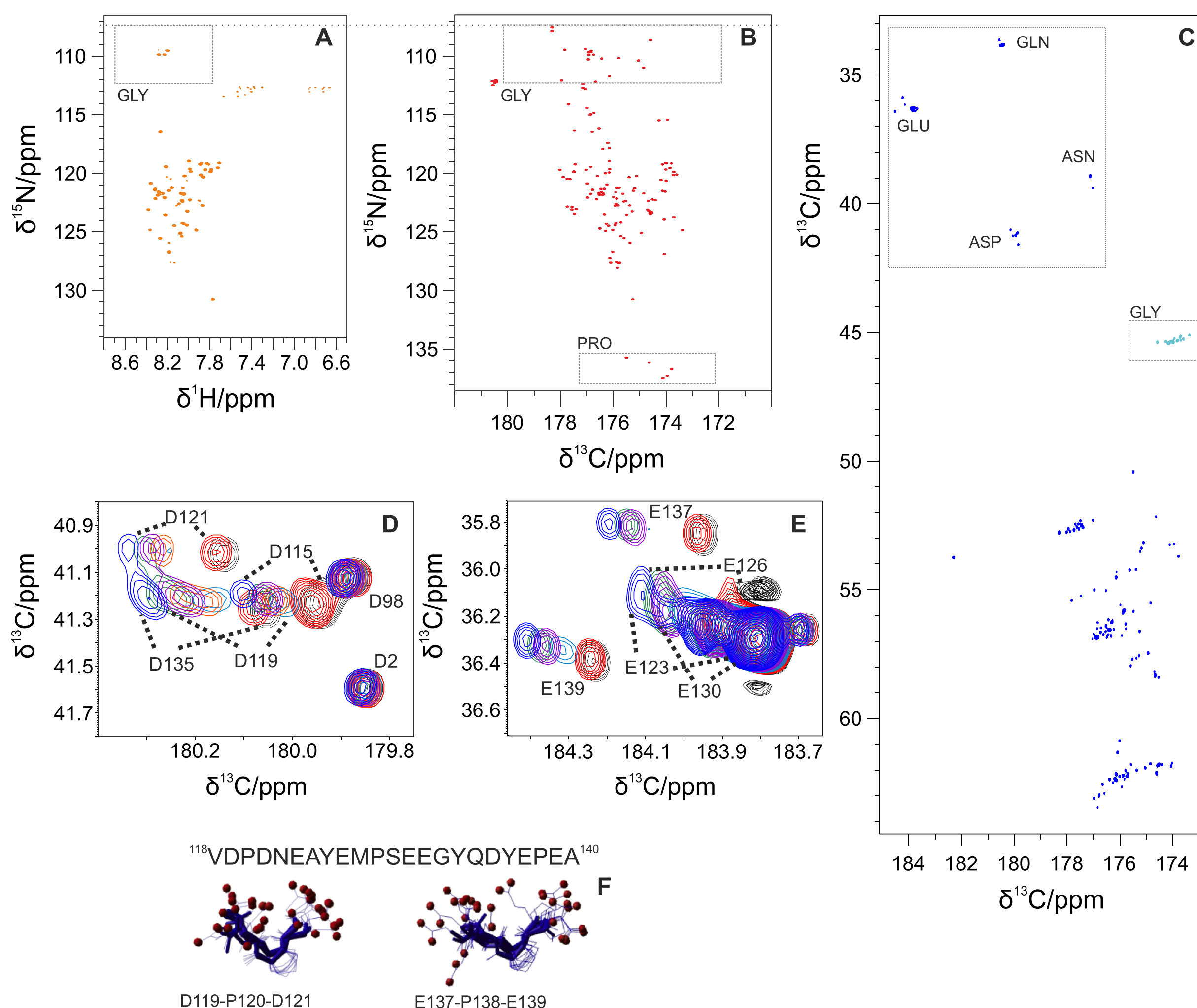

Fig. 7. Following the interaction of α-synuclein with calcium ions.
Panel A, B and C show 2D NMR spectra (2D HN, 2D CON and 2D CACO) acquired on 0.6 mM α-synuclein in TRIS 20 mM at pH 7.4 and 308 K with a 16.4 T NMR spectrometer. The information that can be derived by these three spectra provides a complete fingerprint of the protein with information about all the backbone nuclear spins as well as of some side chains (C^{δ}, C^{γ} of Glu and Gln C^{γ}, C^{β} of Asp and Asn, highlighted in the box in panel C). Panel D and E illustrate enlargements of 2D CACO spectra containing the cross peaks of Asp and Glu side chains respectively and their shifts upon addition of calcium ions. This suite of 2D NMR spectra is useful to follow interactions at the atomic level; they reveal particular motifs of α-synuclein involved in the interaction of with calcium ions (model in panel F). The overlay of the CON spectra in the proline region (not reported) also revealed two proline residues (P120, P138) flanked by negatively charged residues involved in the interaction with calcium ions [79].

the interaction indicating that, even in a disordered protein such as α-synuclein only specific regions of the protein are involved in the interaction (panels D and E in Fig. 7). The 2D CON also reveals additional information: two out of the five proline residues present in α-synuclein are found to be highly perturbed by the addition of calcium ions [79], a surprising finding considering that prolines do not have pronounced metal ion binding properties. Interestingly the two perturbed proline residues lie in between two negatively charged amino acids, revealing a local motif involved in the interaction (DPD, EPE, panel F in Fig. 7). The region involved in the interaction is also rich in tyrosine residues, which were found to be perturbed upon addition of Ca^{2+} [79]. These findings highlight particular motives in the C-terminal region of the protein involved in the interaction with metal ions, even in the presence of high flexibility and disorder [79].

3. Multidomain proteins: a key perspective in the field

Proteins are often investigated focusing on a single domain, neglecting the fact that they are usually composed of a sequence of different domains each with its own function. This “divide-and-conquer” approach has been widely exploited, allowing important features to be understood [112]. However, the interplay between the different domains is not captured through this approach.

The study of multidomain proteins in their entirety requires different NMR strategies that should consider the structural heterogeneity of each domain. Indeed, these modular proteins are usually composed of a succession of globular and disordered domains which can be investigated using different NMR approaches. As discussed above, the selection of the most appropriate NMR experiment as well as the optimization of the relevant parameters depends on the structural and dynamic properties of each domain rendering the investigation of multidomain proteins very challenging. Moreover, the complexity of the NMR spectra, in terms of the number of expected signals, obviously increases when focusing on multidomain proteins rather than on single isolated domains. Needless to say innovations that allow to study increasingly complex protein constructs are very important to fully understand also mutual interactions between the different domains themselves and to clarify how their interplay influences protein function.

The idea is thus to exploit the different spectral characteristics of globular and disordered domains to discriminate resonances from the

two domain types in the spectra or to simplify spectra in order to detect correlations from one type of domain only. The complementarity of ^{1}H and ^{13}C detected experiments is a valuable source of information. For example, the 2D HN spectrum, the first one that is often acquired when starting a protein investigation, provides signals from both domain types. The well-dispersed signals, arising from the globular domains can be easily identified whereas those of the disordered regions are clustered in a small central region of the spectra featuring extensive cross-peak overlap, as also evident from the two examples reported in Fig. 1. Depending on the experimental conditions (pH, temperature, ionic strength), some of the HN resonances of disordered protein regions can be difficult to observe because of line broadening. On the other hand, in the absence of extreme solvent exchange processes the signals of the disordered regions that can be observed in the spectra are generally more intense with respect to the ones deriving from globular components because of the intrinsically different transverse relaxation properties. However, as discussed above, they are generally characterized by significant resonance overlap, due to the reduced chemical shift dispersion typical of IDPs/IDRs. In this context, experiments based on carbonyl carbon direct detection offer a useful tool to selectively highlight the cross peaks deriving from the IDRs also when part of complex multidomain proteins. Indeed, the favorable chemical shift dispersion of heteronuclei provides a large contribution to increasing the resolution. In addition, the relaxation properties of carbonyl nuclear spins in the different motional regimes, combined with long delays in which transverse carbonyl relaxation is operative in CON-based experiments, results in a large difference in experimental sensitivity for globular and disordered regions. These differences are so pronounced that only cross peaks deriving from IDRs are generally observed in CON-based spectra. This constitutes a useful property to selectively highlight cross peaks deriving from disordered regions in complex multidomain proteins in a clean and complete way.

