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New Advances in Fast Methods of 2D NMR Experiments

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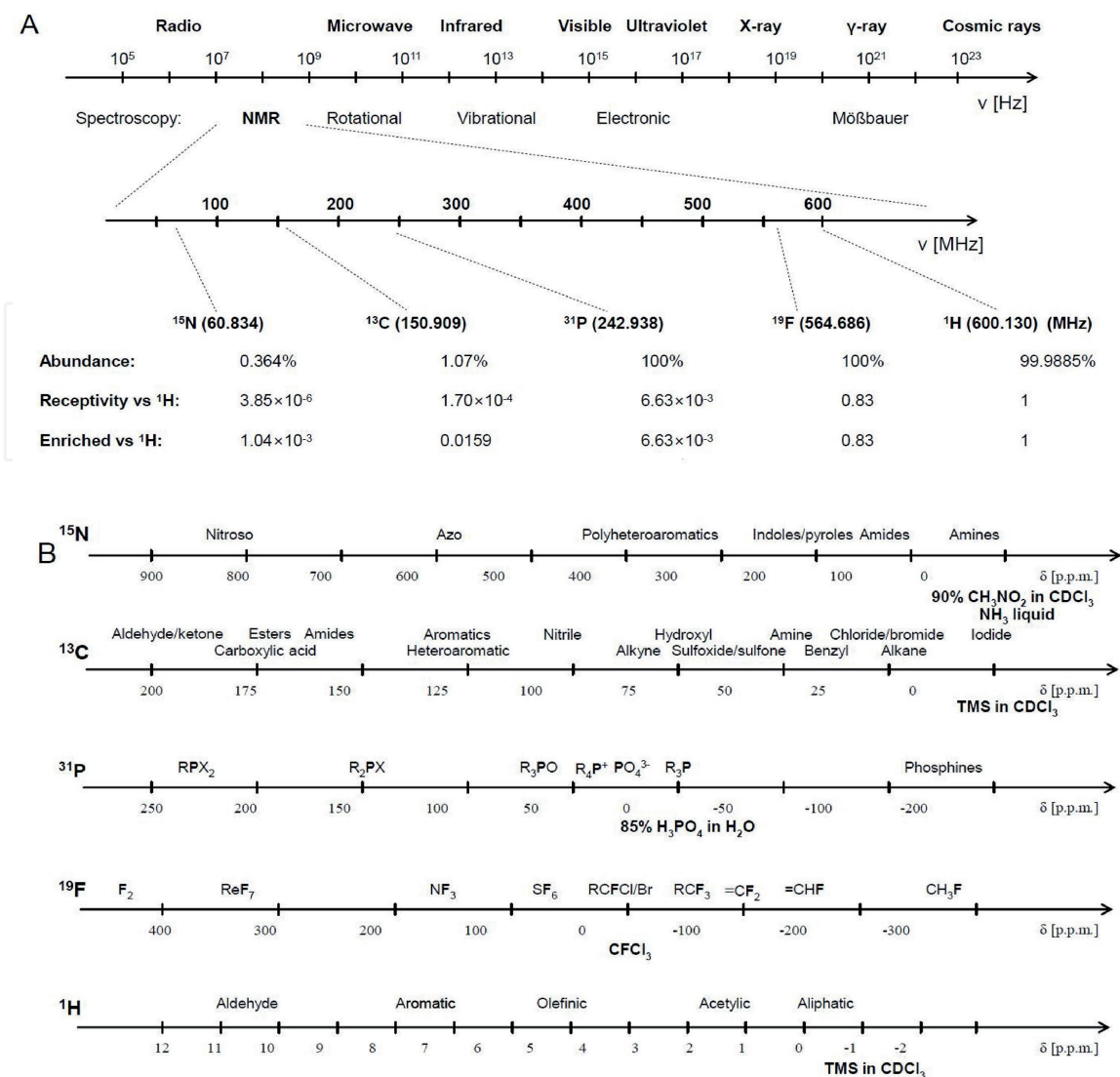
Abstract

Although nuclear magnetic resonance spectroscopy is a potent analytical tool for identification, quantification, and structural elucidation, it suffers from inherently low sensitivity limitations. This chapter focuses on recently reported methods that enable quick acquisition of NMR spectra, as well as new methods of faster, efficient, and informative two-dimensional (2D) NMR methods. Fast and efficient data acquisition has risen in response to an increasing need to investigate chemical and biological processes in real time. Several new techniques have been successfully introduced. One example of this is band-selective optimized-flip-angle short-transient (SOFAST) NMR, which has opened the door to studying the kinetics of biological processes such as the phosphorylation of proteins. The fast recording of NMR spectra allows researchers to investigate time sensitive molecules that have limited stability under experimental conditions. The increasing awareness that molecular structures are dynamic, rather than static, has pushed some researchers to find alternatives to standard, time-consuming methods of ^{15}N relaxation observables acquisition.

Keywords: NMR, 2D NMR, ultrafast data processing, SOFAST, relaxation

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical tool for identifying, quantifying, and elucidating structures of molecules. Moreover, NMR offers several approaches to studying molecular dynamics and to probing the interactions of inert molecules such as protein-protein, protein-DNA, and protein-ligand. The main strength of NMR spectroscopy is its ability to discern or resolve individual resonance frequencies of a wide range of different nuclei (e.g., ^1H , ^{13}C , ^{15}N , ^{29}Si , ^{30}Al , ^{31}P to ^{235}U). As the resonating frequency is unique for each type of nuclei, one can envision an NMR for each nucleus as a separate spectroscopy such as ^1H NMR spectroscopy, ^{13}C NMR spectroscopy, ^{235}U NMR spectroscopy (**Figure 1**), etc. More importantly, NMR has the ability to evaluate information about the environment of each atom and their neighbor's nuclei (both through space and through bond), allowing researchers to differentiate the unique magnetic environments of the same nuclei in different positions of a single molecule. Thus, NMR spectroscopy is extensively used for the identification and in the structural elucidation in a wide

**Figure 1.**

Frequency scale ranges and types of spectroscopies that correspond to them, respectively. The natural abundances and chemical shifts of the most common (¹⁵N, ¹³C, ³¹P, ¹⁹F, and ¹H nuclei) nuclei biomolecules studies with respect to the scale of the 600 MHz proton frequency adapted from [1].

range of applications in gas [2, 3], liquid [4–16], and solid-state samples [17–28]. Nowadays, NMR spectroscopy is one of the most important analytical tools that has been used in several fields. These fields include structural biology [29–38], organic chemistry [39–52], polymer characterization [40, 46, 53–64], inorganic chemistry [65–75], and physics [76–83].

