NMR spectroscopy of large molecules and multimolecular assemblies in solution

Gerhard Wider and Kurt Wüthrich

New strategies and technical advances in NMR spectroscopy and biochemical methods for isotope labeling have enabled solution NMR studies of biomacromolecular systems of 100 kDa and larger. Recent progress has been made, in particular, with techniques for sequential resonance assignments, novel approaches for the direct observation of hydrogen bonds in nucleic acids and proteins, and segmental isotope labeling.

Address
Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Hönggerberg, CH-8093 Zürich, Switzerland
Correspondence: Kurt Wüthrich

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Abbreviations

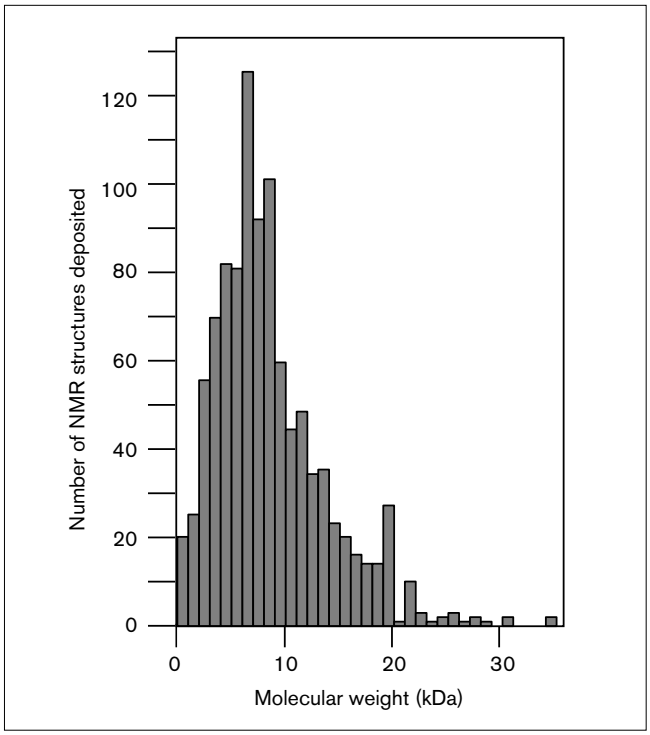
2D	two-dimensional
3D	three-dimensional
COSY	correlated spectroscopy
CRINEPT	cross-correlated relaxation-enhanced polarization transfer
CSA	chemical shift anisotropy
INEPT	insensitive nuclei enhanced by polarization transfer
NOE	nuclear Overhauser enhancement
NOESY	NOE spectroscopy
PDB	Protein Data Bank
SAR	structure/activity relationship
TROSY	transverse relaxation-optimized spectroscopy

Introduction

The Brookhaven Protein Data Bank (PDB) [1] contains more than 1000 NMR structures of proteins and nucleic acids. The size distribution of these deposits (Figure 1) shows that most of the NMR structures are in the 2 to 25 kDa range, with a maximum at 8 to 10 kDa and only few structures with molecular weights above 25 kDa [2]. The scarcity of NMR structures above 25 kDa reflects the increasing effort and cost involved in the structure determination, but it is also a manifestation of the fact that, in the field of macromolecular structure determination, NMR spectroscopy is largely used as a complement to X-ray crystallography [3]. The availability of solution NMR techniques for the study of larger molecular sizes is nonetheless of considerable interest [4–6], for example, for the structure determination of large proteins that resist crystallization or for investigations into intermolecular interactions in solution, including the delineation of intermolecular contacts in stable supramolecular structures [7•] and 'SAR (structure/activity relationship) by NMR' [8].

The foundations of NMR structural studies are high-quality NMR spectra recorded with good sensitivity and spectral resolution. With increasing molecular weight, these basic requirements are harder to achieve [9–12]. Limiting factors are low sensitivity and line broadening due to rapid transverse spin relaxation and extensive signal overlap due to the high complexity of the spectra. Recent advances have been achieved with both novel NMR techniques and new biochemical approaches. In particular, using the NMR techniques TROSY (transverse relaxation-optimized spectroscopy) [13] and CRINEPT (cross-correlated relaxation-enhanced polarization transfer) [14••] in combination with suitable isotope labeling schemes [10], the size limit for the observation of NMR signals in solution has been extended severalfold. In the following sections, a description of the use of these new techniques in the observation of high-quality NMR spectra of particles with molecular sizes beyond 100 kDa will be followed by a discussion of recent advances in both resonance assignment techniques and the collection of structural constraints.