The improvement of hardware components allowed for the development of consoles equipped with multiple receivers for the simultaneous detection of different FIDs. With this technology it becomes possible to easily design experiments that involve different nuclear spins and different magnetization transfer pathways in order to acquire more experiments in the time needed for a single one [113–117]. These experiments, known as “multiple receivers” experiments, enable to detect, within the same transient, two or more different FIDs deriving from

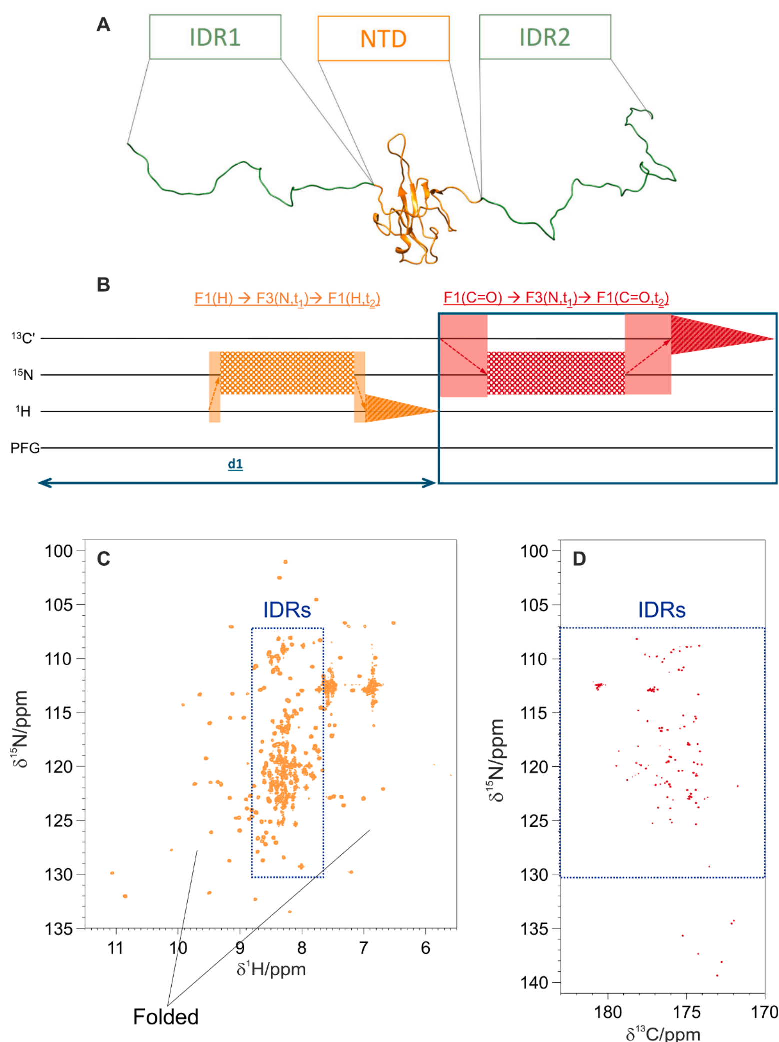

Fig. 8. Multiple receiver NMR experiments: different views on complex multi domain proteins.
Panel A shows NTR, the 1–248 residues construct of the nucleocapsid protein from SARS-CoV-2, is a modular protein constituted by the presence of two disordered regions (IDRs, green) flanking a globular domain (NTD, orange). A possible strategy for the investigation of such a system is represented by multiple receiver experiments. The mr_CON//HN scheme is schematically reported in panel B. The recycle delay needed for the 2D CON experiment is used to acquire a 2D HN experiment. The resulting spectra, acquired on NTR are reported in panel C (2D HN) and D (2D CON) [118]. It is worthy of note that the 2D HN experiment allows for the detection of the residues in the folded and in the disordered regions. The 2D CON experiments work as a relaxation filter and allow for a clear characterization of the IDRs also in the context of multidomain proteins.

different nuclear spins in the same experiment. Fig. 8 shows an example of how fruitfully the MR receiver strategy can be implemented to study a modular protein, the Nucleocapsid protein from SARS-CoV-2.

The nucleocapsid protein from SARS-CoV-2, expressed in high copy-number by the virus upon infection, has an important role in packaging the RNA genome of the virus, but it also plays several important roles in the transcription and assembly of the virion within the infected host [119]. Constituted by alternating disordered and globular domains, it is characterized by heterogeneous structural and dynamic properties, important for its function. The 3D structure of the globular domains is known: the N-terminal domain (NTD) is responsible for RNA binding and the C-terminal one (CTD) is responsible for homo-dimerization [120]. The three disordered linkers (IDR1, IDR2 and IDR3) are believed to be responsible for an intricate mechanism involved in the formation of the ribonucleoprotein complex [118,121–124]. They are also engaged in many interactions with other viral proteins or host proteins. The N protein from different coronaviruses seems to be genetically more stable than other structural proteins of the virus, which makes it an excellent candidate for developing antiviral therapies [125].

The NTR construct, which comprises the NTD flanked by two intrinsically disordered regions (IDR1 and IDR2), has been studied to understand how the flanking disordered regions contribute to the overall properties of this protein domain as well as to investigate their role in interactions with possible partners, such as RNA and other polyanions [118,126]. The multiple receivers experiment used to investigate NTR, is a CON-based variant in which an HSQC is included during the CON interscan delay. The two experiments exploit different nuclear spins as starting polarization source. It is thus quite straightforward to include the instructions to acquire a 2D HN HSQC within the relaxation delay of the CON experiment. Care should be taken to use ^{15}N-^{13}C decoupling strategies during the HN HSQC evolution time that allow to restore carbonyl carbon polarization to equilibrium before the beginning of the CON experiment. This can be easily accomplished by the use of 180° ^{13}C pulses for decoupling during ^{15}N chemical shift evolution, flanked by a second 180° ^{13}C pulse to restore the ^{13}C polarization to the z-axis right after or right before the ^{15}N evolution time. As a result, two spectra, the 2D CON and 2D HN HSQC, can be collected in the time needed for the 2D CON experiment, without essentially any compromise with respect to their independent acquisition [117].

The results obtained on the NTR construct are reported in Fig. 8. The 2D HN HSQC shows a number of well dispersed signals that derive from the globular NTD domain together with other signals clustered in a very narrow region of the spectrum, deriving from the flanking intrinsically disordered regions. While it is quite straightforward to identify the signals of the NTD domain, the situation becomes more complicated in the central region of the spectrum. The simultaneous acquisition of the 2D CON spectrum allows for selective highlighting of the signals from the flanking disordered regions (IDR1, IDR2). The availability of resonance assignment of these cross-peaks allows to follow at the atomic level, perturbations deriving from the addition of potential partners to the solution. A particularly interesting interaction in this context is that with RNA, the polymer that is packaged inside the virion thanks to the activity of the Nucleocapsid protein itself. While several interaction studies of NTD with RNA indicated a quite extended interaction region centred on the flexible positively charged "finger" of the globular domain [127–130], no information at atomic resolution was available on the role of the flanking disordered regions in this interaction. Several studies with other techniques however indicated the importance of linkers for the interaction but, in general, only overall data were reported without going into the details of specific regions because of extensive resonance overlap in the 2D HN NMR spectra. The use of 2D CON spectra, in particular when acquired simultaneously with 2D HN ones through the multiple receivers approach, revealed interesting atomic resolution information highlighting selected regions within the IDR1 and IDR2 involved in the interaction. These comprise positively charged regions that, together with the highly flexible finger region of NTD, also positively charged, form a "flexible" ridge that is particularly well suited to sense and interact with negatively charged RNA backbones. The construct thus seems well suited to interact with long polyanions, such as RNA fragments [118].