Despite its significant advantages, NMR suffers from some limitations, of which the relatively low sensitivity seems to be the most severe. An NMR sample can be treated as a collection of many nuclear spins of magnetically active nuclei that act as small bar magnets. These nuclear spins have two possible orientations with different energy levels that adapt when placed within the strong magnetic field. The number of nuclear spins occupying each energy level is determined by the Boltzmann distribution equation:

$$N_U/N_L = e^{-\Delta E/kT} = e^{-h\nu/kT}$$

where N_U and N_L are the nuclei (expressed in numbers) in the upper and lower energy states, respectively, k is the Boltzmann constant, ΔE is the energy gap between two energy states of the spins, higher (N_U) and lower (N_L) energy, respectively, and T is the temperature expressed in Kelvin (K). Many of the nuclei

are at 298 K, the most common temperature in which biomolecular NMR measurements are performed. At this temperature, there is miniscule excess of the nuclei in the lower energy state that could be excited to the higher energy state by absorbing the energy given by the radio frequency (RF) pulse and later detected successfully during the data acquisition process. Therefore, the relatively low sensitivity of the NMR measurements seems to be the most severe limitation of this method. Another limitation comes from each measured nuclei having a defined range of frequencies at which it resonates (for practical reasons, frequencies are recalculated into the “unit-less” ppm scale in order to make it independent of the applied external magnetic field, B_0). This in turn depends on its magnetic neighborhood, and, therefore, the whole range must be covered with good resolution.

In earlier days, most NMR experiments only recorded one-dimensional (1D) spectra of which several were of ^1H or ^{13}C nuclei. For complex biomacromolecules, such as proteins, nucleic acids and their complexes, and complex mixtures, this technique has a particularly low spectral range of ^1H resonance, what leads to the low resolution of the spectra. This low resolution proves insufficient for analysis.

NMR signals depend on several nuclear properties such as the natural abundance of investigated isotopes, nuclear spin (I), gyromagnetic ratio (γ), quadrupolar moment (Q), and spin relaxation rates. Although most elements have an NMR active isotope, NMR spectroscopy is still not a practical approach for detecting many elements. For example, quadrupolar nuclei with nuclear spin $> \frac{1}{2}$ possess a quadrupolar moment that causes an effective spin relaxation mechanism, causing dramatic line broadening in the NMR spectra. Unfortunately, the nuclear spin for important elements such as carbon ^{12}C isotope and ^{16}O isotope is zero; thus, no NMR signals can be detected for such nuclei. For example, the NMR active isotope of oxygen, ^{17}O has a low natural abundance (0.037%), low receptivity, quadrupolar nuclear spin $= 5/2$, and very short spin relaxation T_1 values. These factors make ^{17}O NMR spectroscopy an unfeasible approach. Thus, nuclei with nuclear spin $= \frac{1}{2}$; high natural abundance similar to those of ^1H , ^{31}P , and ^{19}F ; and high γ are the most accessible nuclei for NMR studies in organic and biological samples. Even though the 1D ^1H NMR spectroscopy is the most common method, it suffers from the peak overlap as the spectra is very narrow (about 10 ppm). To overcome the challenge of spectra overlap, the multidimensional techniques which encode the indirect dimensions were proposed (i.e., Ernst, 2D, Bax 3D 90s, Clore 4D, Michal, etc.). Multidimensional NMR spectra have efficiently tackled the resolution problems for the spectra of large molecules such functional biomolecules and their respective native complexes. Applying two-dimensional (2D) or multidimensional NMR experiments are usually used to resolve overlapping peaks. Nevertheless, 2D experiments require considerably larger amounts of time than the usual 1D experiments do. The coincidence of simultaneous increase in measurement time with increase in dimensionality is directly correlated with the exact mechanism of how exactly the additional dimension is built in NMR. In an $n\text{D}$ NMR experiment, n dimensions are created by n independent time increments which encode chemical-shift information. In 1D NMR, chemical shifts are encoded during acquisition time when data is written referred to as direct dimension hereafter. There we have no restriction in putting the number of complex points as it will not increase the measurement time significantly. The scenario changes drastically when moving to multidimensional NMR. In multidimensional NMR, first there is a prepare period and then a mixing period where the magnetization is transferred to another dimension. Here both homonuclear or heteronuclear magnetization can be generated which can be frequency labeled. This frequency labeling is the evolutionary period, and here the number of pints will determine the resolution of this dimension and the total measurement time. Each time increment is equivalent to record one additional

experiment. Hence, for nD experiment, we can construct n dimensions, and in each dimension, some points have to be recorded to achieve the desired resolution in that dimension. This is the main reason the measurement time increases in a multiplicative manner with respect to the number of points in each dimension one needs to acquire to achieve the desired resolution. In the simplest multidimensional NMR, two-dimensional NMR thus uses two frequency axes. These are namely direct as explained earlier and indirect axis which is created by time increments after mixing. Now each time increment encodes one 1D spectra individually, and the second frequency is generated by the second Fourier transformation. Signal intensity is usually presented on the spectra as contours, bearing resemblance to topographic maps, or as an intensity plot. Thus, some studies (such as untargeted metabolomics) do not fully utilize 2D NMR because there are a large number of samples that would require more time to complete. Several approaches have been continuously developed to reduce the experimental time and to enhance the NMR sensitivity. These approaches include hyperpolarization methods such as dynamic nuclear polarization (DNP), cryoprobes, and new ultrahigh magnetic fields. These methods, however, are costly and need substantial hardware additions.

