High-resolution NMR techniques for solid-phase synthesis and combinatorial chemistry

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Recent developments in NMR spectroscopy have created two complementary categories of analysis for drug discovery processes. The first category includes techniques for obtaining high-resolution (especially ^{1}H) NMR spectra of compounds still bound to solid-phase synthesis resins. The second category includes an automated high-throughput spectroscopy technique for acquiring high-resolution NMR spectra of liquids, directly sampled from microtiter plates, in a 'tubeless' mode, without the use of expensive deuterated solvents. This review summarizes these recent developments.

Combinatorial chemistry is influenced by the desire to produce more compounds – and a wider variety – faster. Developments such as solid-phase synthesis (SPS) and high-throughput screening have played significant roles in accelerating both the synthesis and testing phases of the drug discovery process[1]. Sample analysis, however, is still a rate-limiting step. When using SPS resins, the difficulties of analysis caused by the physical heterogeneity of a resin sample are well recognized[2–4]. For combinatorial chemistry methods in general, however, especially when the term 'combinatorial chemistry' refers to the use of a large chemical library (as seen in programs that use multiple-parallel synthesis to produce collections of pure compounds), the difficulties of chemical analysis are primarily caused by the very large numbers of samples.

Scope of NMR

High-resolution nuclear magnetic resonance (NMR) is often considered to be one of the most information-rich analytical techniques available, but it is perceived to have certain drawbacks, such as the following:

- it requires homogeneous samples,
- it is too insensitive,
- it requires large sample sizes,
- it is too slow, and
- expensive deuterated solvents are required.

Each of these issues will be dealt with here in more detail to assess how recent developments in NMR spectroscopy are allowing NMR analyses to play new and different roles within a modern drug discovery program.

Combinatorial chemistry has embraced SPS as a routine tool, if not actually the cornerstone technique[5,6]. However, until recently there were no suitable analytical techniques available for use with compounds still bound to SPS resins[7,8]. Conventional analytical techniques, such as mass spectrometry (MS), HPLC, and high-resolution NMR, usually required homogeneous samples, meaning that samples for analysis had first to be cleaved from the SPS resin. This presented a significant limitation either to the monitoring of a reaction in progress or the characterization of an intermediate

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in a multi-step synthesis; every cleavage reaction took time, potentially altered the product, was probably irreversible and by definition lowered the product's yield. Recent developments in NMR spectroscopy have removed the need to cleave the samples, and this allows high-resolution NMR spectra to be obtained for samples still bound to SPS resins; these developments are the subject of the first half of this review.

The second half of this review will focus on the new tools being used to acquire NMR spectra of solution-state samples rapidly. Although these tools were originally designed for LC-NMR, and so may initially appear to be unrelated to combinatorial chemistry, they have recently been combined in several different ways to create tools that can acquire NMR spectra on combinatorial chemistry samples in a rapid and automated fashion. This capability begs to be called 'high-throughput NMR spectroscopy'.

SPS resin samples: gel-phase NMR

Organic compounds are most effectively analyzed by NMR spectroscopy only if the NMR spectra exhibit good spectral resolution (narrow linewidths). This is typically achieved by analyzing solution-phase samples that are physically homogeneous, and then 'shimming' the static field of the NMR magnet to make the sample magnetically homogeneous (to within ppb).

All substances have a physical property called 'magnetic susceptibility' that provides a measure of how that substance affects a surrounding magnetic field. A uniform (homogeneous) substance will cause only a uniform change of the applied (static) magnetic field; this change can be easily 'shimmed' out, especially if the sample forms a cylindrical geometry that is infinitely long relative to the detector coil. A heterogeneous sample, however (like a slurry of a SPS resin), will have regions of differing magnetic susceptibility throughout the sample (because the resin and the solvent will have different susceptibilities) that will cause localized variations (distortions) in the magnetic field that are *not* correctable by 'shimming' the field[9,10]. These magnetic susceptibility mismatches create additional linebroadenings that add to the normal NMR linewidths; the width of this broadening scales up directly with the NMR observation frequency (see below).

