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Methyl groups as NMR probes for biomolecular interactions

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Intermolecular interactions are indispensible for biological function. Here we discuss how novel NMR techniques can provide unique insights into the assembly, dynamics and regulation of biomolecular complexes. We focus on applications that exploit the methyl TROSY effect and show that methodological advances and biological insights go hand in hand. We envision that future methyl TROSY applications will continue to provide unique information regarding intermolecular interactions, even for very large eukaryotic protein complexes that are often highly asymmetric.

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Introduction

Protein interactions form the basis of biological function and elucidating the underlying atomic details is thus an important field of research in structural biology. The exquisite sensitivity of chemical shifts makes NMR spectroscopy an ideal tool to study intermolecular interactions [1]. However, this technique has long been limited to low-molecular weight systems. Recently developed methodology, such as methyl group labeling [2,3] and relaxation-optimized techniques (methyl TROSY [4]), have overcome these restrictions and enabled studies of numerous large, supra-molecular complexes. Here, we review selected methyl NMR studies that have provided import insights into the function and intermolecular interactions of high-molecular weight systems (Figure 1) and discuss the future potential of NMR for studying biomolecular interactions.

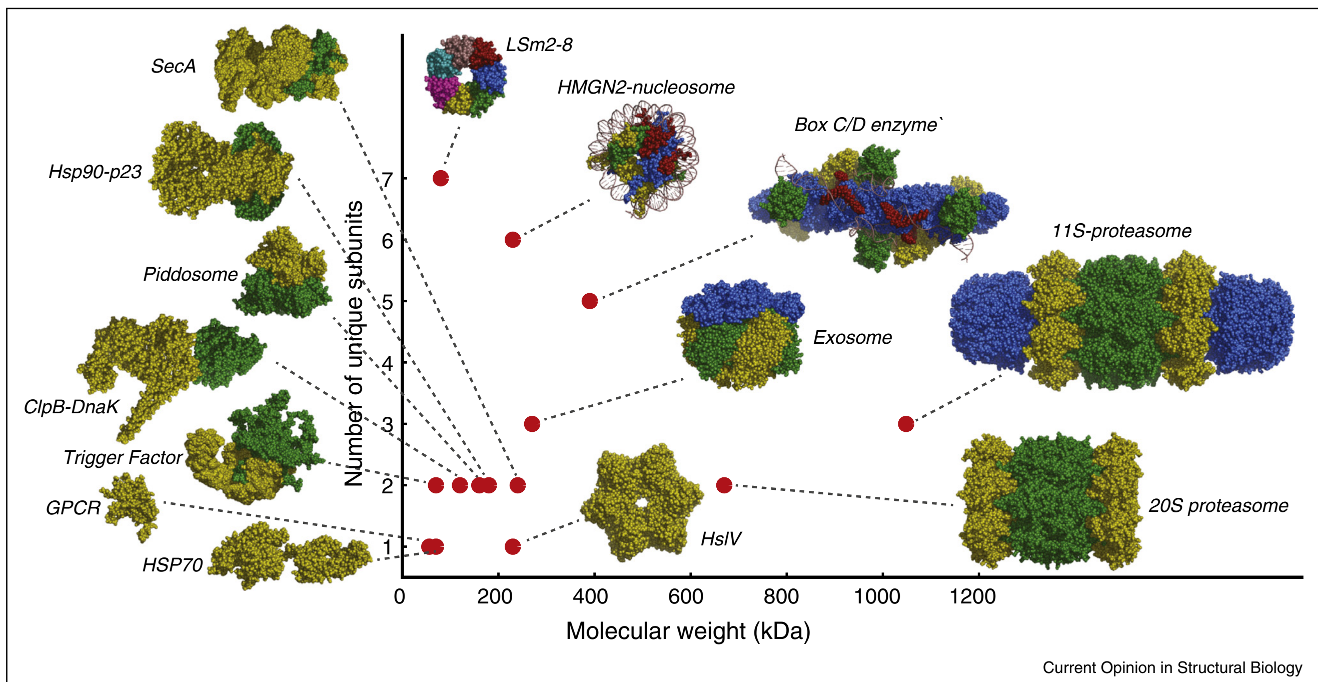
Methyl TROSY

Methyl NMR utilizes the advantageous spectroscopic properties of CH_3-groups [5] that arise from their three-fold symmetry and fast rotation around the connecting C–C bond. This results in highly favorable relaxation properties that are exploited in methyl TROSY HMQC experiments [4,6,7] that yield well-resolved and sensitive NMR spectra even for high-molecular weight systems. Introduction of NMR-active methyl groups can be achieved with metabolic precursors to label the Ile-δ1 or Leu-δ and Val-γ positions in proteins expressed in *Escherichia coli* [8–13]. However, labeled methyl groups can also be introduced in Thr [14,15], Ile-γ2 [16,17], Ala [18,19] and Met residues [20–24], and in Leu and Val in a residue-specific and stereospecific manner [25–28]. A labeling strategy that bypasses protein expression in NMR-active media and thus is applicable, for example, to proteins purified from insect cell lysates is the chemical modification of Cys [29] or Lys side chains [30–34] with NMR-active methyl groups. Of note, many of these labeling options can be combined [35•] to produce proteins exclusively labeled at the sites of interest. Here, we focus on the use of fully protonated methyl groups (as opposed to CHD_2 and CH_2D isotopomers) as these provide optimal sensitivity for large complexes [36].

Resonance assignment

The assignment of the NMR methyl resonances to specific residues in the protein is a prerequisite for extracting site-specific information regarding intermolecular interactions. Methyl group assignment can, however, be challenging and time-consuming. For intermediate size proteins (<~50 kDa), methyl resonances can be assigned through correlation with aliphatic and carbonyl resonances of the same residue [37–39]. For larger proteins, where traditional backbone assignment is not feasible, a number of complementary strategies have been proposed: A divide-and-conquer approach where a large protein complex is split into smaller parts that are then assigned traditionally [40,41] (Figure 2a); single-amino acid substitutions and subsequent comparison of WT and mutant HMQC spectra [41–43] (Figure 2b); comparison of methyl–methyl distances derived from NOE spectra with known crystal structures and, finally, site-specific introduction of paramagnetic tags leading to pseudocontact shifts (PCSs) [44] or paramagnetic relaxation enhancement (PRE) in spatially close residues [45]. In practice, combining several of

Figure 1



Summary of a number of biological assemblies that have been studied using methyl TROSY spectroscopy and that are mentioned in this review. Systems with increasing molecular weight (*x*-axis) and systems that contain multiple unique biological units (*y*-axis) are now accessible to detailed NMR studies. Novel methods (e.g. methionine scanning, LEGO NMR) will allow the study of complexes that are both large and highly asymmetric.

these strategies will increase the completeness of methyl group assignments [46].