Figure 1

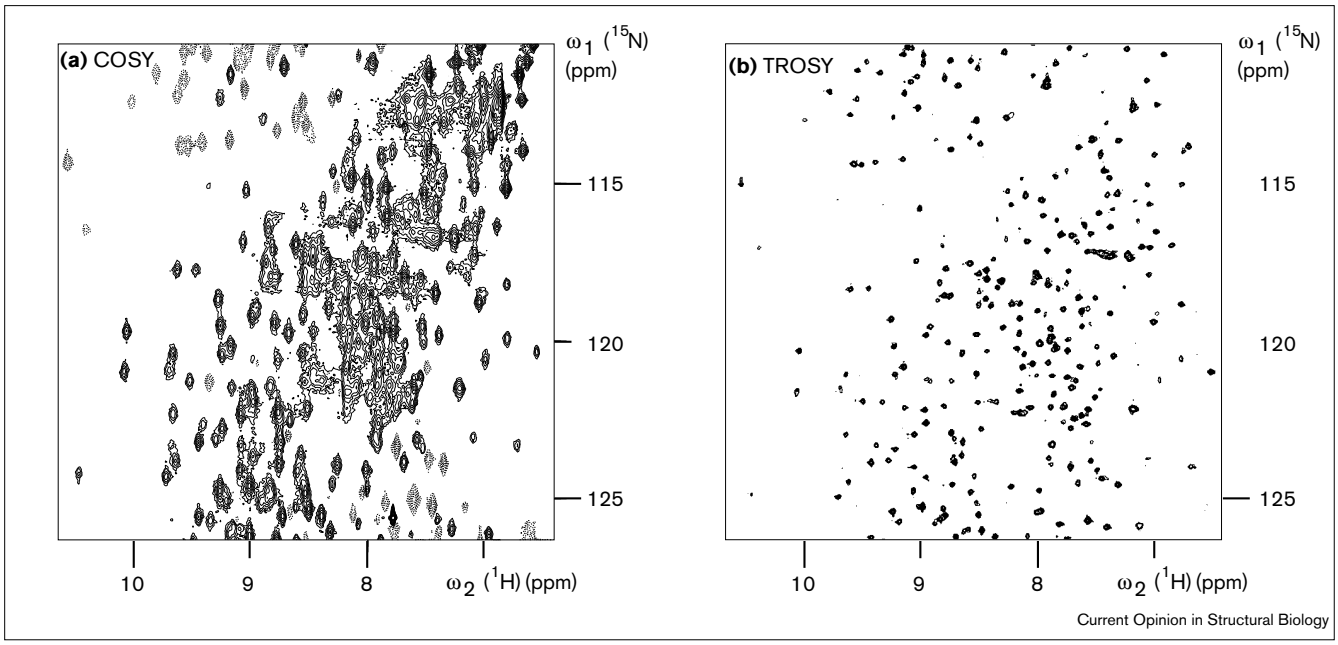


Molecular weight distribution of the NMR structures deposited in the Brookhaven PDB as of December 1997. The number of structures deposited is plotted versus the molecular weight. Reproduced with permission from [2].

NMR sensitivity and spectral resolution with large molecules in solution

During the past 20 years, the highest polarizing magnetic field available for high-resolution NMR has increased in several steps corresponding to proton resonance frequencies

Figure 2



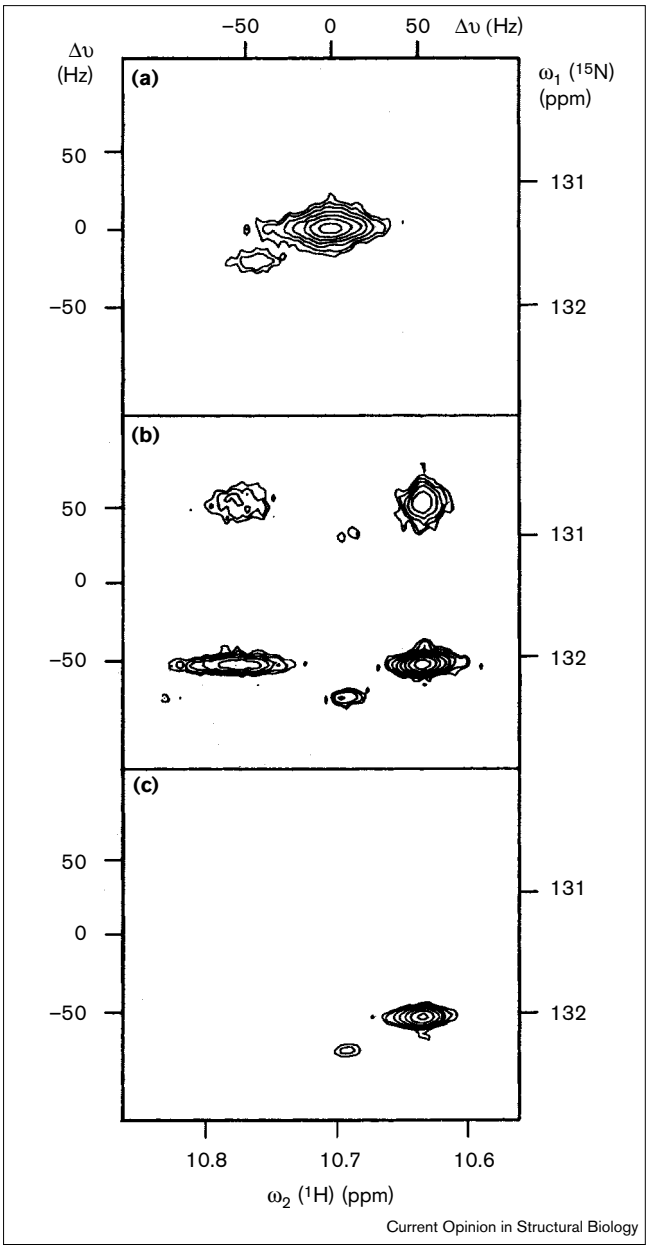
A comparison of the ^{15}N–^{1}H correlation spectra of a protein with a molecular weight of 45 kDa recorded using **(a)** conventional procedures ('COSY') and **(b)** TROSY. Both spectra were measured at a proton resonance frequency of 750 MHz, using a 0.8 mM sample of uniformly ^{15}N- and ^{2}H-labeled gyrase-45 from *Staphylococcus aureus* in water at 25°C and pH 8.6.

of 400, 500, 600, 750 and 800 MHz. Each of these advances in magnet design benefitted biomolecular NMR through improved intrinsic sensitivity and peak separation [5,9,12], but for commonly used heteronuclear experiments (e.g. [10,11,15]), the advantages of using higher magnetic fields were partly offset by field-dependent line broadening, which again causes loss of sensitivity and spectral resolution. Using the TROSY technique [13,16•,17•], line broadening at higher magnetic fields, which is a manifestation of increased transverse relaxation rates that also cause deterioration of the sensitivity in complex NMR experiments, has been largely suppressed. This results in improved spectral resolution (Figure 2), improved effective sensitivity or a combination of both, depending on the experiment used (see below).