Along these lines, another interaction that was studied was the one with heparin, one of the most negatively charged, natural, linear polyanions. Heparin was also used, for different reasons, in the treatment of severely ill covid19 patients and it was thus interesting to see whether a direct interaction with the N protein could occur. The use of the mr_CON//HN approach allowed to monitor the interaction with atomic resolution and reveal again an important role played by the IDRs in the interaction [126].

4. Conclusions and perspectives

As the number of investigated IDPs/IDRs increases, their important role in protein function is becoming evident. A strong link is also emerging between the misfunction of IDPs/IDRs and several diseases that are difficult to treat nowadays. NMR is playing a crucial role in revealing molecular details that encode specific features for protein function. These are already beginning to highlight functional modules associated to a high extent of flexibility and disorder.

The investigation of complex protein constructs that comprise both globular and disordered domains is generally very challenging, but it is also expected to reveal the interplay between the different modules. By understanding how globular and disordered domains interact, we can learn more about how complex proteins work and how they are regulated. This knowledge could lead to the development of new drugs and therapies for a variety of diseases.

A variety of NMR methods optimized for the investigation of IDPs/IDRs has been proposed in recent years and is extensively used in many NMR labs. However, there is still wide room for further improvements thanks to the great versatility and potential of NMR spectroscopy, in particular when stimulated by challenging examples, such as the increasing complexity of the IDPs/IDRs under investigation, their involvement in a broad range of different types of interactions, their important role in promoting liquid-liquid phase separation (LLPS).

CRediT authorship contribution statement

Marco Schiavina: Writing – original draft, Writing – review & editing. **Lorenzo Bracaglia:** Writing – original draft, Writing – review & editing. **Tessa Bolognesi:** Writing – original draft, Writing – review & editing. **Maria Anna Rodella:** Writing – original draft, Writing – review & editing. **Giuseppe Tagliaferro:** Writing – original draft, Writing – review & editing. **Angela Sofia Tino:** Writing – original draft, Writing – review & editing. **Roberta Pierattelli:** Conceptualization, Writing – original draft, Writing – review & editing. **Isabella C. Felli:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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References

[1] M. Varadi, S. Anyango, M. Deshpande, S. Nair, C. Natassia, G. Yordanova, D. Yuan, O. Stroe, G. Wood, A. Laydon, A. Žídek, T. Green, K. Tunyasuvunakool, S. Petersen, J. Jumper, E. Clancy, R. Green, A. Vora, M. Lutfi, M. Figurnov, A. Cowie, N. Hobbs, P. Kohli, G. Kleywegt, E. Birney, D. Hassabis, S. Velankar, AlphaFold protein structure database: massively expanding the structural coverage of protein-sequence space with high-accuracy models, Nucl. Acids Res. 50 (2022) D439–D444, https://doi.org/10.1093/nar/gkab1061.

[2] K.M. Ruff, R.V. Pappu, AlphaFold and implications for intrinsically disordered proteins, J. Mol. Biol. 433 (2021) 167208, https://doi.org/10.1016/j.jmb.2021.167208.

[3] V.N. Uversky, Introduction to intrinsically disordered proteins (IDPs), Chem. Rev. 114 (2014) 6557–6560, https://doi.org/10.1021/cr500288y.

[4] J. Habchi, P. Tompa, S. Longhi, V.N. Uversky, Introducing protein intrinsic disorder, Chem. Rev. 114 (2014) 6561–6588, https://doi.org/10.1021/cr400514h.

[5] V.N. Uversky, C.J. Oldfield, A.K. Dunker, Intrinsically disordered proteins in human diseases: introducing the D^2 concept, Annu. Rev. Biophys. 37 (2008) 215–246, https://doi.org/10.1146/annurev.biophys.37.032807.125924.

[6] D. Kurzbach, T.C. Schwarz, G. Platzer, S. Höfler, D. Hinderberger, R. Konrat, Compensatory adaptations of structural dynamics in an intrinsically disordered protein complex, Angew. Chem. Int. Ed. 53 (2014) 3840–3843, https://doi.org/10.1002/anie.201308389.

[7] R. Konrat, NMR contributions to structural dynamics studies of intrinsically disordered proteins, J. Magn. Reson. 241 (2014) 74–85, https://doi.org/10.1016/j.jmr.2013.11.011.

[8] U. Jakob, R. Kriwacki, V.N. Uversky, Conditionally and transiently disordered proteins: awakening cryptic disorder to regulate protein function, Chem. Rev. 114 (2014) 6779–6805, https://doi.org/10.1021/cr400459c.

[9] M. Fuxreiter, Á. Tóth-Petróczy, D.A. Kraut, A.T. Matouschek, R.Y.H. Lim, B. Xue, L. Kurgan, V.N. Uversky, Disordered proteinaceous machines, Chem. Rev. 114 (2014) 6806–6843, https://doi.org/10.1021/cr4007329.

[10] P. Tompa, E. Schad, A. Tantos, L. Kalmar, Intrinsically disordered proteins: emerging interaction specialists, Curr. Opin. Struct. Biol. 35 (2015) 49–59, https://doi.org/10.1016/j.sbi.2015.08.009.

[11] V. Csizmok, A.V. Follis, R.W. Kriwacki, J.D. Forman-Kay, Dynamic protein interaction networks and new structural paradigms in signaling, Chem. Rev. 116 (2016) 6424–6462, https://doi.org/10.1021/acs.chemrev.5b00548.

[12] M. Arbesú, M. Pons, Integrating disorder in globular multidomain proteins: fuzzy sensors and the role of SH3 domains, Arch. Biochem. Biophys. 677 (2019) 108161, https://doi.org/10.1016/j.abb.2019.108161.

[13] A.R. Camacho-Zarco, V. Schnapka, S. Guseva, A. Abyzov, W. Adamski, S. Milles, M.R. Jensen, L. Zidek, N. Salvi, M. Blackledge, NMR provides unique insight into the functional dynamics and interactions of intrinsically disordered proteins, Chem. Rev. 122 (2022) 9331–9356, https://doi.org/10.1021/acs.chemrev.1c01023.

[14] P. Tompa, M. Fuxreiter, Fuzzy complexes: polymorphism and structural disorder in protein–protein interactions, Trends Biochem. Sci. 33 (2008) 2–8, https://doi.org/10.1016/j.tibs.2007.10.003.

[15] M. Fuxreiter, Fuzziness: linking regulation to protein dynamics, Mol. BioSyst. 8 (2012) 168–177, https://doi.org/10.1039/C1MB05234A.

[16] P.E. Wright, H.J. Dyson, Intrinsically disordered proteins in cellular signalling and regulation, Nat. Rev. Mol. Cell Biol. 16 (2015) 18–29, https://doi.org/10.1038/nrm3920.

[17] A.K. Dunker, M.S. Cortese, P. Romero, L.M. Iakoucheva, V.N. Uversky, Flexible nets. The roles of intrinsic disorder in protein interaction networks, FEBS J. 272 (2005) 5129–5148, https://doi.org/10.1111/j.1742-4658.2005.04948.x.

[18] M. Arbesú, M. Maffei, T.N. Cordeiro, J.M.C. Teixeira, Y. Pérez, P. Bernadó, S. Roche, M. Pons, The unique domain forms a fuzzy intramolecular complex in Src family kinases, Structure 25 (2017) 630–640.e4, https://doi.org/10.1016/j.str.2017.02.011.