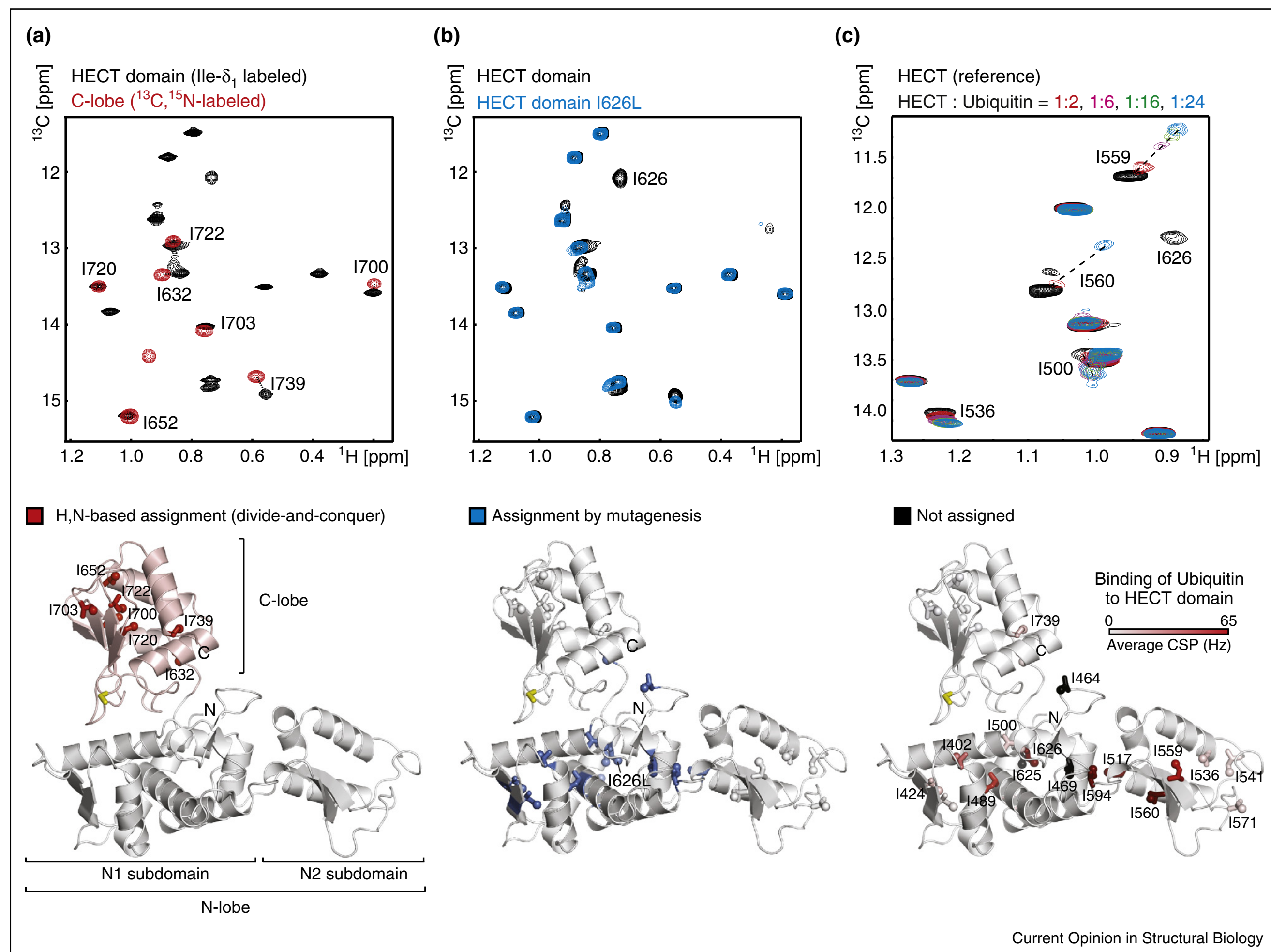
Identification of binding sites using CSPs: methyl fingerprinting

Most protein interaction surfaces are enriched in methyl groups [47]. Together with their favorable NMR properties, methyl groups are thus excellent probes for studying biomolecular interactions in high-molecular weight complexes [12,43]. In brief, methyl groups involved in biomolecular interactions will experience a change in their chemical environment and as a consequence undergo chemical shift perturbations (CSP) upon ligand binding (Figure 2c). Of note, CSPs can provide not only detailed qualitative, but also quantitative insights into interactions. As an example, recently it was shown that the mRNA decapping enzyme DcpS interacts with two substrates in a sequential manner, where the affinity for the first and second binding event differ by three orders of magnitude [48•].

The chaperone Hsp90 forms a 180 kDa dimer that can open and close to assist protein folding in an ATP-dependent manner. Each Hsp90 monomer contains an N-terminal, a middle and a C-terminal domain, a feature that has been exploited for resonance assignment of the Ile-δ1 methyl groups by a divide-and-conquer approach [49]. Addition of ATP resulted in methyl CSPs that were restricted to the N-terminal domain, whereas the Hsp90 activator p23 interacts with both the N-terminal and the middle domain. These results suggested that two p23 monomers bind co-operatively to one Hsp90 dimer to enforce a closed conformation [49]. An important function of Hsp90 is to counteract the aggregation of the protein Tau, a cause of neurodegeneration. Methyl CSP studies have revealed the molecular basis for the interaction between the Hsp90 chaperone and the Tau substrate that consists of a >100 Å long substrate-binding interface that allows for multiple low-affinity interactions [50••]. In sum, methyl TROSY studies have provided important insights into the mechanism of Hsp90 function. In addition, the Hsp70 chaperone that interacts with Hsp90, has been subject to detailed methyl TROSY studies [51].