At high magnetic fields, chemical shift anisotropy (CSA) of ^{1}H, ^{15}N and ^{13}C nuclei can be a significant source of transverse relaxation, in addition to the omnipresent relaxation as a result of dipole–dipole coupling. TROSY exploits constructive interference between dipole–dipole coupling and CSA relaxation, and actually uses CSA relaxation at higher fields to cancel field-independent dipolar relaxation. Technically, the TROSY approach is based on the following: in heteronuclear two-spin systems, such as ^{15}N–^{1}H in the amide groups of proteins or in nucleic acid bases, the NMR signal of each nucleus is split into two components by the scalar spin–spin coupling. In 2D correlation experiments, one therefore observes a four-line fine structure (Figure 3b). With the advent of modern multidimensional NMR, this four-line pattern routinely collapses into a single, centrally located line by broad-band decoupling techniques (Figure 3a) [12,15,18], with the expectation of obtaining a simplified spectrum and improved sensitivity. As has long been known (see [13] for a literature survey), however, the individual multiplet components have different transverse relaxation times and, hence, different line widths (Figure 3b), which are mixed by the aforementioned decoupling. For large molecules studied at higher magnetic fields, the differential line broadening of the individual multiplet components is very pronounced and results in the deterioration of the averaged signal (Figure 3a,b). Using the TROSY technique, the multiplet structure is not decoupled (Figure 4) and only the narrowest, most slowly relaxing line of each multiplet is retained (Figure 3c). The sensitivity loss as a result of the use of only one out of the four multiplet components is partially recovered both by using a new polarization transfer element that retains 50% (not only 25%) of the original proton polarization [17•] and by the fact that the absence of decoupling allows the use of the steady-state heteronuclear magnetization, in addition to the proton polarization [16•,17•]. Overall, when working with molecular sizes above 20 kDa, a superior ratio of peak height to noise is readily achieved with TROSY compared with corresponding conventional experiments.

The absence of any mixing of the individual NMR transitions that correspond to the four fine structure

Figure 3



Contour plots of a tryptophan indole ^{15}N–^{1}H cross-peak from different types of ^{15}N–^{1}H correlation spectra. **(a)** Conventional broad-band decoupled [^{15}N,^{1}H]-COSY spectrum. **(b)** Same as (a), without decoupling during the evolution and detection periods (see Figure 4). **(c)** [^{15}N,^{1}H]-TROSY spectrum.

components in Figure 3b enables a straightforward description of TROSY using single-transition basis operators [13]. Equation 1 describes the time evolution of the two single transitions, $<S_{12}^{\pm}>$ and $<S_{34}^{\pm}>$, of spin S, which represents ^{15}N in ^{15}N–^{1}H groups. Spin I represents ^{1}H and corresponding equations can be written for $<I_{13}^{\pm}>$ and $<I_{24}^{\pm}>$ [13]. The frequencies of the two S transitions, ω_S^{12} and ω_S^{34}, differ by the scalar coupling constant between I and S:

$$\frac{\mathrm{d}}{\mathrm{dt}}\begin{bmatrix}\langle S_{12}^{\pm}\rangle\\ \langle S_{34}^{\pm}\rangle\end{bmatrix} = -\begin{bmatrix}c_{11}\,c_{12}\\ c_{21}\,c_{22}\end{bmatrix}\bullet\begin{bmatrix}\langle S_{12}^{\pm}\rangle\\ \langle S_{34}^{\pm}\rangle\end{bmatrix}$$

$$c_{11} = \pm\,\mathrm{i}\omega_S^{12} + \mathrm{R}_{1212} + \frac{1}{\mathrm{T}_{2S}} + \frac{1}{2\mathrm{T}_{1I}}$$

$$c_{12} = 3\left(\mathrm{p}^2 - \delta_I{}^2\right)J(\omega_I) - \frac{1}{2\mathrm{T}_{1I}} \qquad (1)$$

$$c_{21} = 3\left(\mathrm{p}^2 - \delta_I{}^2\right)J(\omega_I) - \frac{1}{2\mathrm{T}_{1I}}$$

$$c_{22} = \pm\,\mathrm{i}\omega_S^{34} + \mathrm{R}_{3434} + \frac{1}{\mathrm{T}_{2S}} + \frac{1}{2\mathrm{T}_{1I}}$$

This equation contains two types of relaxation terms, that is, the transverse relaxation rates, R_{1212} and R_{3434}, of the spin S that arise from interactions within the ^{15}N–^{1}H moiety and $1/T_{2S}$ and $1/T_{1I}$, which account for the transverse relaxation of spin S and the longitudinal relaxation of spin I by all other mechanisms of relaxation, respectively. For studies of large spherical molecules at higher magnetic fields, the terms $3(p^2–\delta_I{}^2)\,J(\omega_I)$ vanish and the R terms can be approximated as:

$$R_{3434} = (\mathrm{p} + \delta_S)^2\, 4J(0) \qquad (2)$$

$$R_{1212} = (\mathrm{p} - \delta_S)^2\, 4J(0) \qquad (3)$$

where the spectral density at zero frequency is given by:

$$J(0) = 0.4\ \tau_c \qquad (4)$$

where τ_c is the rotational correlation time for a spherical molecule, p is the dipolar interaction energy and δ_S is the ^{15}N-CSA interaction. The terms $(p + \delta_S)^2$ and $(p–\delta_S)^2$ describe the energy that is available to induce relaxation, and $J(0)$ accounts for the Brownian molecular motions that modulate this energy in such a way that it can be exchanged with the spin system at the frequency needed to promote relaxation processes.

In TROSY-type experiments, transverse relaxation is quenched by minimizing the interaction energy that might induce relaxation at a given value of $J(0)$ [see below for possibilities of varying $J(0)$ to achieve reduced relaxation rates]. From Equations 2 and 3, it is clear that the two fine structure components of the ^{15}N doublet have different relaxation rates for nonvanishing δ_S values. As p is field-independent, whereas δ_S increases proportionally to the field strength, there is actually a 'magic field' at which R_{1212} will be near zero, while R_{3434} is very large. For ^{15}N–^{1}H groups, one approaches this situation at the highest presently available ^{1}H frequencies of 750 or 800 MHz, and a minimum of R_{1212} is expected in the ^{1}H frequency range of about 950 to 1050 MHz. The ideal situation for TROSY, whereby the transverse relaxation characterized by R_{1212} would be completely quenched (Equation 3), will not foreseeably be attained in practice, for two reasons. It

appears that δ_S is slightly variable depending on the residue type, sequence effects and, possibly, 3D structure effects (e.g. [19,20]), so there is no common 'magic field' for all of the residues in a protein. All the assumptions made about the CSA tensor used in deriving Equation 3, in particular concerning the collinearity of the ^{15}N–^{1}H bond and the principal tensor axis, are not strictly valid and, in practice, one expects some residual T_2 relaxation, even at the magic field for a given residue [5,13].