Other methods such as cross-polarization, INEPT, DEPT, and fast methods provide good enhancements without hardware additions and are available in most existing NMR systems. Fast 2D NMR methods and band-selective optimized-flip-angle short-transient (SOFAST) 2D NMR methods provide good NMR spectra with a short experimental time, which in turn enables the researchers to carry out powerful 2D NMR experiments in a few minutes. Increasing the number of spectral dimensions increases the spectra acquisition time dramatically. In order to overcome this issue and reduce the demand for the experimental time needed to perform the spectra measurement, different methods of acquisition of the spectra have been proposed. In this chapter, we introduce the relaxation mechanisms, and then we briefly discuss a few methods of fast methods including SOFAST, ultrafast (UF) 2D NMR, pure shift NMR, and post-processing methods. The pure shift NMR method produces spectra that only contain chemical-shift data and is void of any coupling information. This reduces the spectral complexities to a large extent as all multiplets collapse in singlets. This saves a lot of analysis time. Pure shift as such is not a fast method. However, we have incorporated this as it is indeed an important milestone in achieving high spectral resolution and a very important step towards minimizing the spectral analysis time.

2. Fast 2D NMR approaches

The power of NMR is well realized because it is the only noninvasive technique which has an atomic level resolution and can work well for both solution and solid-state samples. Although one-dimensional NMR in principle contains a wealth of information, the situation becomes severely limited due to signal overlap for small organic molecules onwards. This requires spreading the signals in other dimensions which can be the same nuclei or different NMR active nuclei. Here, concepts of 2D or multidimensional NMR arise, and with the development of 2D, 3D, and even higher-dimensional NMR, the overlap problem is solved even for large biomolecules. The price we pay is an increase in measurement time. Any higher-dimensional spectra require certain time increments to achieve certain spectral resolution, and as a result, the measurement time increases enormously as the number of dimensions increases. Generally, for the same sample moving from 1D NMR to 2D, the measurement time increases by at least two orders of magnitudes. Moving from 2D to 3D the experimental time increases further by more than one

order of magnitude. Similarly, 4D and 5D become so time-consuming that they are hardly used in practice. This is the main reason for steady research in the direction to overcome the time limitation so that speedy acquisition can be feasible. Here we will discuss some of these advancements and will limit the discussion to 2D. The basic building block of the 2D or any multidimensional experiment consists of a preparation period, evolution period that incorporates incremental time delay, mixing period, and one recycle delay which is required for getting longitudinal magnetization back to equilibrium. There have been numerous methodological developments focusing on each aspect of these building blocks. Nonuniform sampling (NUS) reduces the number of incremental delays. FAST or SOFAST methods reduce recycle delay which is on the order of seconds. Ultrafast works on preparation and mixing components, and with the help of slice selection gradient, single-scan experiments are designed. We will go through each aspect separately. Lastly, there is pure shift NMR which focuses on enhancement of resolution.

2.1 SOFAST 2D NMR

We first focus on SOFAST method that aims to reduce the inter-scan delay and eventually manages to lower the 2D acquisition times [84]. This technique is optimized in order to obtain fast and sensitive 2D NMR HMQC with ^1H - ^{15}N and ^1H - ^{13}C correlation of biomolecules with a different size range [84]. The SOFAST-HMQC experiment makes use of selective ^1H pulses affecting only a portion of proton spins while keeping a large pool of proton spins unperturbed at Z axis. These unperturbed spins enhance the spin-lattice relaxation (T_1) rate via dipolar interactions with the perturbed spins and effective recycle delay decreases. Additionally, selective excitation is achieved with Ernst angle excitation which helps in acquiring more sensitivity [85] at lower recycle delay. Therefore, the concerted use of Ernst angle excitation and higher repetition rates of the pulse sequence yield high signal-to-noise ratio compared to conventional experiments. This strongly is recommended in biomolecular NMR, to study protein modifications and real-time molecular kinetics [85] (**Figure 2**). Numerous versions of SOFAST-HMQC experiments have been proposed for different purposes depending on the experimental conditions [86, 87]. As an example, ^{13}C methyl SOFAST experiment has been developed, allowing the recording of high-quality methyl ^1H - ^{13}C correlation spectra of protein with high

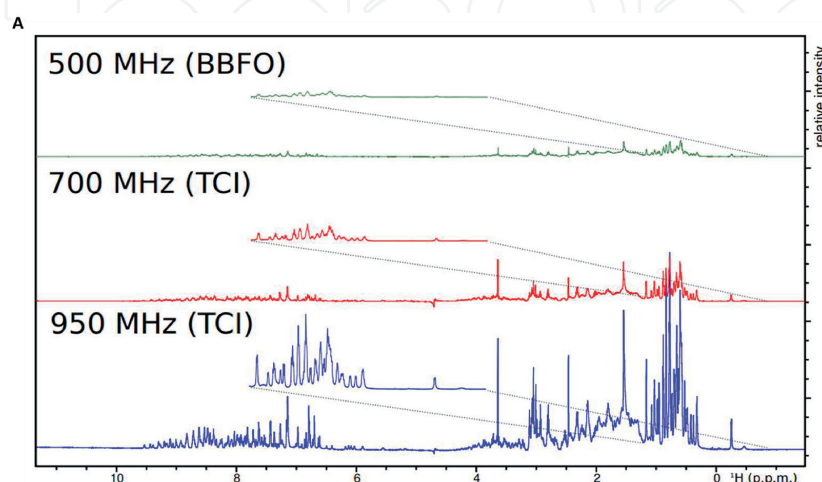


Figure 2. Profits of the high-field NMR magnetic fields on spectral resolution and sensitivity. High-resolution 1D ^1H NMR spectral of a representative small well-folded protein (A) U- ^{15}N human ubiquitin of 1 mM concentration in 20 mM phosphate buffer, 10% D_2O , and 0.02% NaN_3 , recorded with the same parameter set at three spectrometers working at 500, 700, and 950 MHz proton frequencies at 24°C .

molecular weight in a few second acquisition time [88]. Methyl groups are used as spectroscopic probes of protein structure, dynamics, and kinetics because they are dispersed naturally throughout regions of folded protein. It has been shown that the use of methyl groups as a probe is very useful, particularly for drug binding and molecular interactions studies [88].