Restricted molecular motions within a sample can also broaden the NMR linewidths; this is one of the reasons why solid samples usually exhibit broad NMR resonances[11]. Because significant motional freedom is required to generate narrow linewidths, all SPS resins are swollen in solvent as much as possible before acquiring the NMR spectra. The term 'gel-phase' NMR is used to describe this technique, because the resulting solvent-swollen slurries are neither fully solid nor fully liquid, and the solvent swelling is required to obtain usable data. Unfortunately, the term 'gel-phase' NMR has also come to refer specifically to the technique of using only a conventional liquids probe – where the sample-spinning axis is aligned vertically along the magnet bore – to acquire the SPS-resin NMR data.

Limitations in utility

The utility of this 'gel-phase' NMR technique is limited in that the proton NMR spectra it generates are still composed of very broad lines (≥100–300 Hz)[12,13]. Some researchers, however, have exploited the lessened magnetic-susceptibility linebroadenings of lower frequency nuclei, and combined it with the observation of nuclei having a wide range of chemical shifts (like ^{13}C) to generate 'gel-phase' NMR spectra in which, although the linewidths may still be 25–75 Hz or more, the resonances are still resolved well enough to make structural assignments. This technique has been used primarily for ^{13}C NMR (Refs 3,14–23), although NMR spectra of other nuclei such as ^{19}F (Refs 18,24,25), ^{2}H (Ref. 20), and ^{31}P (Refs 26–28) have also been reported (in addition to ^{1}H; Refs 12,13). In most cases, the broad linewidths limited the data analysis; useful information was obtainable only by using nuclei with significant chemical-shift dispersion (wide spectral widths; for example ^{13}C or ^{19}F) or by using extensive deconvolution to enhance the spectral resolution (for ^{1}H spectra). Another strategy includes using the favorably wide chemical-shift range of the gel-phase ^{13}C NMR spectra, but compensates for the poor sensitivity of the technique by incorporating ^{13}C labels near the reaction site of interest; this 'fast ^{13}C NMR' has proven itself useful in monitoring the progress of reactions[17,29–32].

Cleave-and-analyze method

The limited utility of these lower-resolution (especially ^{1}H) gel-phase NMR spectra has caused many users to adopt an alternate strategy, called the 'cleave-and-analyze' method[8], in which the compounds are cleaved from the resin and then analyzed by conventional solution-state NMR[15,27,33–35]. One drawback is that the cleavage reaction itself can modify the compounds being monitored. Other combinatorial chemistry strategies that allow the ease of solution-state NMR to be exploited include the use of soluble polymeric supports like polyoxyethylene (POE) or polyethylene glycol (PEG)[2,36,37] (which can be purified by ether precipitation), or the use of dendrimer supports[38] (which can be purified by size exclusion chromatography or ultrafiltration). Fortunately, studies indicate that the chemical shifts of compounds, regardless of whether they are bound to resins or in solution, are virtually identical[3,17,24] and integratable[17,27,39]. Because many SPS resins contain unbound materials that should be washed from the resin before use, solution-state ^{1}H NMR has also been used to monitor both the impurity levels leached into each successive wash[16] and the leachable impurities after cleavage[40].

SPS resin samples: high-resolution NMR by MAS

Hardware requirements

It was recognized almost 30 years ago that magic-angle-spinning (MAS) NMR could remove the magnetic-susceptibility-induced linebroadenings found in heterogeneous samples[41,42]. This was first demonstrated on a wide variety of physically heterogeneous samples, including powdered solids, and eventually extended to heterogeneous solid–liquid mixtures, compartmentalized liquid samples, membranes, seeds, leaves[9], and emulsions (see reference citations 30–36 in Ref. 12). MAS has also provided substantial line narrowing in one-dimensional ^{13}C and ^{1}H NMR spectra of solvent-swollen polymer gels[43–48] (where MAS removes either the susceptibility broadenings, or the homonuclear dipolar couplings, or both), but conventional MAS probes were used in all of these studies, and these probes are capable of providing only moderate resolution at best (because of the materials used to make the probe).