The relaxation of the spins in ^{15}N–^{1}H moieties by mechanisms other than the mutual interactions considered in Equations 2 and 3 is dominated by dipole–dipole coupling with 'remote' protons, that is, all the protons outside the ^{15}N–^{1}H group. This relaxation pathway cannot be influenced by TROSY. Therefore, an optimal TROSY effect is obtained with uniformly deuterated proteins, for which remote couplings are limited to nearby amide protons [10]. It is of practical importance that ^{15}N relaxation is only slightly affected by remote dipolar coupling, whereas the amide proton is subject to a large effect (see the discussion of TROSY-type triple-resonance experiments below).

For a spherical particle with radius r, the rotational correlation time (τ_c) of Equation 4 is given by:

$$\tau_c = \frac{4\pi r^3 \eta}{3kT} \qquad (5)$$

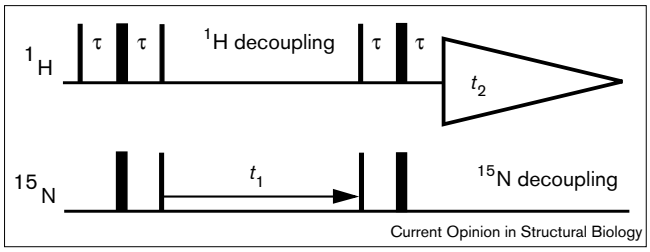
where η is the viscosity of the solvent, T is the temperature in degrees Kelvin and k is the Boltzmann constant. Equations 4 and 5 show that, for a given solvent at a given temperature, the value of $J(0)$ is entirely dictated by the molecular size. An original approach has recently been advanced, whereby reduced effective τ_c values were achieved for the protein ubiquitin when it was encapsulated in reverse micelles that were dissolved in a low-viscosity solvent [21•]. Based on this experimental observation, the authors predict that, compared with the tumbling of the free protein in water, the tumbling rate of a 100 kDa protein could be increased three- to sixfold when using an inverse micelle system in a nonaqueous solvent.

TROSY and CRINEPT in [^{15}N,^{1}H] correlation experiments

A simple scheme for the application of the TROSY principle is the ^{15}N–^{1}H correlation experiment, which has already been used as an illustration in the preceding section (Figures 2 and 3). For brevity, we refer to this basic experiment as 2D [^{15}N,^{1}H]-TROSY. For large proteins, 2D [^{15}N,^{1}H]-TROSY spectra show narrower lines and higher sensitivity compared with conventional 2D [^{15}N,^{1}H]-COSY (correlated spectroscopy) (Figure 2).

The 2D [^{15}N,^{1}H]-TROSY experiment affords a 'fingerprint' of the protein that is highly sensitive to changes in the protein environment. It thus presents a many-parameter NMR probe for studying intermolecular interactions of the protein. Such chemical shift mapping of the amide groups forms the basis of SAR by NMR [8], which is a highly promising screening method for discovering high-affinity ligands for macromolecular receptors. With the improved quality of 2D [^{15}N,^{1}H]-TROSY spectra (Figure 2), this technique will now be applicable to much bigger receptor molecules. A comparison of the fingerprints of individual proteins in the free state and incorporated in supramolecular structures enables the mapping of the intermolecular contacts of these proteins in the higher order structures [7•]. 2D [^{15}N,^{1}H]-TROSY is, in this way, applicable to studies of proteins in supramolecular structures that have accrued molecular weights of 100,000 or larger and, thus, the introduction of the TROSY technique opens a wide field of new applications for solution NMR.

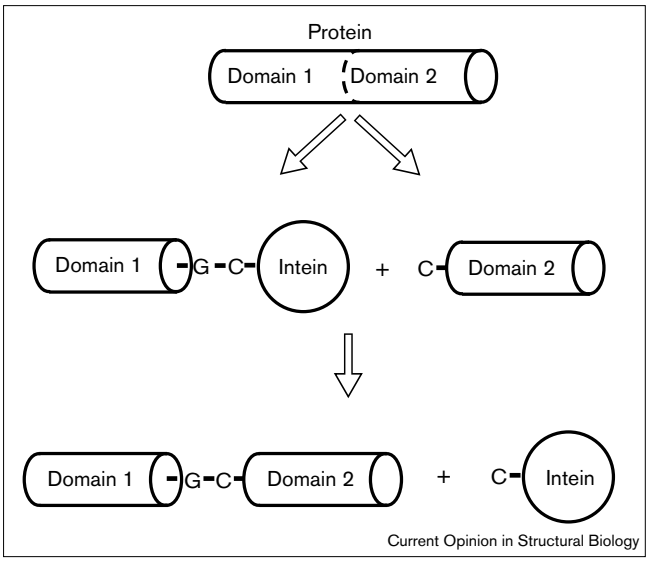
Figure 4



The basic elements of the experimental scheme for 2D ^{15}N–^{1}H correlation spectroscopy. On the lines marked ^{1}H and ^{15}N, narrow and wide bars stand for nonselective 90° and 180° radio-frequency pulses, respectively. The delay τ is set to 2.7 ms. t_1 and t_2 are the evolution and detection periods, respectively. Without decoupling during t_1 and t_2, a spectrum of the type shown in Figure 3b is obtained. A TROSY spectrum consisting of the sharp component in the lower right of Figure 3b is obtained by the inclusion of additional pulses and delays, and by proper coherence pathway selection using phase cycling and pulsed-field gradients [13,17•].