For biomolecular NMR applications, the use of longitudinal relaxation optimized experiments permits the acceleration of 2D NMR data acquisition which provides a tool for high-throughput screening for macromolecules like proteins or nucleic acids. Such experiments allow one to obtain a high signal-to-noise ratio compared to its counterpart, as mentioned previously. Theillet et al. present an example of the use of SOFAST-HMQC experiment in order to monitor protein phosphorylation reactions [89]. In this study, the N-terminal transactivation domain of human p53 ^{15}N -labeled protein was phosphorylated by either recombinant enzyme or endogenous kinases present in cell extracts. The protein phosphorylation reactions then were monitored by SOFAST-HMQC NMR experiments in real time to obtain a high atomic-resolution understating of phosphorylations level of serine and threonine residues in kinase reactions [89].

2.2 Ultrafast 2D NMR

Secondly, we focus on ultrafast 2D NMR that is able to deliver any kind of 2D NMR spectra involving homo- or heteronuclear correlation in a single scan and, therefore, in a fraction of a second. The basis of this approach relies on the concept of revealing the spin interactions measured by spatiotemporal encoding [90]. The strategy is essentially that, instead of measuring N_1 successive experiments multiple times on the sample with an independent increase of time, a better way to do it, as in the case of UF spectroscopy, is the one that “divides” the sample into N_1 fractions and records all of them simultaneously within one scan (Figure 3) [90]. The application of slice-selective gradients encodes different phases in different slices similarly to incremental decay. As a result, the entire spectrum can be built from a single scan. However, the cost is reduced sensitivity.

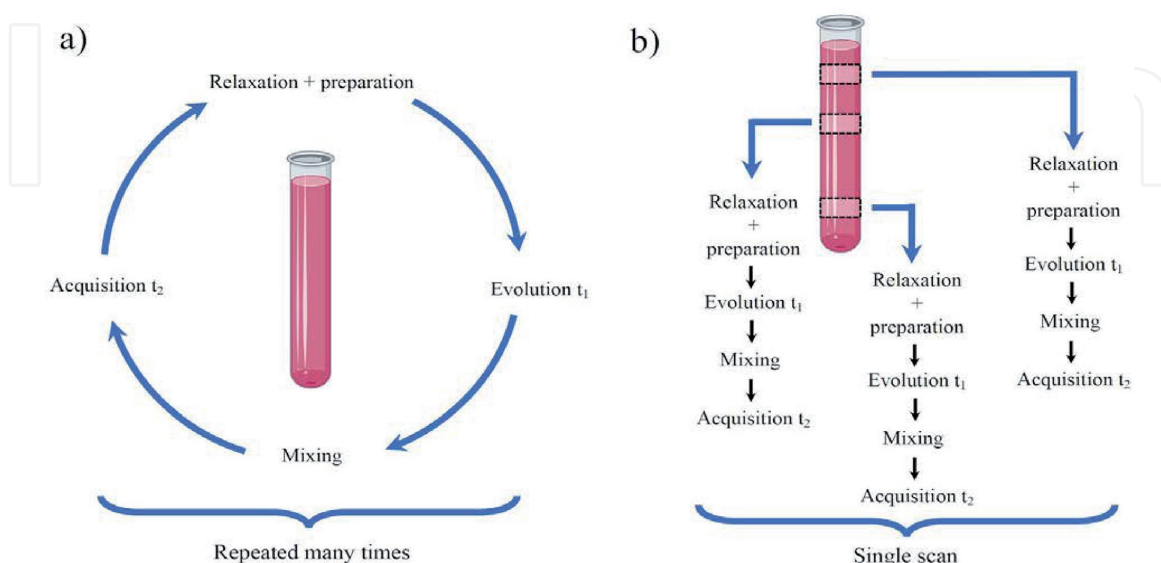


Figure 3.

Comparison between the conventional and UF 2D NMR approaches. (a) Conventional approach of 2D NMR is done on the sample by measuring N_1 successive experiments multiple times with an independent increase of time. (b) UF 2D NMR approach can be conceptualized as “dividing” the sample to fractions and recording all of them simultaneously within one scan [adapted and modified from ref. [90]].

The so-called fast 2D NMR spectroscopy has found multiple applications in the field of metabolomics [86, 91, 92], as well as in the analysis of natural products [92–94]. The ultrafast quantitative 2D NMR spectroscopy is also an appropriate choice for measuring ^{13}C enrichments in a single scan (with ultrafast COSY and zTOCSY experiments). The authors of this study have reported an accuracy of 1–2%, an average precision of 3%, which is believed to be better than those previously achieved with different approaches [95]. This approach is also used in acquiring R_1 longitudinal relaxation rates [4].

2.3 Nonuniform sampling and processing

Multidimensional experiments have two significant limitations: sensitivity and low resolution, especially for large molecules. For obtaining good sensitivity, we need a very high number of scans for ^{13}C , ^{15}N -labeled samples, and J-coupling transfer, and dipole-dipole interactions were utilized. For instance, metabolomics research is based on the analysis of the natural abundance of carbon and hydrogen nuclei; therefore, the sensitivity remains the main issue because isotope labeling cannot be applied. Enhancing the sensitivity and reducing the acquisition time may require the concerted use of methods such as SOFAST method and nonuniform sampling while decreasing the number of recorded points for indirect dimensions. Sometimes the single-scan approach can be used [96–98]. The main limitation of single-scan methods, however, is recording only ^1H plane, even for 2D experiments [99].

Some studies demonstrated that a combination of fast methods with reducing the number of data points can benefit multidimensional NMR spectra like BEST-Trosy, which provide magnification of sensitivity and resolution for structural and kinetic investigation of biomolecular and complex systems [100].