[19] F.-X. Theillet, H.M. Rose, S. Liokatis, A. Binolfi, R. Thongwichian, M. Stuiver, P. Selenko, Site-specific NMR mapping and time-resolved monitoring of serine and threonine phosphorylation in reconstituted kinase reactions and mammalian cell extracts, Nat. Protoc. 8 (2013) 1416–1432, https://doi.org/10.1038/nprot.2013.083.

[20] A. Bah, J.D. Forman-Kay, Modulation of intrinsically disordered protein function by post-translational modifications, J. Biol. Chem. 291 (2016) 6696–6705, https://doi.org/10.1074/jbc.R115.695056.

[21] P.R. Banerjee, D.M. Mitrea, R.W. Kriwacki, A.A. Deniz, Asymmetric modulation of protein order-disorder transitions by phosphorylation and partner binding, Angew. Chem. Int. Ed. 55 (2016) 1675–1679, https://doi.org/10.1002/anie.201507728.

[22] P.R. Romero, S. Zaidi, Y.Y. Fang, V.N. Uversky, P. Radivojac, C.J. Oldfield, M. S. Cortese, M. Sickmeier, T. LeGall, Z. Obradovic, A.K. Dunker, Alternative splicing in concert with protein intrinsic disorder enables increased functional diversity in multicellular organisms, Proc. Natl. Acad. Sci. USA 103 (2006) 8390–8395, https://doi.org/10.1073/pnas.0507916103.

[23] M. Buljan, G. Chalancon, A.K. Dunker, A. Bateman, S. Balaji, M. Fuxreiter, M. M. Babu, Alternative splicing of intrinsically disordered regions and rewiring of protein interactions, Curr. Opin. Struct. Biol. 23 (2013) 443–450, https://doi.org/10.1016/j.sbi.2013.03.006.

[24] N.E. Davey, G. Travé, T.J. Gibson, How viruses hijack cell regulation, Trends Biochem. Sci. 36 (2011) 159–169, https://doi.org/10.1016/j.tibs.2010.10.002.

[25] K. Van Roey, B. Uyar, R.J. Weatheritt, H. Dinkel, M. Seiler, A. Budd, T.J. Gibson, N.E. Davey, Short linear motifs: ubiquitous and functionally diverse protein interaction modules directing cell regulation, Chem. Rev. 114 (2014) 6733–6778, https://doi.org/10.1021/cr400585q.

[26] M. Kumar, S. Michael, J. Alvarado-Valverde, B. Mészáros, H. Sámano-Sánchez, A. Zeke, L. Dobson, T. Lazar, M. Örd, A. Nagpal, N. Farahi, M. Käser, R. Kraleti, N. E. Davey, R. Pancsa, L.B. Chemes, T.J. Gibson, The eukaryotic linear motif resource: 2022 release, Nucl. Acids Res. 50 (2022) D497–D508, https://doi.org/10.1093/nar/gkab975.

[27] S. Brocca, R. Grandori, S. Longhi, V. Uversky, Liquid–Liquid phase separation by intrinsically disordered protein regions of viruses: roles in viral life cycle and control of virus–host interactions, Int. J. Mol. Sci. 21 (2020) 9045, https://doi.org/10.3390/ijms21239045.

[28] C.A. Elena-Real, P. Mier, N. Sibille, M.A. Andrade-Navarro, P. Bernadó, Structure–function relationships in protein homorepeats, Curr. Opin. Struct. Biol. 83 (2023) 102726, https://doi.org/10.1016/j.sbi.2023.102726.

[29] K. Kazimierczuk, A. Zawadzka, W. Koźmiński, Narrow peaks and high dimensionalities: exploiting the advantages of random sampling, J. Magn. Reson. 197 (2009) 219–228, https://doi.org/10.1016/j.jmr.2009.01.003.

[30] J. Nováček, A. Zawadzka-Kazimierczuk, V. Papoušková, L. Žídek, H. Šanderová, L. Krásný, W. Koźmiński, V. Sklenář, 5D ^{13}C-detected experiments for backbone assignment of unstructured proteins with a very low signal dispersion, J. Biomol. NMR 50 (2011) 1–11, https://doi.org/10.1007/s10858-011-9496-2.

[31] W. Bermel, I.C. Felli, L. Gonnelli, W. Koźmiński, A. Piai, R. Pierattelli, A. Zawadzka-Kazimierczuk, High-dimensionality ^{13}C direct-detected NMR experiments for the automatic assignment of intrinsically disordered proteins, J. Biomol. NMR 57 (2013) 353–361, https://doi.org/10.1007/s10858-013-9793-z.

[32] B. Brutscher, I.C. Felli, S. Gil-Caballero, T. Hošek, R. Kümmerle, A. Piai, R. Pierattelli, Z. Sólyom, NMR methods for the study of instrinsically disordered proteins structure, dynamics, and interactions: general overview and practical guidelines, Adv. Exp. Med. Biol. 870 (2015) 49–122, https://doi.org/10.1007/978-3-319-20164-1_3.

[33] M.G. Murrali, M. Schiavina, V. Sainati, W. Bermel, R. Pierattelli, I.C. Felli, ^{13}C APSY-NMR for sequential assignment of intrinsically disordered proteins, J. Biomol. NMR 70 (2018) 167–175, https://doi.org/10.1007/s10858-018-0167-4.

[34] S.-T.D. Hsu, C.W. Bertoncini, C.M. Dobson, Use of protonless NMR spectroscopy to alleviate the loss of information resulting from exchange-broadening, J. Am. Chem. Soc. 131 (2009) 7222–7223, https://doi.org/10.1021/ja902307q.

[35] S. Gil, T. Hošek, Z. Solyom, R. Kümmerle, B. Brutscher, R. Pierattelli, I.C. Felli, NMR spectroscopic studies of intrinsically disordered proteins at near-physiological conditions, Angew. Chem. Int. Ed. 52 (2013) 11808–11812, https://doi.org/10.1002/anie.201304272.

[36] Q. Wang, J. Zheng, S. Pettersson, R. Reynolds, E.-K. Tan, The link between neuroinflammation and the neurovascular unit in synucleinopathies, Sci. Adv. 9 (2023), https://doi.org/10.1126/sciadv.abq1141.

[37] S. Chandra, X. Chen, J. Rizo, R. Jahn, T.C. Südhof, A broken α-helix in folded α-synuclein, J. Biol. Chem. 278 (2003) 15313–15318, https://doi.org/10.1074/jbc.M213128200.

[38] T.S. Ulmer, A. Bax, N.B. Cole, R.L. Nussbaum, Structure and dynamics of micelle-bound human α-synuclein, J. Biol. Chem. 280 (2005) 9595–9603, https://doi.org/10.1074/jbc.M411805200.

[39] C.R. Bodner, C.M. Dobson, A. Bax, Multiple tight phospholipid-binding modes of α-synuclein revealed by solution NMR spectroscopy, J. Mol. Biol. 390 (2009) 775–790, https://doi.org/10.1016/j.jmb.2009.05.066.

[40] G. Fusco, T. Pape, A.D. Stephens, P. Mahou, A.R. Costa, C.F. Kaminski, G. S. Kaminski Schierle, M. Vendruscolo, G. Veglia, C.M. Dobson, A. De Simone, Structural basis of synaptic vesicle assembly promoted by α-synuclein, Nat. Commun. 7 (2016) 12563, https://doi.org/10.1038/ncomms12563.

[41] T.C. Schwarz, A. Beier, K. Ledolter, T. Gossenreiter, T. Höfurthner, M. Hartl, T. S. Baker, R.J. Taylor, R. Konrat, High-resolution structural information of membrane-bound α-synuclein provides insight into the MoA of the anti-Parkinson drug UCB0599, Proc. Natl. Acad. Sci. USA 120 (2023), https://doi.org/10.1073/pnas.2201910120.