In heteronuclear NMR experiments, magnetization is transferred between the different types of nuclei via spin–spin couplings in so-called insensitive nuclei enhanced by polarization transfers (INEPTs) [12,15]. Thereby, the time periods required for the INEPT transfers are comparable to the evolution times in multidimensional NMR experiments. As TROSY is applied only during the evolution and detection periods [13], transverse relaxation during INEPT transfers may become a limiting factor for very large particles, that is, for molecular weights beyond 100,000. The new CRINEPT technique overcomes this limitation by combining INEPT with cross-correlated relaxation-induced polarization transfer (CRIPT) [14••]. CRIPT is proportional to the size of the molecule, so it becomes a highly efficient transfer mechanism for molecular sizes above 200 kDa in aqueous solution at ambient temperature, yielding a two- to threefold gain in sensitivity, in addition to the sensitivity gain due to TROSY [13,22••,23].

Figure 5



Schematic drawing of the procedure used by Xu *et al.* [27••] for the *in vitro* ligation of two separately expressed, folded protein domains into one polypeptide chain, enabling the selective labeling of individual domains in intact multidomain proteins. Domain 1 is expressed as a fusion protein with an intein, the presence of which allows the chemical ligation of the two domains using mild chemicals.

New isotope labeling strategies for high molecular weight structures

TROSY and CRINEPT offer avenues for the recording of high-quality solution NMR spectra of particles with molecular weights well beyond 100 kDa. Many large systems may give spectra of manageable complexity, for example, as a result of either symmetry in uniformly labeled oligomeric proteins or selective isotope labeling of individual molecules in supramolecular structures, such as labeled proteins in protein–nucleic acid complexes or in association with unlabeled lipids and detergents. For proteins without inherent symmetry, the complexity of the NMR spectra increases with increasing size, which may prevent a detailed analysis, in spite of the favorable line shapes obtained with TROSY. New biochemical approaches, however, now promise to reduce the spectral complexity to a manageable level, even for large proteins without inherent symmetry, using segmental isotope labeling [24,25••,26,27••].

Segmental isotope labeling for protein NMR spectroscopy was first achieved using *trans*-splicing to combine protein domains [24,25••]. *Trans*-splicing is a variation of a natural linking process that occurs during the biosynthesis of some proteins, for which the nascent form includes 'intein' peptide segments that are spliced out of the polypeptide chain in a post-translational process. The method may introduce some foreign 'linker amino acids' at the location where two polypeptide fragments are combined. As an alternative, chemical ligation [26,27••] has been used to assemble proteins from two or more independently generated fragments. For the best yield, a Gly–Cys dipeptide should form the junction, which may be achieved by amino acid replacements in the wild-type protein. The protein fragments are separately cloned and expressed in bacteria, and can thus be obtained with and without isotope labeling. Using intermediate linkage to an intein moiety, which can be obtained by expressing one domain as a fusion protein with the intein (Figure 5), they are then chemically linked to obtain the intact protein. The solution structure of the labeled domain can be determined by NMR spectroscopy without interference from NMR lines originating from the other domains. In multiple experiments with isotope labeling of different individual protein domains, the intact protein, which would otherwise produce very crowded NMR spectra, can thus be made accessible to structural studies by solution NMR in spite of its large size, provided that TROSY and, possibly, CRINEPT are implemented in the NMR experiments used.

Resonance assignments in big molecules

Most of the information contained in the high-quality NMR spectra obtained using TROSY and CRINEPT can only be extracted and exploited on the basis of individual resonance assignments and it is therefore of keen interest to develop NMR techniques that can provide this information for very large proteins. Assignment both by sequential nuclear Overhauser enhancements (NOEs) [28] with $^{2}H,^{15}N$- or ^{15}N-labeled proteins and by triple-resonance experiments with $^{2}H,^{13}C,^{15}N$- or $^{13}C,^{15}N$-labeled proteins [10,12,15] appears to be amenable to this task, but most of the work so far has been carried out on the adaptation of triple-resonance experiments for assignment in large molecules. In triple-resonance experiments with large proteins, the implementation of TROSY can prevent fast transverse relaxation of ^{15}N during extended time periods with transverse ^{15}N magnetization, as well as preventing amide proton line broadening. As triple-resonance experiments of interest usually contain a so-called 'constant time evolution period' for ^{15}N [12,15], the implementation of TROSY does not affect the ^{15}N line shape, but yields important gains in sensitivity, which are dependent on the size of the molecule studied. For a protein molecular weight of about 25,000, a two- to threefold gain in sensitivity can be expected [22••,23], but for the individual ^{15}N–^{1}H groups in a $^{2}H,^{13}C,^{15}N$-labeled protein with a molecular weight of 110,000, we registered sensitivity gains of one to two orders of magnitude [29]. This outstanding improvement now enables the sequential assignment of proteins with molecular weights above 100,000. On top of this 'basic TROSY effect', the modified triple-resonance experiments can be further optimized, for example, for improved spectral resolution through the use of ^{13}C constant time evolution [30] and through the introduction of a variety of sensitivity enhancement schemes that have, in part, also been used in [$^{15}N,^{1}H$]-TROSY and [$^{13}C,^{1}H$]-TROSY [31,32,33•,34–36,37•]. For very large molecules, the implementation of CRINEPT [14••] for part of the magnetization transfers, in addition to TROSY

during evolution and detection, should further extend applications of triple-resonance experiments.

Outlook to structural data on large biomacromolecular systems

A first level of structural information can be based on the recording of [^{15}N,^{1}H]-TROSY spectra and sequence-specific resonance assignments for polypeptide backbones. A comparison of these data for globular proteins free in solution and incorporated into bimolecular complexes or higher order aggregates can yield a map of intermolecular contacts in supramolecular structures, either along the amino acid sequence or, for example, when an X-ray crystal structure of the protein is available, on the surface of the assigned protein [7•]. The same foundations will support the extension of SAR by NMR [8] to large receptors, including membrane-standing proteins solubilized in detergent or lipid micelles. As described in some detail in the preceding sections, NMR techniques for these types of studies on structures with molecular weight beyond 100 kDa are now available and greatly expand the potential of solution NMR spectroscopy in studies of intermolecular interactions with biological macromolecules.