Normal 2D experiments yield 2D spectra, while during data acquisition in the indirect dimension (marked as t_1), the second “vertical” dimension has incremental time delay. This time delay is linear, and the gap between the time points is the same. In contrast, NUS approaches the t_1 change in a semi-random way. In NUS, only some of the time increments are recorded, and then the complete spectra are constructed after reconstructing the missing data points. Various algorithms are designed to select which data points to acquire and what will lead to successful reconstruction. The advantage of NUS is that the same level of resolution can be achieved with significantly fewer t_1 increments. Thus, overall measurement time decreases. This is practical for NMR spectroscopist because this means one can obtain well-resolved 2D NMR spectra in less time.

There has been extensive research on the optimization of finding suitable combinations for sampling schemes and reconstruction algorithms [98]. NUS sampling conventionally selects random acquisition points in the indirect dimensions. The “randomness” of NUS is restricted by factors such as the transverse relaxation rate. Exponentially weighted random sampling is designed to overcome the T_2 decay as more signals decay incrementally in the initial phase. Poisson gap sampling has also been used to yield excellent results as described later. Processing the recorded data can be classified in several ways [101]. For example, the sparse time domain data may be directly transformed with Fourier transform (FT) algorithms, and subsequently the missing data are added by interpolation and then followed by conventional Fourier transform [101] in the second dimension. This can also be reversed for reconstruction, followed by FT in both dimensions.

NUS can be a good tool, especially for triple resonance experiments, which have a low dynamic range. An almost identical intensity and a small number of peaks allow the use of low-density sampling. For a highly dynamic range of signals in experiments like NOESY (which provides information on the restraint through

the space between atoms), reconstruction will be better with less mixing time. The positive results of reconstructed spectra were demonstrated with 20% and more sparse incremental time [102]. NUS methods have also been widely used for resonance backbone and side-chain assignments of macromolecules, determination of cross-correlated relaxation rates (CCR), kinetic processes, posttranslational modifications, and metabolomics [103].

The determinative criteria of spectra quality have a big gap in the random sampling schedule, especially at the beginning and at the end of dataset points. To overcome this limitation, the Poisson distribution [104] was used with sinusoidal variation of average gap length. In particular, sinusoidal variation of gap length keeps a degree of order in the sampling schedule, and it condenses points at the beginning of the time domain data. This provides significant benefits for time domain data with exponential decay [104].

The approach for the reconstruction of traditional uniform sampling is FT [105]. The processing spectra from NUS acquisition experiments, however, demand different types of reconstruction methods because regular discrete FT leads to artifacts as several data points are missing. Among the reconstruction algorithms, multidimensional decomposition (MDD) is the model where the full matrix of different dimensions can be identified as a sum of one-dimensional vectors (components), in which each of the components is coherent to a peak or group of peaks. MDD essentially breaks multidimensional data into sets of one-dimensional data, which are much easier to analyze and solve. The overlap in multidimensional NMR is common, and since MDD is able to resolve the overlapping resonances, MDD is well equipped for resolution enhancement of crowded datasets. MDD typically only needs a subset (20–30%) of the full dataset in order to reconstruct the full NMR signal [106, 107]. **Figure 4** gives a visual demonstration of the sampling of the full dataset to make a smaller dataset and create the spectra. One paper reports a method demonstrated on nonuniformly sampled [16] N-NOESY-HSQC datasets recorded for the 14 kDa protein azurin [108]. MDD typically only needs a subset (20–30%) of the full dataset in order to reconstruct the full NMR signal [106, 107].

Another algorithm is compressed sensing (CS) which is one of the best reconstruction methods available. Maximum entropy method (Max Ent) is another algorithm. CS is very useful in under sampled multidimensional experiments for solid-state NMR, fast metabolomics studies, and chemical-shift imaging [109, 110]. CS was demonstrated to give better reconstruction of weaker peaks, compared to an existing Max Ent implementation [110]. In one paper, two time-equivalent 3D

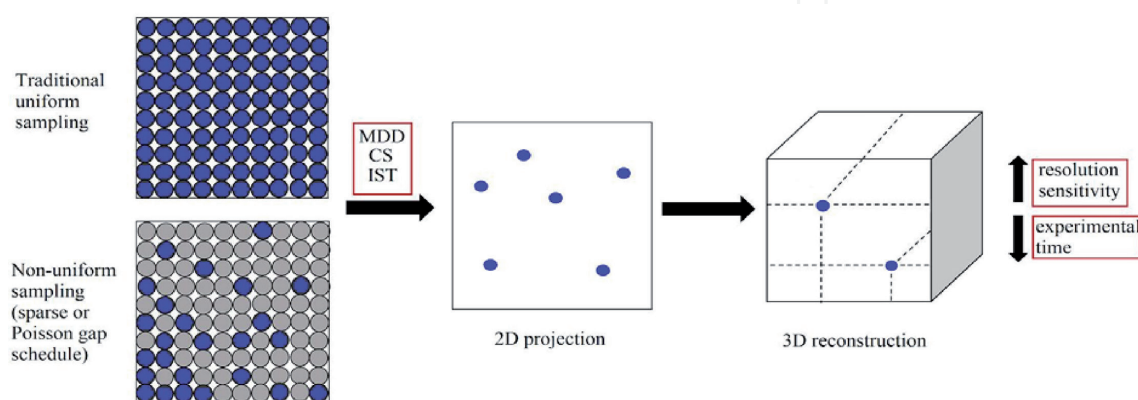


Figure 4. Visual demonstration of NUS for obtaining NMR spectra. NUS takes a small subset of the total dataset (crowded blue dots) and uses that comparatively small dataset (20–30% of the total dataset) to create an accurate NMR spectra of the molecule of interest.

NUS TROSY-HNCA semi-constant time experiments were recorded, with CS giving better overall peak resolution [111].

Iterative soft threshold (IST) is another type of reconstruction algorithm. Like other methods, IST is able to “clean up” the NMR data by filtering the spectra. It does this by setting a threshold value and then, as its name suggests, runs through a fixed number of iterations until the procedure is complete. The best approach to IST is to use it in sync with an optimized NUS schedule. IST computational methods only take a reasonable amount of time and greatly reduce the analysis time required to obtain NMR spectra [112, 113]. One paper reports successful application of the IST approach to the ubiquitin protein. On a dual core, 2.2GHz CPU, the overall time required to process the sampled NMR dataset took under 60 seconds [114], a significant contribution to decreasing total analysis time.