[42] C.W. Bertoncini, Y.-S. Jung, C.O. Fernandez, W. Hoyer, C. Griesinger, T.M. Jovin, M. Zweckstetter, Release of long-range tertiary interactions potentiates aggregation of natively unstructured α-synuclein, Proc. Natl. Acad. Sci. USA 102 (2005) 1430–1435, https://doi.org/10.1073/pnas.0407146102.

[43] J.R. Allison, P. Varnai, C.M. Dobson, M. Vendruscolo, Determination of the free energy landscape of α-synuclein using spin label nuclear magnetic resonance measurements, J. Am. Chem. Soc. 131 (2009) 18314–18326, https://doi.org/10.1021/ja904716h.

[44] V.N. Uversky, A Protein-Chameleon: conformational plasticity of α-synuclein, a disordered protein involved in neurodegenerative disorders, J. Biomol. Struct. Dyn. 21 (2003) 211–234, https://doi.org/10.1080/07391102.2003.10506918.
[45] J. Yao, H.J. Dyson, P.E. Wright, Chemical shift dispersion and secondary structure prediction in unfolded and partly folded proteins, FEBS Lett. 419 (1997) 285–289, https://doi.org/10.1016/S0014-5793(97)01474-9.
[46] M. Kjaergaard, F.M. Poulsen, Disordered proteins studied by chemical shifts, Prog. Nucl. Magn. Reson. Spectrosc. 60 (2012) 42–51, https://doi.org/10.1016/j.pnmrs.2011.10.001.
[47] J.W. Peng, G. Wagner, Investigation of protein motions via relaxation measurements, in: T.L. James, N.J. Oppenheimer (Eds.), Methods in Enzymology, 1994, pp. 563–596, https://doi.org/10.1016/S0076-6879(94)39022-3.
[48] M. Buck, J. Boyd, C. Redfield, D.A. MacKenzie, D.J. Jeenes, D.B. Archer, C. M. Dobson, Structural determinants of protein dynamics: analysis of ^{15}N NMR relaxation measurements for main-chain and side-chain nuclei of hen egg white lysozyme, Biochemistry 34 (1995) 4041–4055, https://doi.org/10.1021/bi00012a023.
[49] K. Tamiola, B. Acar, F.A.A. Mulder, Sequence-specific random coil chemical shifts of intrinsically disordered proteins, J. Am. Chem. Soc. 132 (2010) 18000–18003, https://doi.org/10.1021/ja105656t.
[50] F. Sebák, P. Ecsédi, W. Bermel, B. Luy, L. Nyitray, A. Bodor, Selective ^{1}H$^{\alpha}$ NMR methods reveal functionally relevant proline *cis/trans* isomers in intrinsically disordered proteins: characterization of minor forms, effects of phosphorylation, and occurrence in proteome, Angew. Chem. Int. Ed. 61 (2022), https://doi.org/10.1002/anie.202108361.
[51] W. Bermel, I. Bertini, I.C. Felli, Y.-M. Lee, C. Luchinat, R. Pierattelli, Protonless NMR experiments for sequence-specific assignment of backbone nuclei in unfolded proteins, J. Am. Chem. Soc. 128 (2006) 3918–3919, https://doi.org/10.1021/ja0582206.
[52] T.E. Machonkin, W.M. Westler, J.L. Markley, ^{13}C{^{13}C} 2D NMR: A novel strategy for the study of paramagnetic proteins with slow electronic relaxation rates, J. Am. Chem. Soc. 124 (2002) 3204–3205, https://doi.org/10.1021/ja017733j.
[53] W. Bermel, I. Bertini, I.C. Felli, R. Kümmerle, R. Pierattelli, ^{13}C Direct detection experiments on the paramagnetic oxidized monomeric copper, zinc superoxide dismutase, J. Am. Chem. Soc. 125 (2003) 16423–16429, https://doi.org/10.1021/ja037676p.
[54] H. Kovacs, D. Moskau, M. Spraul, Cryogenically cooled probes—A leap in NMR technology, Prog. Nucl. Magn. Reson. Spectrosc. 46 (2005) 131–155, https://doi.org/10.1016/j.pnmrs.2005.03.001.
[55] M.G. Murrali, A. Piai, W. Bermel, I.C. Felli, R. Pierattelli, Proline fingerprint in intrinsically disordered proteins, ChemBioChem 19 (2018) 1625–1629, https://doi.org/10.1002/cbic.201800172.
[56] B. Mateos, C. Conrad-Billroth, M. Schiavina, A. Beier, G. Kontaxis, R. Konrat, I. C. Felli, R. Pierattelli, The ambivalent role of proline residues in an intrinsically disordered protein: from disorder promoters to compaction facilitators, J. Mol. Biol. 432 (2020) 3093–3111, https://doi.org/10.1016/j.jmb.2019.11.015.
[57] M. Schiavina, L. Bracaglia, M.A. Rodella, R. Kümmerle, R. Konrat, I.C. Felli, R. Pierattelli, Optimal ^{13}C NMR investigation of intrinsically disordered proteins at 1.2 GHz, Nat. Protoc.3 (2023), https://doi.org/10.1038/s41596-023-00921-9.
[58] I.C. Felli, R. Pierattelli, ^{13}C direct detected NMR for challenging systems, Chem. Rev. 122 (2022) 9468–9496, https://doi.org/10.1021/acs.chemrev.1c00871.
[59] W. Bermel, I. Bertini, I.C. Felli, R. Kümmerle, R. Pierattelli, Novel ^{13}C direct detection experiments, including extension to the third dimension, to perform the complete assignment of proteins, J. Magn. Reson. 178 (2006) 56–64, https://doi.org/10.1016/j.jmr.2005.08.011.
[60] W. Bermel, M. Bruix, I.C. Felli, M.V. V Kumar, R. Pierattelli, S. Serrano, Improving the chemical shift dispersion of multidimensional NMR spectra of intrinsically disordered proteins, J. Biomol. NMR 55 (2013) 231–237, https://doi.org/10.1007/s10858-013-9704-3.
[61] A. Piai, E.O. Calçada, T. Tarenzi, A. Del Grande, M. Varadi, P. Tompa, I.C. Felli, R. Pierattelli, Just a flexible Linker? The structural and dynamic properties of CBP-ID4 revealed by NMR spectroscopy, Biophys. J. 110 (2016) 372–381, https://doi.org/10.1016/j.bpj.2015.11.3516.
[62] P.S. Arora, A.Z. Ansari, T.P. Best, M. Ptashne, P.B. Dervan, Design of artificial transcriptional activators with rigid poly- L-proline linkers, J. Am. Chem. Soc. 124 (2002) 13067–13071, https://doi.org/10.1021/ja0208355.
[63] S. Sato, Y. Kwon, S. Kamisuki, N. Srivastava, Q. Mao, Y. Kawazoe, M. Uesugi, Polyproline-rod approach to isolating protein targets of bioactive small molecules: isolation of a new target of indomethacin, J. Am. Chem. Soc. 129 (2007) 873–880, https://doi.org/10.1021/ja0655643.
[64] T. Tanaka, W. Nomura, T. Narumi, A. Masuda, H. Tamamura, Bivalent ligands of CXCR4 with rigid linkers for elucidation of the dimerization state in cells, J. Am. Chem. Soc. 132 (2010) 15899–15901, https://doi.org/10.1021/ja107447w.
[65] A. Urbanek, M. Popovic, C.A. Elena-Real, A. Morató, A. Estaña, A. Fournet, F. Allemand, A.M. Gil, C. Cativiela, J. Cortés, A.I. Jiménez, N. Sibille, P. Bernadó, Evidence of the reduced abundance of proline cis conformation in protein poly proline tracts, J. Am. Chem. Soc. 142 (2020) 7976–7986, https://doi.org/10.1021/jacs.0c02263.
[66] R.B. Best, K.A. Merchant, I.V. Gopich, B. Schuler, A. Bax, W.A. Eaton, Effect of flexibility and *cis* residues in single-molecule FRET studies of polyproline, Proc. Natl. Acad. Sci. 104 (2007) 18964–18969, https://doi.org/10.1073/pnas.0709567104.