A second level of structural data can also be accessed with currently available techniques, that is, the *de novo* determination of secondary polypeptide structure based on the chemical shifts obtained from sequence-specific backbone resonance assignments [38,39]. Further data on the secondary structures of large systems can be obtained using [^{15}N,^{1}H]-TROSY for studies of amide proton exchange [28]. The implementation of [^{15}N,^{1}H]-TROSY routines into 3D ^{15}N-resolved NOE spectroscopy (NOESY) schemes [40,41••] will enable the collection of NOE distance constraints and allow the further characterization of regular secondary structures, in particular when using experimental schemes with transverse relaxation optimization in both ^{1}H dimensions, in addition to the ^{15}N dimension [41••].

The *de novo* 3D structure determination of proteins in large molecular systems will rely on the aforementioned TROSY-enhanced NOESY experiments to obtain at least a minimal set of distance constraints to define the global polypeptide fold [28]. As was discussed extensively in a review previously published in this journal [10], the density of the NOE constraints in the large systems considered here may be limited by a variety of factors, in particular by the requirement for extensive deuterium labeling. Therefore, supplementary conformational constraints will most probably be needed to obtain good quality structures. Where applicable, the measurement of residual dipolar couplings [42–45,46•,47•], which has been recently reviewed in this journal [48], may contribute to the refinement of large structures. Additional novel conformational restraints may result from the use of improved NMR techniques, as exemplified by the observation of scalar couplings across hydrogen bonds (^{h}J) in RNA [49••], DNA [50••,51] and proteins [52••,53••]. These new parameters, which became accessible with the improved resolution resulting from TROSY, promise to revolutionize NMR structural studies of nucleic acids. The ^{h}J couplings reported so far for proteins are about an order of magnitude smaller than those for nucleic acids, that is, about 1 Hz, which may limit its practical use, in spite of its obvious potential in novel extensions of NMR structural studies of proteins and their intermolecular interactions.

Conclusions

Solution NMR spectroscopy of biological molecular systems of 100 kDa and beyond is now a reality. Even if practical applications were limited to correlation experiments for chemical shift measurement and triple-resonance schemes for resonance assignment, an interesting role for NMR spectroscopy in studies of supramolecular structures would be assured. Most certainly, NMR of large systems will go beyond this basic level. With the immediate advent of spectrometers operating at 900 MHz and the prospect of further advances in selective isotope labeling, as well as in NMR techniques, we look forward to continued excitement in biomolecular NMR studies in the immediate and the more distant future.

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References and recommended reading

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

- • of special interest
- •• of outstanding interest

1. Bernstein FC, Koetzle TF, Williams GJB, Meyer EF, Brice MD, Rodgers JR, Kennard O, Shimanoucghi T: **The protein data bank: a computer-based archival file for macromolecular structures.** *J Mol Biol* 1977, **112**:535-542.

2. Güntert P: **Structure calculation of biological macromolecules from NMR data.** *Q Rev Biophys* 1998, **31**:145-237.

3. Wüthrich K: **NMR – this other method for protein and nucleic acid structure determination.** *Acta Crystallogr D* 1995, **51**:249-270.

4. Borman S: **Scaling up protein NMR.** *Chem Eng News* 1999, February 15:65-67.

5. Wüthrich K: **The second decade – into the third millennium.** *Nat Struct Biol* 1998, **5**:492-495.

6. Yu H: **Extending the size limit of protein nuclear magnetic resonance.** *Proc Natl Acad Sci USA* 1999, **96**:332-334.

7. • Pellecchia M, Sebbel P, Hermanns U, Wüthrich K, Glockshuber R: **Pilus chaperone FimC-adhesin FimH interactions mapped by TROSY-NMR.** *Nat Struct Biol* 1999, **6**:336–339.

First application of transverse relaxation-optimized spectroscopy (TROSY) chemical shift mapping of intermolecular contacts between two proteins in a complex of molecular weight 51 kDa, which could previously not be achieved with conventional NMR methods. This use of TROSY opens a new avenue for NMR studies of supramolecular structures with high molecular weights.

8. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW: **Discovering high-affinity ligands for proteins: SAR by NMR.** *Science* 1996, **274**:1531-1534.

9. Wagner G: **Prospects for NMR of large proteins.** *J Biomol NMR* 1993, **3**:375-385.

10. Kay LE, Gardner KH: **Solution NMR spectroscopy beyond 25 kDa.** *Curr Opin Struct Biol* 1997, **7**:722-731.

11. Clore GM, Gronenborn AM: **NMR structure determination of proteins and protein complexes larger than 20 kDa.** *Curr Opin Chem Biol* 1998, **2**:564-570.

12. Wider G: **Technical aspects of NMR spectroscopy with biological macromolecules and studies of hydration in solution.** *Prog Nucl Magn Reson Spectros* 1998, **32**:193-275.

13. Pervushin K, Riek R, Wider G, Wüthrich K: **Attenuated T_2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution.** *Proc Natl Acad Sci USA* 1997, **94**:12366-12371.

14. •• Riek R, Wider G, Pervushin K, Wüthrich K: **Polarization transfer by cross-correlated relaxation in solution NMR with very large proteins.** *Proc Natl Acad Sci USA* 1999, **96**:4918-4923.
For structures with molecular weights beyond 100 kDa, transverse relaxation during magnetization transfers using scalar spin–spin couplings becomes a limiting factor. Cross-correlated relaxation-enhanced polarization transfer (CRINEPT) makes use of the fact that the rate of polarization transfer by cross-correlated relaxation is proportional to the rotational correlation time and thus generates efficient transfer for solution NMR spectroscopy of very high molecular weight structures.

15. Bax A, Grzesiek S: **Methodological advances in protein NMR.** *Accounts Chem Res* 1993, **26**:131-138.

16. • Pervushin K, Riek R, Wider G, Wüthrich K: **Transverse relaxation optimized spectroscopy (TROSY) for NMR studies of aromatic spin systems in ^{13}C-labeled proteins.** *J Am Chem Soc* 1998, **120**:6394-6400.
TROSY yields four- to tenfold improved sensitivity for multidimensional NMR experiments on aromatic spin systems in ^{13}C-labeled proteins. This paper gives details of the use of the heavy-atom, steady-state magnetization, in addition to the ^{1}H polarization.