The archaeal 20S proteasome is a 670 kDa-complex and one of the largest complexes, for which extensive Ile-δ1, Leu-δ and Val-γ methyl assignments have been obtained [40]. The proteasome degrades unfolded proteins and consists of four homo-heptameric rings that form a barrel-shaped structure. The two outer α-rings can each interact with a 150 kDa 11S regulatory particle (RP), resulting in a >1 MDa-complex. NMR titration experiments with a

Figure 2



Methyl resonance assignment strategies for binding site mapping. In the divide-and-conquer approach **(a)**, assignments of smaller subunits are obtained individually by traditional H,N-based NMR methods and then transferred to the full complex. **(b)** If specific subunits cannot be expressed individually, they can be assigned in a mutational approach. The obtained assignments can then be used to map observed CSPs onto a structural model **(c)**. Of note, the 45 kDa Smurf2 HECT domain is not amenable for H–N based experiments due to limited solubility of the protein, but can be studied using methyl TROSY techniques [41,80].

proteasome containing NMR-active α-rings revealed CSPs for a large number of methyl groups upon addition of 11S that could be used to map the 11S binding-surface and to extract an affinity constant [40]. Recently, methyl-NMR studies of proteasome-substrate interactions have revealed that unfolded proteins strongly associate with the inner walls of the antechamber proteasome ensuring that substrates remain unfolded before degradation [52].

Two methods that can complement CSP information are the analysis of differential relaxation of methyl groups in the presence of protonated versus deuterated binding partners [53] and cross saturation transfer (CST) measurements [54–57]. Both methods are unique in that they can discriminate between direct and indirect (i.e. allosteric) CSPs.

Beyond binding site mapping: NMR structures of large complexes

Methyl TROSY spectroscopy has opened the way for determining 3D structures of large protein complexes, such as the 50 kDa trigger factor-alkaline phosphatase complex [58] and the 204 kDa, homo-dimeric SecA [59]. SecA is an ATP-driven translocase that interacts with signal sequences of proteins destined for secretion [59]. Ile, Val, Leu, and Met methyl assignment of SecA was obtained by a divide-and-conquer approach. Combined with distance information gained from site-specific spin labeling (PREs), transfer-NOESY spectra and differential line-broadening experiments, this showed for the first time that signal peptides bind to a flexible and elongated groove in SecA inducing an α-helical conformation in the signal peptide. Furthermore, SecA was shown to undergo

a large conformational change in solution that might potentially be coupled to the protein translocation mechanism [59].

Kato *et al.* gained structural insights into the interaction between the nucleosome and the high mobility group nucleosomal (HMGN) protein [60] that regulates various chromatin functions, including transcription. The nucleosome is a 200 kDa complex containing two copies of each of the four histone proteins (H2A, H2B, H3 and H4) that are encircled by a long stretch of double-stranded DNA. Almost complete methyl group assignments were obtained for Ile, Leu and Val residues using NOESY spectra and point mutations. The assignments formed the basis for CSP and PRE experiments that provided distance information for structure determination of the nucleosome:HMGN complex. Importantly, the determined NMR structure agrees very well with mutational data and explains how the HMGN interferes with linker histone H1 interactions to regulate chromatin structure [60].

In another example, CSP and PRE data were combined to produce an atomic-resolution model of the 650 kDa ClpB–DnaK chaperone system that reactivates stress-damaged proteins trapped in an aggregated state in bacteria [61•]. The determined structural model has been validated with functional assays, and structure-based mutations interfering with the formation of the chaperone complex showed reduced disaggregation efficiency. This work provides unique insights into the disaggregation cycle, in which the ClpB–DnaK complex plays a crucial role in ClpB-mediated ATP turnover rate and substrate release.

Beyond NMR spectroscopy: hybrid methods

In many cases, NMR-data alone are insufficient to determine 3D structures of high-molecular weight systems and require additional information from complementary methods. Therefore, modeling approaches that integrate (sparse) NMR data with structures of isolated protein (domains), SAXS and SANS, electron microscopy and native mass spectrometry have been developed [62,63].

The 390 kDa box C/D enzyme is one of the most challenging systems whose structures have been determined using hybrid methods that include NMR [64••]. This enzyme methylates ribosomal RNA at the 2′-O-ribose, a step essential for both pre-rRNA processing and ribosome assembly. The complex consists of the L7Ae, Nop5 and fibrillarin proteins and a 72-base guide sRNA. Using known structures of individual building blocks, the structure of the box C/D ribonucleoprotein was solved based on CSPs, intermolecular PRE data, SAXS and contrast matching SANS data. The authors showed that this is a valid approach by using methyl CSPs and PRE experiments that recapitulated the stepwise formation of the complex. Interestingly, addition of substrate RNA resulted in large structural changes in the enzyme that were accompanied by numerous methyl resonances splitting into two signals with a 1:1 ratio. This demonstrated that only two of the four fibrillarin proteins in the complex interact with the substrate RNA, providing a structural basis for the sequential methylation mechanism [64••].