It was not until an NMR probe was made that combined the use of both MAS and 'magnetic-susceptibility-matched' materials, that the acquisition of high-resolution MAS NMR spectra was made possible, especially for high-field ^{1}H studies. The resulting probe, called a Nano•nmr™ probe (Varian NMR Instruments, Palo Alto, CA, USA), was used by Fitch and coworkers to generate the first 500 MHz NMR spectra of organic compounds still bound to SPS resins that had ^{1}H linewidths as narrow as 8 Hz (Ref. 49). Since that time, Nano•nmr probes have also been used to observe ^{1}H linewidths as narrow as 4 Hz for resin-bound samples (at 500 MHz)[50] (see Figure 1) and even 1 Hz (Ref. 51).

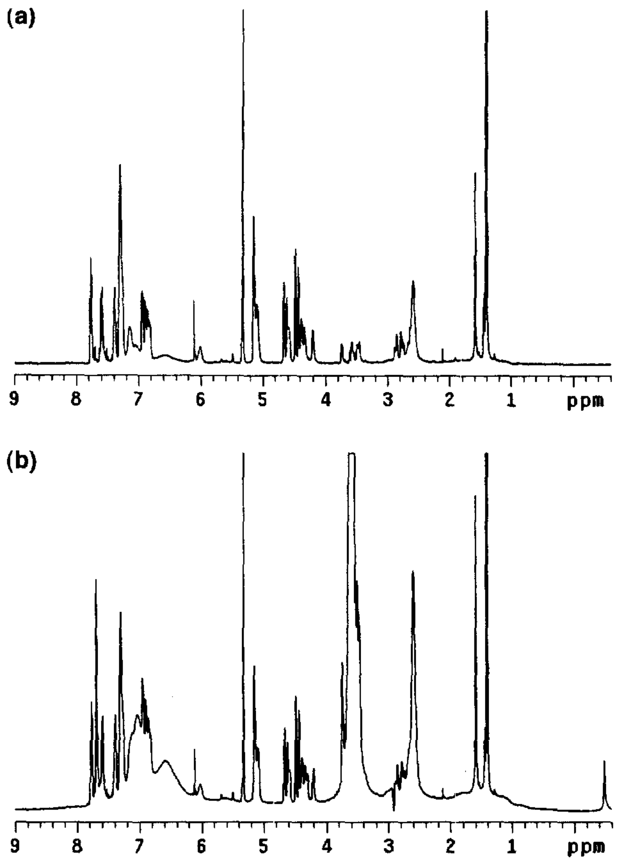

Figure 1. *Proton NMR spectra obtained on a 3 mg sample of Fmoc-Asp(OtBu)-NovaSyn TGA Resin (a Tentagel from Novabiochem) by using a Varian ^{1}H Nanoprobe (500 MHz; 32 scans each). The resin was swollen to a wet slurry in CD$_2$Cl$_2$ and spun at 2050 Hz about the magic-angle axis (54.7° from Z). Data from both a presaturation experiment (a) and a 'one-pulse' experiment (b) are shown (From Ref. 70). This illustrates that presaturation at 3.55 ppm suppresses not only the PEG resonance (at 3.55 ppm) but also the broader polystyrene resonances (at 7.0, 6.6, 2.8 and 1.5 ppm), presumably by spin diffusion. The presaturation experiment works well for all homogeneously broadened lines; the use of presaturation on any probe whose magnetic susceptibility is not properly matched, however, will result in spectra that have broad (and typically asymmetric) residual signals in the baseline (caused by the poor lineshape). In such cases the less discriminating spin-echo-type experiments may be the best alternative.*

The Nano•nmr probe was also the first probe ever designed to place the entire sample within the active region of the receiver coil to achieve the highest possible detection sensitivity. Normally, this sample geometry would generate a bad lineshape that would be both unsymmetric and 'unshimmable' [the lineshape distortions would arise from the elimination of the infinite-cylinder-approximation sample geometry; this creates magnetic-susceptibility discontinuities (at the sample–air and sample–container interfaces) near the receiver coil]. The use of moderate-speed (1–3 kHz) MAS spinning of the sample, however, causes all the magnetic-susceptibility terms in the linebroadening equations for the spinning sample to go to zero[52,53] so the probe can now generate narrow linewidths; hence this Nanoprobe can properly be thought of as a normal high-resolution NMR probe that just happens to use MAS.