[67] P. Ahuja, F.-X. Cantrelle, I. Huvent, X. Hanoulle, J. Lopez, C. Smet, J.-M. Wieruszeski, I. Landrieu, G. Lippens, Proline conformation in a functional tau fragment, J. Mol. Biol. 428 (2016) 79–91, https://doi.org/10.1016/j.jmb.2015.11.023.
[68] E.B. Gibbs, F. Lu, B. Portz, M.J. Fisher, B.P. Medellin, T.N. Laremore, Y.J. Zhang, D.S. Gilmour, S.A. Showalter, Phosphorylation induces sequence-specific conformational switches in the RNA polymerase II C-terminal domain, Nat. Commun. 8 (2017) 15233, https://doi.org/10.1038/ncomms15233.
[69] C.L. Gustafson, N.C. Parsley, H. Asimgil, H.-W. Lee, C. Ahlbach, A.K. Michael, H. Xu, O.L. Williams, T.L. Davis, A.C. Liu, C.L. Partch, A slow conformational switch in the BMAL1 transactivation domain modulates circadian rhythms, Mol. Cell 66 (2017) 447–457.e7, https://doi.org/10.1016/j.molcel.2017.04.011.
[70] L. Banci, L. Barbieri, V. Calderone, F. Cantini, L. Cerofolini, S. Ciofi-Baffoni, I.C. Felli, M. Fragai, M. Lelli, C. Luchinat, E. Luchinat, G. Parigi, M. Piccioli, R. Pierattelli, E. Ravera, A. Rosato, L. Tenori, P. Turano, Biomolecular NMR at 1.2 GHz, arXiv, https://doi.org/10.48550/arXiv.1910.07462.
[71] L. Masino, G. Kelly, K. Leonard, Y. Trottier, A. Pastore, Solution structure of polyglutamine tracts in GST-polyglutamine fusion proteins, FEBS Lett. 513 (2002) 267–272, https://doi.org/10.1016/S0014-5793(02)02335-9.
[72] L.E. Buchanan, J.K. Carr, A.M. Fluitt, A.J. Hoganson, S.D. Moran, J.J. de Pablo, J. L. Skinner, M.T. Zanni, Structural motif of polyglutamine amyloid fibrils discerned with mixed-isotope infrared spectroscopy, Proc. Natl. Acad. Sci. 111 (2014) 5796–5801, https://doi.org/10.1073/pnas.1401587111.
[73] B. Eftekharzadeh, A. Piai, G. Chiesa, D. Mungianu, J. García, R. Pierattelli, I. C. Felli, X. Salvatella, Sequence context influences the structure and aggregation behavior of a polyQ tract, Biophys J. 110 (2016) 2361–2366, https://doi.org/10.1016/j.bpj.2016.04.022.
[74] A. Escobedo, B. Topal, M.B.A. Kunze, J. Aranda, G. Chiesa, D. Mungianu, G. Bernardo-Seisdedos, B. Eftekharzadeh, M. Gairí, R. Pierattelli, I.C. Felli, T. Diercks, O. Millet, J. García, M. Orozco, R. Crehuet, K. Lindorff-Larsen, X. Salvatella, Side chain to main chain hydrogen bonds stabilize a polyglutamine helix in a transcription factor, Nat. Commun. 10 (2019) 2034, https://doi.org/10.1038/s41467-019-09923-2.
[75] L. Baronti, T. Hošek, S. Gil-Caballero, H. Raveh-Amit, E.O. Calçada, I. Ayala, A. Dinnyés, I.C. Felli, R. Pierattelli, B. Brutscher, Fragment-based NMR study of the conformational dynamics in the bHLH transcription factor Ascl1, Biophys J. 112 (2017) 1366–1373, https://doi.org/10.1016/j.bpj.2017.02.025.
[76] S. Kosol, S. Contreras-Martos, A. Piai, M. Varadi, T. Lazar, A. Bekesi, P. Lebrun, I. C. Felli, R. Pierattelli, P. Tompa, Interaction between the scaffold proteins CBP by IQGAP1 provides an interface between gene expression and cytoskeletal activity, Sci. Rep. 10 (2020) 5753, https://doi.org/10.1038/s41598-020-62069-w.
[77] C.A. Elena-Real, A. Urbanek, X.L. Lund, A. Morató, A. Sagar, A. Fournet, A. Estaña, T. Bellande, F. Allemand, J. Cortés, N. Sibille, R. Melki, P. Bernadó, Multi-site-specific isotopic labeling accelerates high-resolution structural investigations of pathogenic huntingtin exon-1, Structure. 31 (6) (2023) 644–650.e5, https://doi.org/10.1016/j.str.2023.04.003.
[78] A. Borgia, M.B. Borgia, K. Bugge, V.M. Kissling, P.O. Heidarsson, C.B. Fernandes, A. Sottini, A. Soranno, K.J. Buholzer, D. Nettels, B.B. Kragelund, R.B. Best, B. Schuler, Extreme disorder in an ultrahigh-affinity protein complex, Nature 555 (2018) 61–66, https://doi.org/10.1038/nature25762.
[79] L. Pontoriero, M. Schiavina, M.G. Murrali, R. Pierattelli, I.C. Felli, Monitoring the interaction of α-synuclein with calcium ions through exclusively heteronuclear nuclear magnetic resonance experiments, Angew. Chem. Int. Ed. 59 (2020) 18537–18545, https://doi.org/10.1002/anie.202008079.
[80] A. Sottini, A. Borgia, M.B. Borgia, K. Bugge, D. Nettels, A. Chowdhury, P. O. Heidarsson, F. Zosel, R.B. Best, B.B. Kragelund, B. Schuler, Polyelectrolyte interactions enable rapid association and dissociation in high-affinity disordered protein complexes, Nat. Commun. 11 (2020) 5736, https://doi.org/10.1038/s41467-020-18859-x.
[81] L.E. Kay, M. Ikura, R. Tschudin, A. Bax, Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins, J. Magn. Reason. 89 (1990) 496–514, https://doi.org/10.1016/0022-2364(90)90333-5 (1969).
[82] M. Sattler, J. Schleucher, C. Griesinger, Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients, Prog. Nucl. Magn. Reson. Spectrosc. 34 (1999) 93–158, https://doi.org/10.1016/S0079-6565(98)00025-9.
[83] Z. Solyom, M. Schwarten, L. Geist, R. Konrat, D. Willbold, B. Brutscher, BEST-TROSY experiments for time-efficient sequential resonance assignment of large disordered proteins, J. Biomol. NMR 55 (2013) 311–321, https://doi.org/10.1007/s10858-013-9715-0.
[84] B. O'Hare, A.J. Benesi, S.A. Showalter, Incorporating ^{1}H chemical shift determination into ^{13}C-direct detected spectroscopy of intrinsically disordered proteins in solution, J. Magn. Reson. 200 (2009) 354–358, https://doi.org/10.1016/j.jmr.2009.07.014.
[85] J. Nováček, N.Y. Haba, J.H. Chill, L. Žídek, V. Sklenář, 4D Non-uniformly sampled HCBCACON and ^{1}J(NC$^{\alpha}$)-selective HCBCANCO experiments for the sequential assignment and chemical shift analysis of intrinsically disordered proteins, J. Biomol. NMR 53 (2012) 139–148, https://doi.org/10.1007/s10858-012-9631-8.
[86] D. Pantoja-Uceda, J. Santoro, Direct correlation of consecutive C′–N groups in proteins: a method for the assignment of intrinsically disordered proteins, J. Biomol. NMR 57 (2013) 57–63, https://doi.org/10.1007/s10858-013-9765-3.
[87] K. Takeuchi, G. Heffron, Z.-Y.J. Sun, D.P. Frueh, G. Wagner, Nitrogen-detected CAN and CON experiments as alternative experiments for main chain NMR resonance assignments, J. Biomol. NMR 47 (2010) 271–282, https://doi.org/10.1007/s10858-010-9430-z.
[88] S. Chhabra, P. Fischer, K. Takeuchi, A. Dubey, J.J. Ziarek, A. Boeszoermenyi, D. Mathieu, W. Bermel, N.E. Davey, G. Wagner, H. Arthanari, ^{15}N detection harnesses the slow relaxation property of nitrogen: delivering enhanced