17. • Pervushin K, Wider G, Wüthrich K: **Single transition-to-single transition polarization transfer (ST2-PT) in (^{15}N,^{1}H)-TROSY.** *J Biomol NMR* 1998, **12**:345-348.
With single transition-to-single transition polarization transfer, the magnetization of the slowly relaxing ^{15}N transition can be completely transferred to the slowly relaxing ^{1}H transition, which affords a $\sqrt{2}$ sensitivity enhancement in transverse relaxation-optimized spectroscopy (TROSY) pulse sequences.

18. Freeman R, Kupce E: **Decoupling: theory and practice I. Current methods and recent concepts.** *NMR Biomed* 1997, **10**:372-380.

19. Fushman D, Tjandra N, Cowburn D: **Direct measurement of ^{15}N chemical shift anisotropy in solution.** *J Am Chem Soc* 1998, **120**:10947-10952.

20. Tessari M, Mulder FAA, Boelens R, Vuister GW: **Determination of amide proton CSA in ^{15}N-labeled proteins using ^{1}H CSA/^{15}N–^{1}H dipolar and ^{15}N CSA/^{15}N–^{1}H dipolar cross-correlation rates.** *J Magn Resonance* 1997, **127**:128-133.

21. • Wand AJ, Ehrhardt MR, Flynn PF: **High-resolution NMR of encapsulated proteins dissolved in low-viscosity fluids.** *Proc Natl Acad Sci USA* 1998, **95**:15299-15302.
In a novel approach to reducing transverse relaxation, the protein ubiquitin was introduced into a reversed micelle, which, in turn, was dissolved in a low viscosity solvent that enables rapid rotational movement of the micelles. For large proteins, the mobility of the micelles could potentially be significantly faster than that of the free protein in water.

22. •• Salzmann M, Pervushin K, Wider G, Senn H, Wüthrich K: **TROSY in triple-resonance experiments: new perspectives for sequential NMR assignment of large proteins.** *Proc Natl Acad Sci USA* 1998, **95**:13585-13590.
With the implementation of transverse relaxation-optimized spectroscopy (TROSY) elements in triple-resonance experiments, a severalfold increase in sensitivity can be obtained for proteins with molecular weights around 25 kDa. Sensitivity enhancements of one to two orders of magnitude are predicted for proteins of 100 kDa or somewhat larger.

23. Salzmann M, Wider G, Pervushin K, Senn H, Wüthrich K: **TROSY type triple-resonance experiments for sequential NMR assignments of large proteins.** *J Am Chem Soc* 1999, **121**:844-848.

24. Yamazaki T, Otomo T, Oda N, Kyogoku Y, Uegaki K, Ito N, Ishino Y, Nakamura H: **Segmental isotope labeling for protein NMR using peptide splicing.** *J Am Chem Soc* 1998, **120**:5591-5592.

25. •• Otomo T, Teruya K, Uegaki K, Yamazaki T, Kyogoku Y: **Improved segmental isotope labeling of proteins and application to a larger protein.** *J Biolmol NMR* 1999, **14**:105-114.
Improved and more versatile techniques for the segmental isotope labeling of proteins using *trans*-splicing are introduced.

26. Muir TW, Sondhi D, Cole PA: **Expressed protein ligation: a general method for protein engineering.** *Proc Natl Acad Sci USA* 1998, **95**:6705-6710.

27. •• Xu R, Ayers B, Cowburn D, Muir TW: **Chemical ligation of folded recombinant proteins: segmental isotopic labeling of domains for NMR studies.** *Proc Natl Acad Sci USA* 1999, **96**:388-393.
Improved and more versatile techniques for the segmental isotope labeling of proteins using chemical semisynthesis are described.

28. Wüthrich K: **NMR of proteins and nucleic acids.** New York: Wiley; 1986.

29. Salzmann M: **The use of TROSY for the sequential NMR assignment of very large proteins.** Zürich: ETH Zürich; no. 13189.

30. Salzmann M, Pervushin K, Wider G, Senn H, Wüthrich K: **[^{13}C]-constant-time [^{15}N,^{1}H]-TROSY-HNCA for sequential assignments of large proteins.** *J Biomol NMR* 1999, **14**:85-88.

31. Weigelt J: **Single scan, sensitivity- and gradient-enhanced TROSY for multidimensional NMR experiments.** *J Am Chem Soc* 1998, **120**:10778-10779.

32. Czisch M, Boelens R: **Sensitivity enhancement in the TROSY experiment.** *J Mag Resonance* 1998, **134**:158-160.

33. • Brutscher B, Boisbouvier J, Pardi A, Marion D, Simorre JP: **Improved sensitivity and resolution in ^{1}H–^{13}C NMR experiments of RNA.** *J Am Chem Soc* 1998, **120**:11845-11851.
[^{13}C,^{1}H]-correlation experiments with improved sensitivity and resolution were obtained by implementing transverse relaxation-optimization spectroscopy (TROSY) and a standard sensitivity enhancement routine. Applications using isotope-labeled RNA yielded spectra of impressive quality.

34. Meissner A, Schulte-Herbrüggen T, Briand J, Sørensen OW: **Double spin-state-selective coherence transfer. Application for two-dimensional selection of multiplet components with long transverse relaxation times.** *Mol Phys* 1998, **95**:1137-1142.

35. Rance M, Loria JP, Palmer AG: **Sensitivity improvement of transverse relaxation- optimized spectroscopy.** *J Magn Resonance* 1999, **136**:92-101.

36. Yang DW, Kay LE: **Improved (^{H}N)-^{1}H-detected triple resonance TROSY-based experiments.** *J Biomol NMR* 1999, **13**:3-10.