Because a slurry of an SPS resin is heterogeneous, it contains magnetic-susceptibility variations within the sample that also cause linebroadenings unless MAS is used. While the advantages of using MAS for SPS-resin NMR are documented above, and Nanoprobes are now being widely used for that purpose[54–57], the Nano•nmr probe is also designed to handle small-volume (≤40 μl) liquid samples. It is the highest-sensitivity-per-nucleus NMR probe commercially available (with the possible exception of high-temperature-superconductor coil probes) and it has proven itself useful in obtaining ^{1}H and ^{13}C NMR data on sample-limited solution-state samples dissolved in both organic solvents and 90:10 H_2O:D_2O (Refs 58–60). This is in contrast to the conventional or 'high-resolution' MAS probes used in other resin studies, which typically:

- produce broader lineshapes[61,62] that do not allow presaturation to be used;
- have lower filling factors and less efficient detection sensitivities[63] that require larger volumes of sample (i.e. 7-mm rotors containing 75 mg of resin[64,65]); and
- cannot handle solution-state samples[61,62] because of solvent leakage.

Although an alternative for acquiring solution-state NMR data on small homogeneous samples is to use either spherical microcells or magnetic-susceptibility-matched polymer plugs or sample tubes, these tools attempt to minimize only the effect of the susceptibility discontinuities, and only those discontinuities that surround the sample. This makes these special cells and plugs useless for acquiring SPS resin spectra. In contrast, MAS completely eliminates the effects of all discontinuities both around and within the sample. The ability to eliminate these linebroadening effects becomes increasingly important at higher magnetic fields and with higher-frequency nuclei (such as ^{1}H), because then the linebroadening becomes more severe, and the chemical-shift dispersion becomes smaller[9,10]. Note that the susceptibility broadening scales with both the observation frequency and the field strength (^{1}H linewidths may be up to four times broader than ^{13}C linewidths, and 600 MHz ^{1}H linewidths may be up to two times broader than 300 MHz ^{1}H linewidths) – assuming that the linewidth is determined primarily by magnetic-susceptibility contributions and not other interactions (such as restricted motion, homonuclear dipolar couplings, or chemical shift heterogeneity)[26,48]. Studies finding that '13-Hz wide ^{13}C lines' obtained on a 300 MHz spectrometer are 'entirely satisfactory'[62] contrast with concerns that this 13-Hz-wide carbon resonance has <25% of the signal-to-noise of a corresponding 3-Hz-wide carbon resonance; a corresponding proton signal at 600 MHz could be as much as eight times wider.

In addition to magnetic-susceptibility discontinuities within the sample, the narrowest NMR linewidths and lineshapes can only be obtained if all the materials used *within the probe* (which don't undergo MAS) are also designed and constructed in a manner that minimizes magnetic-susceptibility discontinuities[66,67]. Although this has long been a critical consideration in building modern high-resolution liquid probes, it has historically not been a consideration in building MAS probes because in their typical application – solid-state NMR – high spinning speeds, high-power handling, and wide variable-temperature-range issues dominate. The recent interest in resin NMR, however, is causing some conventional-design MAS probes to be redesigned to incorporate various degrees of susceptibility matching. The quality of this susceptibility matching is directly reflected in the narrowness of both the linewidth and (especially) the lineshape.

High spin rates do not appear to be a critical issue in acquiring SPS resin spectra[12]; a minimum of ≥1 kHz is typically sufficient (because spin rates greater than the nonspin linewidth are required[42]), although spin rates of 2–3 kHz are often used. If the sample is non-rigid, and if the probe is well susceptibility-matched, the intensity of spinning sidebands should be low (<1%), but if the side bands still complicate the ^{1}H NMR spectrum, the spin rate can be adjusted to move the spinning sidebands of large peaks away from any small peaks of interest. The spin rate is less critical for ^{13}C NMR spectra because the spinning side bands are not normally visible[12]. Transparent sample cells are preferable because they facilitate sample handling (spin rates of up to 5 kHz can be achieved by clear glass 4-mm rotors); the sample cells themselves do not need to be constructed of susceptibility-matched materials (because they will be spinning at the magic angle), but they should provide a liquid-tight seal.