resolution for intrinsically disordered proteins, Proc. Natl. Acad. Sci. USA 115 (2018), https://doi.org/10.1073/pnas.1717560115.
[89] M. Karjalainen, H. Tossavainen, M. Hellman, P. Permi, HACANCOi: a new H^{α}-detected experiment for backbone resonance assignment of intrinsically disordered proteins, J. Biomol. NMR 74 (2020) 741–752, https://doi.org/10.1007/s10858-020-00347-5.
[90] W. Bermel, I. Bertini, V. Csizmok, I.C. Felli, R. Pierattelli, P. Tompa, H-start for exclusively heteronuclear NMR spectroscopy: the case of intrinsically disordered proteins, J. Magn. Reson. 198 (2009) 275–281, https://doi.org/10.1016/j.jmr.2009.02.012.
[91] I.C. Felli, R. Pierattelli, S.J. Glaser, B. Luy, Relaxation-optimised Hartmann–Hahn transfer using a specifically Tailored MOCCA-XY16 mixing sequence for carbonyl–carbonyl correlation spectroscopy in 13C direct detection NMR experiments, J. Biomol. NMR 43 (2009) 187–196, https://doi.org/10.1007/s10858-009-9302-6.
[92] Y. Yoshimura, N.V. Kulminskaya, F.A.A. Mulder, Easy and unambiguous sequential assignments of intrinsically disordered proteins by correlating the backbone ^{15}N or $^{13}C'$ chemical shifts of multiple contiguous residues in highly resolved 3D spectra, J. Biomol. NMR 61 (2015) 109–121, https://doi.org/10.1007/s10858-014-9890-7.
[93] I.C. Felli, W. Bermel, R. Pierattelli, Exclusively heteronuclear NMR experiments for the investigation of intrinsically disordered proteins: focusing on proline residues, Magn. Reson. 2 (2021) 511–522, https://doi.org/10.5194/mr-2-511-2021.
[94] W. Bermel, I. Bertini, I.C. Felli, L. Gonnelli, W. Koźmiński, A. Piai, R. Pierattelli, J. Stanek, Speeding up sequence specific assignment of IDPs, J. Biomol. NMR 53 (2012) 293–301, https://doi.org/10.1007/s10858-012-9639-0.
[95] S. Hiller, F. Fiorito, K. Wüthrich, G. Wider, Automated projection spectroscopy (APSY), Proc. Natl. Acad. Sci. USA 102 (2005) 10876–10881, https://doi.org/10.1073/pnas.0504818102.
[96] J.-G. Jee, Application of non-uniform sampling to three dimensional carbon direct-detection NMR Experiment, Bull. Korean Chem. Soc. 32 (2011) 3551–3552, https://doi.org/10.5012/bkcs.2011.32.10.3551.
[97] M. Nowakowski, S. Saxena, J. Stanek, S. Żerko, W. Koźmiński, Applications of high dimensionality experiments to biomolecular NMR, Prog. Nucl. Magn. Reson. Spectrosc. 90–91 (2015) 49–73, https://doi.org/10.1016/j.pnmrs.2015.07.001.
[98] K. Grudziąż, A. Zawadzka-Kazimierczuk, W. Koźmiński, High-dimensional NMR methods for intrinsically disordered proteins studies, Methods 148 (2018) 81–87, https://doi.org/10.1016/j.ymeth.2018.04.031.
[99] Y. Pustovalova, M. Mayzel, V.Yu. Orekhov, XLSY: extra-large NMR spectroscopy, Angew. Chem. Int. Ed. 57 (2018) 14043–14045, https://doi.org/10.1002/anie.201806144.
[100] M.A. Zambrello, A.D. Schuyler, M.W. Maciejewski, F. Delaglio, I. Bezsonova, J. C. Hoch, Nonuniform sampling in multidimensional NMR for improving spectral sensitivity, Methods 138–139 (2018) 62–68, https://doi.org/10.1016/j.ymeth.2018.03.001.
[101] S. Robson, H. Arthanari, S.G. Hyberts, G. Wagner, Nonuniform sampling for NMR spectroscopy, in: A.J. Wand (Ed.), Methods in Enzymology, 2019, pp. 263–291, https://doi.org/10.1016/bs.mie.2018.09.009.
[102] K. Tamiola, F.A.A. Mulder, Using NMR chemical shifts to calculate the propensity for structural order and disorder in proteins, Biochem. Soc. Trans. 40 (2012) 1014–1020, https://doi.org/10.1042/BST20120171.
[103] C. Camilloni, A. De Simone, W.F. Vranken, M. Vendruscolo, Determination of secondary structure populations in disordered states of proteins using nuclear magnetic resonance chemical shifts, Biochemistry 51 (2012) 2224–2231, https://doi.org/10.1021/bi3001825.
[104] J. Kragelj, V. Ozenne, M. Blackledge, M.R. Jensen, Conformational propensities of intrinsically disordered proteins from NMR chemical shifts, ChemPhysChem 14 (2013) 3034–3045, https://doi.org/10.1002/cphc.201300387.
[105] L. Banci, I. Bertini, F. Cramaro, R. Del Conte, A. Rosato, M.S. Viezzoli, Backbone dynamics of human Cu,Zn superoxide dismutase and of its monomeric F50E/G51E/E133Q mutant: the influence of dimerization on mobility and function, Biochemistry 39 (2000) 9108–9118, https://doi.org/10.1021/bi000067z.
[106] C.W. Lawrence, S.A. Showalter, Carbon-detected ^{15}N NMR spin relaxation of an intrinsically disordered protein: FCP1 dynamics unbound and in complex with RAP74, J. Phys. Chem. Lett. 3 (2012) 1409–1413, https://doi.org/10.1021/jz300432e.
[107] M. Schiavina, R. Konrat, I. Ceccolini, B. Mateos, R. Konrat, I.C. Felli, R. Pierattelli, Studies of proline conformational dynamics in IDPs by ^{13}C-detected cross-correlated NMR relaxation, J. Magn. Reson. 354 (2023) 107539, https://doi.org/10.1016/j.jmr.2023.107539.
[108] G. Pasat, J.S. Zintsmaster, J.W. Peng, Direct ^{13}C-detection for carbonyl relaxation studies of protein dynamics, J. Magn. Reson. 193 (2008) 226–232, https://doi.org/10.1016/j.jmr.2008.05.003.
[109] W. Bermel, I. Bertini, I.C. Felli, R. Peruzzini, R. Pierattelli, Exclusively heteronuclear NMR experiments to obtain structural and dynamic information on proteins, ChemPhysChem 11 (2010) 689–695, https://doi.org/10.1002/cphc.200900772.
[110] I Bertini, I.C. Felli, L. Gonnelli, M.V. Vasantha Kumar, R. Pierattelli, High-resolution characterization of intrinsic disorder in proteins: expanding the suite of (13)C-detected NMR spectroscopy experiments to determine key observables, Chembiochem 12 (15) (2011) 2347–2352, https://doi.org/10.1002/cbic.201100406.
[111] R. Llinás, M. Sugimori, R.B. Silver, Microdomains of high calcium concentration in a presynaptic terminal, Science 256 (1992) 677–679, https://doi.org/10.1126/science.1350109 (1979).