3. Pure shift NMR

Proton NMR suffers from extensive signals splitting due to the presence of many weak and strong couplings, leading to significant spectral overlap, even for small molecules. The problem is severe at low magnetic fields. Although the coupling constant gives useful structural information, difficulty in obtaining them due to strong overlap often becomes a major roadblock. Ideally, a spectrum, where all multiplets collapse in singlets, gives the most straightforward information which can be achieved by pure shift. Subsequently, the analysis of couplings can be introduced in a systematic manner. The problem is simple for ^{13}C NMR as natural isotopic abundance is low and only singlets are present. However, the situation becomes complex for ^{13}C isotope-enriched samples, especially for large biomolecules. In those cases, spin-state-selective decoupling are most commonly used. Both components of the splitting due to ^{13}C - ^{13}C coupling are recorded in a linear combination of inphase mode and antiphase mode. This is known as IPAP (inphase-antiphase) [115] or direct excitation of single-quantum coherences [116]. This method mainly works because of clear ^{13}C signals in different spectral shift regions and different and distinct spin-spin coupling values between those different classes of spins. This is clearly not the case for proton NMR where coupling values spread much wider ranges and also spectral overlap is more severe.

Pure shift NMR focuses on chemical shift and removes homonuclear couplings which lead to complex multiplet structure of individual peaks. This results in severe overlap even for small molecules and particularly for ^1H spectra since the chemical-shift dispersion of the proton is normally lower (~ 10 ppm) compared to other heteronuclei like ^{13}C or ^{15}N . Having the highest gyromagnetic ratio and natural abundance, ^1H NMR is the most obvious choice for almost all routine and conventional small and large molecule analysis. However, the spectral overlap due to homonuclear coupling limiting the resolution presents a major limitation of 1D proton NMR spectroscopy. Pure shift NMR was developed to simplify the overlapped proton NMR spectra by keeping only chemical-shift information. There have been great contributions coming first from Zangger [117–120] and subsequently by Morris and others [117, 121–127] in this direction. Some of the recent advances are presented here.

First experimental demonstration of homonuclear broadband decoupling in proton NMR was reported by Ernst and coworkers [128]. The basic pulse program consists of a 90° excitation pulse followed by spin echo. So, in direction dimension, homonuclear coupling is active, and chemical-shift evolution refocuses at the end of t_1 , while both chemical-shift and homonuclear couplings are active during acquisition. So, the resultant spectrum shows tilted multiplets where the indirect

dimension spreads out the J-coupling. Now, 45° projection of the 2D spectrum yields pure shift spectra where all the scalar couplings have been removed. The main problem of J-resolved spectra is the twisted phase accumulation during t_1 which leads to both absorptive and dispersive phase components in the indirect dimension. This results in partial or complete cancelation after taking the projection. To avoid this, normally absolute value mode calculation or power spectrum is taken before the calculation 45° projection. This generates broad peaks with long tails in the final decoupled spectrum.

The most prominent contribution came from Zangger and Sterk [120] who employed selective pulses and gradient encoding simultaneously. In this approach, first a selective 90° pulse was applied, followed by an incremental delay and combination of hard and soft selective 180° pulse with the same incremental delay and subsequent acquisition. The pulses were applied with weak gradient encoding implying spatial resolution in the sample. Hence, the whole sample is now divided in different slices, and the first frequency-selective pulse excites signals of different frequencies in different slices of the sample tube. Most importantly all signals are excited at once, but the price we pay is loss in sensitivity as the effective signal is coming from a single slice. Normally, the first experiment proposed was ~2% sensitive compared to conventional 1D NMR. In each slice, selective signals get excited by the selective first excitation pulse. Subsequently, coupling with all other spins evolves in the incremental delay. Next the selective spins undergo complete 360° rotation, while all other spins undergo 180° rotation initiated by hard 180° pulse. Therefore, after the same incremental delay, the J-coupling between the selected spin with all other spins gets refocused and homonuclear decoupling is achieved. The initially proposed scheme recorded FID with different incremental time delays. Then, from each FID, the first few points (t_c/DW) determined by the incremental delay (t_c) and the dwell time (DW) were concatenated to generate the final FID. The excitation bandwidth is determined by the relative proximity of the scalar-coupled protons. If coupled protons with very similar chemical shifts need to be resolved, then the selective pulses must be highly selective. This also implies the application of higher gradient strengths to generate more slices to cover the full spectral width which decreases sensitivity immensely as the effective slice thickness reduces. There have been considerable developments to overcome the limitations in the first proposed scheme.

First the two-dimensional mode of data acquisition was omitted, and the homonuclear broadband decoupling was performed during acquisition [119]. This generates a single-scan decoupling sequence like normal 1D NMR. This simplified the whole thing as these signals can be stored as regular 1D or as acquisition dimension of multidimensional spectra. Most importantly, the processing of the data becomes a routine, and there is a huge reduction of total measurement time. In this modified scheme shown in **Figure 5**, the acquisition is interrupted approximately every $1/3(^3J_{HH})$ to incorporate decoupling block. Additionally the decoupling of the signals should be placed right in the middle of the data chunk. So the first and the last acquisition block length is only half as long of all other blocks sandwiched between two.