Beyond structures: interactions that modulate protein dynamics

Ligand interactions often involve biologically relevant conformational changes remote from the interaction interface [65]. These allosteric conformational changes can be subtle and thus difficult to identify in crystal structures. Methyl NMR studies have revealed allosteric effects in a number of high-molecular weight systems. For example, NMR binding studies have shown that interaction of the 11S RP with the proteasome α-rings not only induces an opening of the pore [66], but also concomitant CSPs at the active sites that are 50 Å remote from the entrance pore [67]. This long-range effect results from an allosteric pathway involving a subtle, rapid exchange of the proteasome between two conformations. RP binding to the entrance pore stabilizes one of these conformations and thereby enhances protein degradation. The drug chloroquin [68] interferes with a central part of this allosteric pathway and thereby inhibits proteasome activity. In another barrel-shaped protease, the 230 kDa HslV [69••], interaction with the activating HslU RP has been shown to dynamically couple to Thr methyl groups in the active site, revealing an allosteric pathway similar to the one observed in proteasome.

G-protein coupled receptors (GPCRs) are an important class of transmembrane proteins that are activated by light-sensitive compounds, odors, pheromones, hormones and neurotransmitters. Interestingly, GPCRs undergo conformational changes that correlate with activity. Numerous compounds that stabilize the active conformation (agonists) or inactive conformation (inverse agonists) have been identified. However, structural studies of GPCRs are hindered by the fact that they cannot be expressed in *E. coli*. In an elegant NMR study, the β2 adrenergic GPCR was produced in insect cells [70] and subsequently labeled with $^{13}CH_3$-methyl groups by chemical modification of lysine side chains. Interestingly, although both, agonists or inverse agonists, bind to the same site in the trans-membrane region, they cause distinct structural rearrangements at the remote extracellular surface providing a basis for their effects on GPCR activity.

Additional insights regarding the correlation between motions in the GPCR and small compounds were obtained through NMR analysis of methionine methyl groups [71]. To that end, Sf9 cells were grown in methionine-deficient media to which ^{13}C methyl-labeled methionine was added. To further improve spectral quality, Shimada and colleagues developed a deuteration method for insect cells [72•], which led to an impressive five-fold

increase in sensitivity of GPCR NMR spectra. Together, these data on the highly challenging GPCR systems have revealed a complex picture regarding the relation between conformational changes and ligand binding that modulates GPCR-mediated signal transduction.

Structural information invisible in crystal structures

High-resolution crystal structures are indispensible for the analysis of large protein complexes by NMR spectroscopy. One of the major future applications of methyl TROSY spectroscopy will be to complement these static structures with information regarding protein motions and interactions. Methyl TROSY studies of complexes of known structure have proven that unique and unexpected insights into protein assembly and dynamics can be gained in solution.

Binding events can take place through conformational selection with one of the binding partners sampling the structure of the bound state in the absence of a ligand. Recently, the archaea exosome core complex that functions in mRNA degradation has been shown to sample two states in solution, although only one conformation was visible in the high-resolution crystal structure [73•]. Interestingly, one of these conformations is important for the interaction with activator proteins, which indicates that binding events can be more complex than presumed based on static structures.

Furthermore, Driscoll and colleagues have shown in two independent studies that methyl TROSY spectroscopy, in combination with native mass spectrometery, can provide unique insights into the assembly of large complexes [74•,75,76]. In one example they studied the complex formation between that the death domains (DD) of CD95 and FADD [75]. A crystal structure of the complex containing four chains of each of the two proteins was solved at a acidic pH and revealed a symmetric complex. However, based on methyl TROSY experiments the two proteins form an asymmetric complex at neutral pH in solution with a 5:5 or 5:4 ratio of the two proteins.