Resin and solvent requirements

One measure of the quality of the NMR spectra of resin-bound materials is the achievable linewidths. The first high-resolution ^1H studies of resin-bound compounds[49], in which ^1H linewidths as narrow as 8 Hz were obtained (at 500 MHz), used compounds bound to Tentagel resin (Rapp Polymere)[26,39] (5 mg) slurried in DMSO-d_6. Subsequently, ^1H linewidths of <6 Hz and ^{13}C linewidths of <3 Hz were obtained on a 2 mg sample of Tentagel S PHB Cys(Trt)Fmoc (Novabiochem) in CD_2Cl_2 (unpublished data), and 500-MHz ^1H linewidths as narrow as 4 Hz (Ref. 50) and 1 Hz (Ref. 51) have been observed for some resins.

In practice, variable-quality spectra are often obtained, depending upon the resin and solvent used. It is well known that different resins swell to different degrees in various solvents[18,20,68,69] and it is tempting to assume that the greater the extent of swelling, the narrower the NMR linewidths will be, as a result of increased motion within the swollen resin[26]. Systematic studies of the influence of different solvents upon the ^1H NMR spectra of different resin structures[50,57,65,70] have demonstrated, however, that this theory is far too simplistic to account for the observed linewidths.

Solvent choice and specificity

The achievable NMR linewidths depend primarily upon the choice of resin used. (Note that the choice of resin is usually based upon its synthetic utility rather than its NMR properties.) Resins that offer the bound compounds the greatest mobility, such as those resins containing long PEG tethers, produce the narrowest ^1H NMR linewidths[50,57]. The choice of solvent then becomes the second-most important issue, but the solvent can still have a huge impact upon the quality of the NMR spectra (Figure 2). Unfortunately, the choice of an optimal solvent depends not only on the type of resin used, but also on the type of compound bound to the resin (because all resins require solvent swelling to produce acceptable spectra). Regardless of the probe, resin or solvent used, however, virtually every ^1H spectrum will still consist of narrow resonances (1–25 Hz) for the bound compounds of interest, superimposed upon a range of broader resonances (50–300 Hz) arising from the cross-linked polymer core (Figures 1b, 2a and 2c).

One explanation for this solvent specificity is that narrow NMR resonances can be generated only if both the compound and its supporting structures (the cross-linked polymer and any tethers) are well solvated. When a hydrophilic compound is bound to a hydrophobic polymer, any solvent that swells the resin bead is probably solvating the larger internal core of the resin (the polystyrene), but this will not necessarily correspond to increased solvation (and hence motion) of the compounds *bound* to the resin core, especially if they are bound to the resin core via a long hydrophilic PEG tether (as in the Tentagel resins). If a single solvent is used to slurry the resin, that solvent must solvate all parts of the resin; this suggests that the choice of solvent also depends upon the compound bound to the resin. Dichloromethane-d_2 is a good first choice of solvent for the following reasons:

- the swelling properties are good;
- it usually produces narrow linewidths for resins;
- its solvent resonances are in uncongested regions of the NMR spectra (^1H triplet at 5.32 ppm, ^{13}C quintuplet at 53.8 ppm); and
- it is easy to remove by evaporation (which facilitates the preparation of the NMR sample).

DMF-d_7 and $CDCl_3$ are good second choices, with DMSO-d_6 producing variable results.

A common phenomenon in many NMR spectra is a second set of solvent resonances[12,25,50,71]. These are known to arise from solvent molecules entrained in a different environment within the solvent-swollen polymer bead. This means that resin slurries should be made quite 'wet' with a significant excess of solvent to maintain proper referencing (and locking) and to avoid a mis-set chemical-shift scale. Despite concerns to the contrary[62], numerous experiments (including some unpublished work) have shown that even vast excesses of solvent do not cause any linebroadenings caused by 'phase separations'[12,72]; in fact, the stronger (and narrower) field/frequency lock signal created by an excess of deuterated solvent actually ensures the narrowest NMR linewidths.