Since relaxation is always there, some chunking artifacts arise after Fourier transform as FID gets interrupted in between. Further, to reduce the overall measurement time, this slice-selective excitation was combined with fast pulsing by shifting the frequency of the selective pulse between individual scans [117]. In slice-selective decoupling, in each slice only the excited magnetization is used for the acquisition. So, by shifting the offset of the 90° excitation pulse and also the selective 180° refocusing pulse after every scan, the unused equilibrium magnetization can be used without any recycle delay. The next scan or the subsequent scans with different offset act as recycle delay for the first set of spins which were excited in the first scan. This makes repetition much faster, and overall signal-to-noise ratio

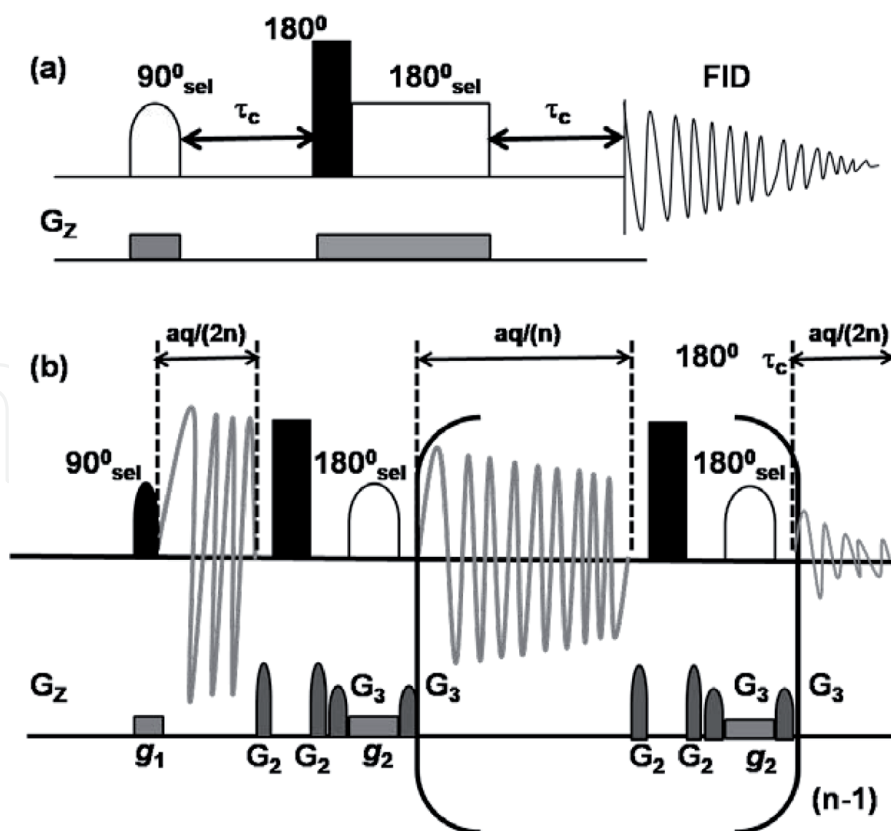


Figure 5.
 (a) Pulse sequence of first Zangger-Sterk experiment. The delay τ_c was incremented by several milliseconds. The first of each FID was concatenated to produce the final FID as outlined in the text. (b) the real-time slice-selective broadband homodecoupling approach. The data chunking times are variable which is represented by number n . frequency shifting is achieved for all selective pulses in parallel after each transient. The slice-selective gradients are represented by g_1 and g_2 ; G_2 and G_3 clear any imperfection of the refocusing pulse.

increases per unit time as shown in **Figure 6**. Apart from varying offset in between different transients, FID chunk size also varied to suppress the decoupling sidebands. The decoupling sidebands arise due to truncation of FID, and the longer the FID chunk, the more dominant are the artifacts as more antiphase magnetization can build up. On the contrary, if keeping the individual FID blocks, short leads to broad signals as more relaxation losses occur during the more frequent acquisition interruptions. The variation of chunking time between subsequent scans leads to smearing out of the decoupling sidebands which otherwise add up if they are kept the same. The slice-selective decoupling has been employed for two-dimensional homonuclear TOCSY [127, 129] and NOESY [122] spectra. The enhanced resolution in the direct dimension can be propagated to the indirect dimension by applying covariance processing which yield pure shift spectra in both the dimension for TOCSY spectrum. Additionally for heteronuclear HSQC, the slice-selective excitation was employed for intrinsically disordered proteins (IDPs) [130] where proton spectral dispersion is severely low. Additionally slice-selective excitation has been used for fast data acquisition [131] and was able to monitor chemical reaction from different spatial location of the sample tube [132].

Recently a new method named as pure shift yielded by chirp excitation (PSYCHE) experiment has been proposed by Morris et al. [125] which is based on anti Z-COSY experiment. This experiment employs two small flip angle chirp pulses which selectively refocus a small proportion of spins, namely, active spins, while leaving the majority ones undisturbed, namely, passive spins. Now this selection of active and passive spins are purely statistical which leads to an obvious advantage over ZS method as there the selection was based on selective pulse. The sequence is closely related to anti Z-COSY except to the fact that instead of using two hard small