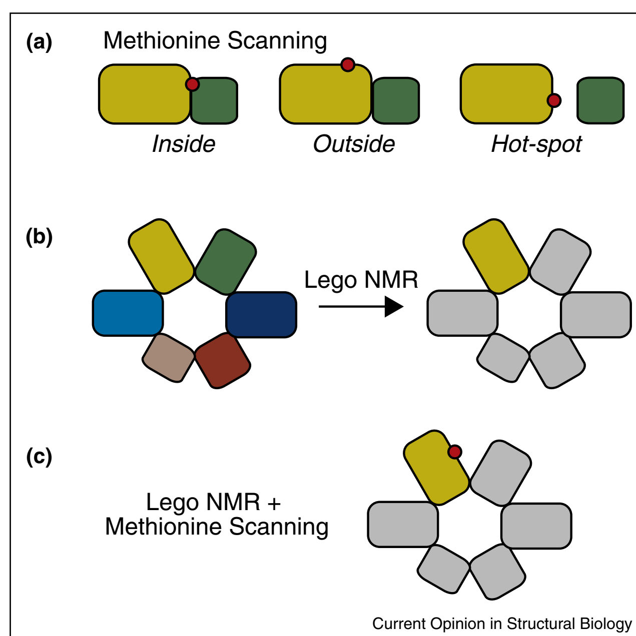
Recent advances in methyl NMR methodology

Relaxation rates in complexes larger than ~2 MDa are so fast that methyl TROSY spectra signals can be broadened beyond detection. In some cases, structural information on complex formation can nevertheless be obtained using dark-state exchange saturation transfer (DEST) methods [77,78••]. DEST and lifetime line-broadening experiments exploit that the relaxation properties of small proteins can be influenced by transient complex formation with very large, NMR-invisible ('dark') assemblies, such as aggregates. This effect was shown for Alzheimer's disease related amyloid β (Aβ) monomers that exchange with large (2–80 MDa) protofibrillar Aβ aggregates. Using methyl groups as NMR probes, insight into the interaction between hydrophobic side chains of the Aβ monomers and the protofibril surface were obtained [78••].

A bottleneck of methyl TROSY spectroscopy is that it is blind to protein regions devoid of methyl groups. This limitation can be overcome with the recently introduced methionine scanning method [79••], where single reporter methionine residues are introduced at specific sites of interest (Figure 3a). This results in the appearance of a novel NMR resonance that can be assigned instantaneously. In a second step, a ligand is added to the protein containing the introduced Met reporter. If CSPs are observed for the Met reporter, this residue must be inside the ligand-binding surface. If the Met reporter experiences no CSP, it resides either outside the binding interface, or the mutation interferes with the interaction. Importantly, native Met and Ile residues can distinguish between the latter two scenarios. Methionine scanning can thus determine on a per-residue basis whether an amino acid is located inside or outside a binding pocket, or whether it is crucial for interaction [79••]. Using methionine scanning, important insights into the binding mode and regulation of HECT-type ubiquitin ligases has been gained [80].

The vast majority of methyl NMR studies of large complexes exploited the favorable properties of highly symmetric assemblies. These include advantages for sample

Figure 3



Schematic representation of methionine scanning and LEGO NMR. **(a)** In methionine scanning a methionine reporter (red) can be identified to be inside or outside a binding interface or as being a hot-spot. **(b)** In LEGO NMR specific subunits of large asymmetric complexes can be made NMR active (colored) in an otherwise NMR inactive background (grey). **(c)** A combination of methionine scanning and LEGO NMR can visualize a specific region of interest in a large, asymmetric complex.

preparation as well as simplification of NMR spectra with a concomitant higher signal-to-noise ratio. Most eukaryotic complexes are, however, asymmetric and thus difficult to assess using NMR spectroscopy. To make such complexes amenable to NMR spectroscopy, a LEGO NMR approach [81••] was recently introduced (Figure 3b). In this method, a sequential co-expression protocol is exploited that allows for the preparation of highly asymmetric complexes that are NMR active in only a subset of the subunits. This significantly reduces spectral overlap and simplifies sample preparation. Using LEGO NMR, hetero-heptameric LSm complexes could be prepared that only contained single NMR active subunit, which allowed for the identification of residues that are involved in RNA binding [81••].

Conclusions

Methyl TROSY NMR spectroscopy allows for the study of biomolecular interactions in large molecular assemblies. Here, we reviewed unique biological insights that could only be gained from methyl NMR experiments. Given the recent success of this methodology, we anticipate that the number of applications will increase rapidly. In this regard, it is worth mentioning that LEGO NMR and methionine scanning are fully compatible strategies and their combination holds great promise for tackling fully asymmetric, high-molecular weight systems by methyl NMR (Figure 3c).

Conflict of interest statement

Nothing declared.

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