[112] T. Wiegand, C. Gardiennet, R. Cadalbert, D. Lacabanne, B. Kunert, L. Terradot, A. Böckmann, B.H. Meier, Variability and conservation of structural domains in divide-and-conquer approaches, J. Biomol. NMR 65 (2016) 79–86, https://doi.org/10.1007/s10858-016-0039-8.
[113] Ē. Kupče, R. Freeman, B.K. John, Parallel acquisition of two-dimensional NMR spectra of several nuclear species, J. Am. Chem. Soc. 128 (2006) 9606–9607, https://doi.org/10.1021/ja0634876.
[114] S. Chakraborty, S. Paul, R.V. Hosur, Simultaneous acquisition of $^{13}C^{\alpha}$–^{15}N and ^{1}H–^{15}N–^{15}N sequential correlations in proteins: application of dual receivers in 3D HNN, J. Biomol. NMR 52 (2012) 5–10, https://doi.org/10.1007/s10858-011-9596-z.
[115] Ē. Kupče, L.E. Kay, Parallel acquisition of multi-dimensional spectra in protein NMR, J. Biomol. NMR 54 (2012) 1–7, https://doi.org/10.1007/s10858-012-9646-1.
[116] A. Viegas, T. Viennet, T.-Y. Yu, F. Schumann, W. Bermel, G. Wagner, M. Etzkorn, UTOPIA NMR: activating unexploited magnetization using interleaved low-gamma detection, J. Biomol. NMR 64 (2016) 9–15, https://doi.org/10.1007/s10858-015-0008-7.
[117] M. Schiavina, M.G. Murrali, L. Pontoriero, V. Sainati, R. Kümmerle, W. Bermel, R. Pierattelli, I.C. Felli, Taking simultaneous snapshots of intrinsically disordered proteins in action, Biophys. J 117 (2019) 46–55, https://doi.org/10.1016/j.bpj.2019.05.017.
[118] L. Pontoriero, M. Schiavina, S.M. Korn, A. Schlundt, R. Pierattelli, I.C. Felli, NMR reveals specific tracts within the intrinsically disordered regions of the SARS-CoV-2 nucleocapsid protein involved in RNA encountering, Biomolecules 12 (2022) 929, https://doi.org/10.3390/biom12070929.
[119] C. Chang, M.-H. Hou, C.-F. Chang, C.-D. Hsiao, T. Huang, The SARS coronavirus nucleocapsid protein—Forms and functions, Antivir. Res. 103 (2014) 39–50, https://doi.org/10.1016/j.antiviral.2013.12.009.
[120] C. Chang, S.-C. Sue, T. Yu, C.-M. Hsieh, C.-K. Tsai, Y.-C. Chiang, S. Lee, H. Hsiao, W.-J. Wu, W.-L. Chang, C.-H. Lin, T. Huang, Modular organization of SARS coronavirus nucleocapsid protein, J. Biomed. Sci. 13 (2006) 59–72, https://doi.org/10.1007/s11373-005-9035-9.
[121] C.-K. Chang, Y.-L. Hsu, Y.-H. Chang, F.-A. Chao, M.-C. Wu, Y.-S. Huang, C.-K. Hu, T.-H. Huang, Multiple nucleic acid binding sites and intrinsic disorder of severe acute respiratory syndrome coronavirus nucleocapsid protein: implications for ribonucleocapsid protein packaging, J. Virol. 83 (2009) 2255–2264, https://doi.org/10.1128/JVI.02001-08.
[122] A. Savastano, A. Ibáñez de Opakua, M. Rankovic, M. Zweckstetter, Nucleocapsid protein of SARS-CoV-2 phase separates into RNA-rich polymerase-containing condensates, Nat. Commun. 11 (2020) 6041, https://doi.org/10.1038/s41467-020-19843-1.
[123] H.M. Forsythe, J.R. Galvan, Z. Yu, S. Pinckney, P. Reardon, R.B. Cooley, P. Zhu, A. D. Rolland, J.S. Prell, E. Barbar, Multivalent binding of the partially disordered SARS-CoV-2 nucleocapsid phosphoprotein dimer to RNA, Biophys. J 120 (2021) 2890–2901, https://doi.org/10.1016/j.bpj.2021.03.023.
[124] L.M. Bessa, S. Guseva, A.R. Camacho-Zarco, N. Salvi, D. Maurin, L.M. Perez, M. Botova, A. Malki, M. Nanao, M.R. Jensen, R.W.H. Ruigrok, M. Blackledge, The intrinsically disordered SARS-CoV-2 nucleoprotein in dynamic complex with its viral partner nsp3a, Sci. Adv. 8 (2022), https://doi.org/10.1126/sciadv.abm4034.
[125] R. Giri, T. Bhardwaj, M. Shegane, B.R. Gehi, P. Kumar, K. Gadhave, C.J. Oldfield, V.N. Uversky, Understanding COVID-19 via comparative analysis of dark proteomes of SARS-CoV-2, human SARS and bat SARS-like coronaviruses, Cell. Mol. Life Sci. 78 (2021) 1655–1688, https://doi.org/10.1007/s00018-020-03603-x.
[126] M. Schiavina, L. Pontoriero, G. Tagliaferro, R. Pierattelli, I.C. Felli, The role of disordered regions in orchestrating the properties of multidomain proteins: the SARS-CoV-2 nucleocapsid protein and its interaction with enoxaparin, Biomolecules 12 (2022) 1302, https://doi.org/10.3390/biom12091302.
[127] D.C. Dinesh, D. Chalupska, J. Silhan, E. Koutna, R. Nencka, V. Veverka, E. Boura, Structural basis of RNA recognition by the SARS-CoV-2 nucleocapsid phosphoprotein, PLoS Pathog. 16 (2020) e1009100, https://doi.org/10.1371/journal.ppat.1009100.
[128] J.S. Redzic, E. Lee, A. Born, A. Issaian, M.A. Henen, P.J. Nichols, A. Blue, K. C. Hansen, A. D'Alessandro, B. Vögeli, E.Z. Eisenmesser, The inherent dynamics and interaction sites of the SARS-CoV-2 nucleocapsid N-terminal region, J. Mol. Biol. 433 (2021) 167108, https://doi.org/10.1016/j.jmb.2021.167108.
[129] I.P. Caruso, V. dos Santos Almeida, M.J. do Amaral, G.C. de Andrade, G.R. de Araújo, T.S. de Araújo, J.M. de Azevedo, G.M. Barbosa, L. Bartkevihi, P. R. Bezerra, K.M. dos Santos Cabral, I.O. de Lourenço, C.L.F. Malizia-Motta, A. de Luna Marques, N.C. Mebus-Antunes, T.C. Neves-Martins, J.M. de Sá, K. Sanches, M.C. Santana-Silva, A.A. Vasconcelos, M. da Silva Almeida, G.C. de Amorim, C. D. Anobom, A.T. Da Poian, F. Gomes-Neto, A.S. Pinheiro, F.C.L. Almeida, Insights into the specificity for the interaction of the promiscuous SARS-CoV-2 nucleocapsid protein N-terminal domain with deoxyribonucleic acids, Int. J. Biol. Macromol. 203 (2022) 466–480, https://doi.org/10.1016/j.ijbiomac.2022.01.121.
[130] S.M. Korn, K. Dhamotharan, C.M. Jeffries, A. Schlundt, The preference signature of the SARS-CoV-2 Nucleocapsid NTD for its 5′-genomic RNA elements, Nat. Commun. 14 (2023) 3331, https://doi.org/10.1038/s41467-023-38882-y.