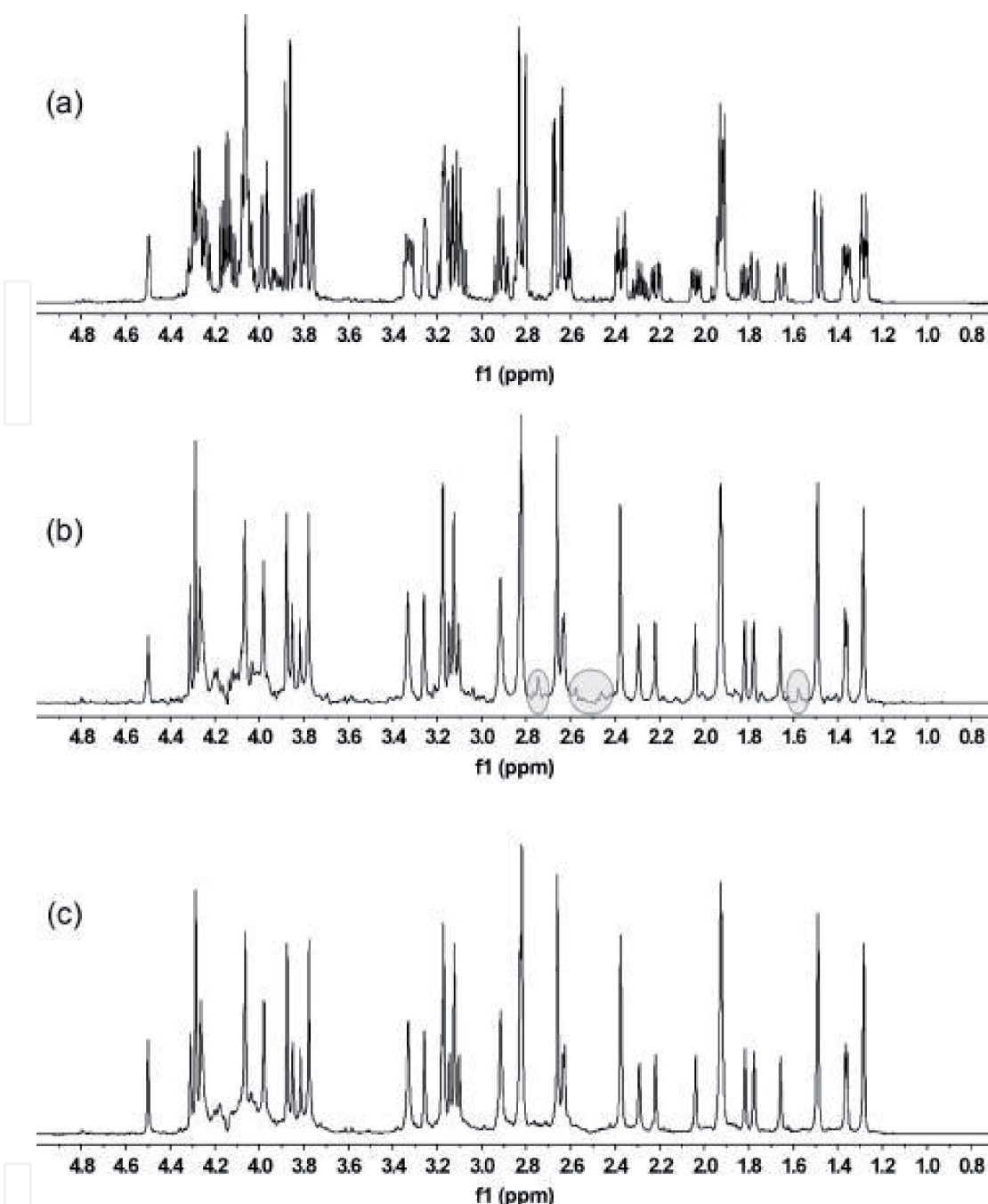


Figure 6.

(a) A regular 1D spectrum, (b) pure shift with fixed chunking time of 25 ms, (c) pure shift with variable chunking time with real-time acquired spectrum of a mixture of strychnine with unknown degradation products in CDCl_3 . Gray circles indicate artifacts resulting from decoupling sidebands. Weak peaks visible in (b) result from additional minor compounds in the mixture, which are way below the level of artifacts in the fixed chunking time spectrum. The figure presented is from [117].

flip angle pulses, it uses two symmetric, low power, frequency-swept chirp pulses along with weak pulsed-field gradient. This gradient employs spatial resolution and suppresses the cross-peak terms as related to anti Z-COSY since they experience different chemical shifts as they are excited at different times during the chirp pulses. The major advantage of PSYCHE is that it is most sensitive among the existing pure shift methods. Several further developments are achieved to obtain ultraclean artifact-free spectra and applied in 2D NMR as well [133, 134]. Very recently one such modification named SAPPHERE-PSYCHE methodology is applied for plant metabolomics study. In that report, single-pulse, PSYCHE, and SAPPHERE-PSYCHE spectra were compared from aqueous extracts of *Physalis peruviana* fruits. It was found using pure shift methodology; many proton NMR signals can be cleanly observed which

were not possible in conventional NMR. These signals belong to amino acids, organic acids, and sugars which are critical components of plant mixture. One example of such metabolite was the identification of glutamic acid from Cape gooseberry which was not possible to isolate due to heavy overlap. Thus, ultra-clean pure shift spectra look very promising and provide new benchmark for metabolomics studies.

4. Summary and future perspectives

High-resolution NMR with high sensitivity is the desired aim for any NMR application. There have been significant hardware advances in achieving high sensitivity. This includes the development of high magnetic field, cryogenically cooled probes [135], recent advent of high-mass-sensitive probe for small volume [136], etc. A combination of these advances results in an increase of sensitivity by more than an order of magnitude. These advancements fueled the developments of fast methodology. Additionally due to the sample instability, which results in the relatively fast decomposition of the biomolecule being an object of the study, the quick methods of 2D spectra acquisition needed to be developed. In this regard, previously mentioned SOFAST, NUS, etc. methodologies were developed and subsequently applied in many different scenarios [90, 93, 94, 137]. Above that, these fast methods can be also used for studying either chemical or biochemical processes that are characterized by the relatively fast kinetics and also carry some structural information, like hydrogen/deuterium (H/D) exchange [138, 139]. With NUS approach, one problem is the generation of very large file size, especially for 4D and 5D experiments. Artifacts are also produced, which generates “unwanted” signals that do not accurately represent the studied molecule. However, there is considerable effort in the scientific community to reduce the amount of produced artifacts. For example, newly proposed MUNIN uses three-way decomposition and a simplified model for NMR spectra based on generally accepted assumptions. This method achieves high-resolution and good sensitivity while avoiding artifacts [106]. Also, a signal separation algorithm is shown to suppress sampling artifacts in high-resolution four-dimensional NMR spectra [140]. It is likely that NUS-based methods will continue to be used in the future, mainly because they will greatly reduce experimental time, and still provide satisfactory results with increased resolution of the spectra what facilitates their analysis. However, with the increase in magnetic field, resolution has been improved only two times and not more than that. Even with the availability of very high-field magnet such as 1.1 GHz and upwards which is limited, resolution was still a problem. Hence, the development of pure shift methods became necessary, and with the techniques great resolution can be achieved uniformly for all scenarios. In this regard, both the methodologies and developments in the direction of reducing measurement time and increasing resolution remain of utmost importance and have become an area of research itself.

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Conflict of interest

The authors declare no conflict of interest.

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