37. • Yang DW, Kay LE: **TROSY triple-resonance four-dimensional NMR spectroscopy of a 46 ns tumbling protein.** *J Am Chem Soc* 1999, **121**:2571-2575.
Improved sensitivity and improved resolution in the ^{1}H dimension were achieved by the implementation of transverse relaxation-optimization spectroscopy (TROSY) and a standard sensitivity enhancement routine in triple-resonance experiments. Four-dimensional spectra of a 46 kDa protein were presented as illustrations for the use of this approach.

38. Spera S, Bax A: **Empirical correlation between protein backbone conformation and C^{α} and C^{β} ^{13}C nuclear-magnetic-resonance chemical-shifts.** *J Am Chem Soc* 1991, **113**:5490-5492.

39. Wishart DS, Sykes BD: **The ^{13}C chemical-shift index - a simple method for the identification of protein secondary structure using ^{13}C chemical-shift data.** *J Biomol NMR* 1994, **4**:171-180.

40. Zhu G, Kong XM, Sze KH: **Gradient and sensitivity enhancement of 2D TROSY with water flip-back, 3D NOESY-TROSY and TOCSY-TROSY experiments.** *J Biomol NMR* 1999, **13**:77-81.

41. •• Pervushin K, Wider G, Riek R, Wüthrich K: **The 3D NOESY-[^{1}H,^{15}N]-ZQ-TROSY NMR experiment with diagonal peak suppression.** *Proc Natl Acad Sci USA* 1999, **96**:9607-9612.
A novel TROSY approach for correlating the ^{15}N and ^{1}H chemical shifts, where zero-quantum (ZQ) coherence is generated and the remote cross-correlation between the ^{1}H and ^{15}N chemical shift anisotropy interactions is used to reduce transverse relaxation during ^{15}N evolution, is applied with 3D ^{15}N-resolved NOESY. The resulting optimization of the transverse relaxation in all three frequency dimensions and the suppression of the diagonal autorelaxation peaks enable resonance assignments by sequential NOEs and the collection of structural constraints in large ^{15}N- or ^{2}H,^{15}N-labeled proteins.

42. Prestegard JH: **New techniques in structural NMR – anisotropic interactions.** *Nat Struct Biol* 1998, **5**:517-522.

43. Gayathra C, Bothner-By AA, van Zijl PCM, MacLean C: **Dipolar magnetic field effects in NMR spectroscopy.** *Chem Phys Lett* 1982, **87**:192-196.

44. Tolman JR, Flanagan JM, Kennedy MA, Prestegard JH: **Nuclear magnetic dipole interactions in field-oriented proteins - information for structure determination in solution.** *Proc Natl Acad Sci USA* 1995, **92**:9279-9283.

45. Tjandra N, Bax A: **Direct measurement of distances and angles in biomolecules by NMR in dilute liquid crystalline medium.** *Science* 1997, **278**:1111-1114.

46. • Hansen MR, Mueller L, Pardi A: **Tunable alignment of macromolecules by filamentous phage yields dipolar coupling interactions.** *Nat Struct Biol* 1998, **5**:1065-1074.
Filamentous phage is introduced as an alternative to dilute liquid-crystal media for the alignment of macromolecules in studies of residual dipole–dipole couplings.

47. • Koenig BW, Hu JS, Ottiger M, Bose S, Hendler RW, Bax A: **NMR measurement of dipolar couplings in proteins aligned by transient binding to purple membrane fragments.** *J Am Chem Soc* 1999, **121**:1385-1386.
Purple membrane fragments are introduced as an alternative medium for the alignment of macromolecules in studies of residual dipole–dipole couplings.

48. Dötsch V, Wagner G: **New approaches to structure determination by NMR spectroscopy.** *Curr Opin Struct Biol* 1998, **8**:619-623.

49. •• Dingley AJ, Grzesiek S: **Direct observation of hydrogen bonds in nucleic acid base pairs by internucleotide $^{2}J_{NN}$ couplings.** *J Am Chem Soc* 1998, **120**:8293-8297.
Two-bond ^{15}N–^{15}N scalar couplings across the hydrogen bonds in RNA base pairs are reported, with values of about 7 Hz. These new NMR parameters have great potential in structural studies of RNAs and are of keen interest with regard to the theory of hydrogen bonds and scalar couplings.

50. •• Pervushin K, Ono A, Fernandez C, Szyperski T, Kainosho M, Wüthrich K: **NMR scalar couplings across Watson-Crick base pair hydrogen bonds in DNA observed by transverse relaxation-optimized spectroscopy.** *Proc Natl Acad Sci USA* 1998, **95**:14147-14151.
Two-bond ^{15}N–^{15}N and one-bond ^{1}H–^{15}N scalar couplings across the hydrogen bonds of Watson–Crick base pairs in DNA were observed in a ^{15}N,^{13}C-labeled DNA duplex, with values of about 7 Hz and 3 Hz, respectively. These new NMR parameters are of keen interest with regard to structural studies of DNA, as well as to theoretical investigations of hydrogen bonds and scalar coupling constants.

51. Majumdar A, Kettani A, Skripkin E: **Observation and measurement of internucleotide $^{2}J_{NN}$ coupling constants between ^{15}N nuclei with widely separated chemical shifts.** *J Biomol NMR* 1999, **14**:67-70.

52. •• Cordier F, Grzesiek S: **Direct observation of hydrogen bonds in proteins by inter-residue $^{3h}J_{NC'}$ scalar couplings.** *J Am Chem Soc* 1999, **121**:1601-1602.
This paper reports the presence of three-bond scalar ^{15}N–$^{13}C'$ couplings of about 0.5 Hz across hydrogen bonds in proteins. This direct detection of hydrogen bonds is of keen interest in structural studies of proteins by NMR spectroscopy.

53. •• Cornilescu G, Hu JS, Bax A: **Identification of the hydrogen bonding network in a protein by scalar couplings.** *J Am Chem Soc* 1999, **121**:2949-2950.
This paper reports the presence of three-bond scalar ^{15}N–$^{13}C'$ couplings of about 0.5 Hz across hydrogen bonds in proteins. This direct detection of hydrogen bonds is of keen interest for structural studies of proteins by NMR spectroscopy.