Sensitivity limitations

One extreme in combinatorial chemistry methodology is the 'one-bead, one-compound' library[4,73]. The ultimate test of NMR sensitivity in this case would be the ability to detect signals arising from only a single bead of SPS resin. This was accomplished when the ^1H resonances from only a single 100 μm bead were detected in less than 3 h by using a Nanoprobe[72]. An estimated 500 picomoles of sample were available for detection. Because fingerprint oils and other solvent contaminants can overwhelm the desired signals from a sample this small, ^{13}C-labeled methoxy groups were

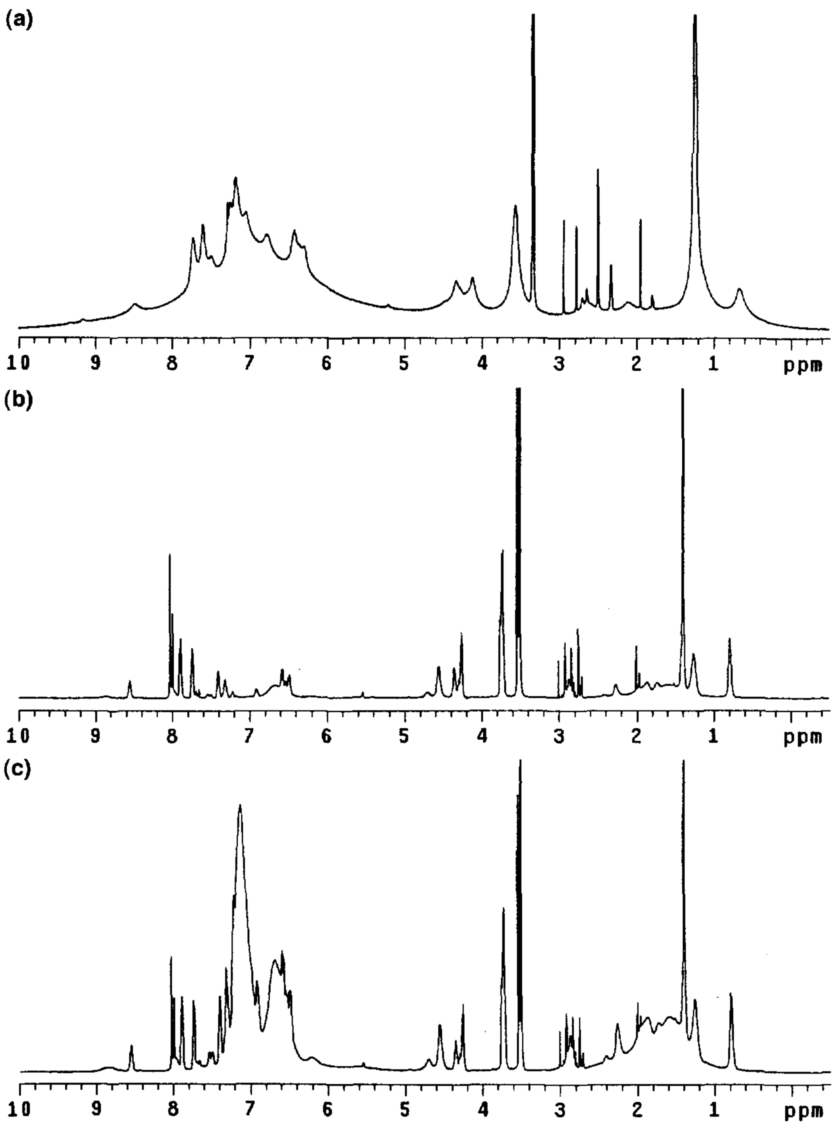

Figure 2. *Proton NMR spectra obtained on a 3 mg sample of Fmoc-Asp(OtBu)-Rink amide MBHA resin (Novabiochem) by using a Varian* ^1H *Nanoprobe (500 MHz; 32 scans each). The top spectrum (a) is the result of a 'one-pulse' experiment on the resin in DMSO-*d_6*. The bottom spectrum (c) is the result of a 'one-pulse' experiment on the resin in DMF-*d_7 *and the middle spectrum (b) additionally used a presaturation pulse on the 7.12 ppm resonance (on the same sample – in DMF-*d_7*). A comparison of spectra (b) and (c) shows the quality of background suppression possible by using presaturation on a polystyrene resin (as compared to the Tentagel resin used in Figure 1), while a comparison of spectra (a) and (c) shows the dramatic influence that an (im)-proper choice of solvent can have on the attainable linewidth. In all three cases, the resin was swollen to a wet slurry in an excess of the appropriate solvent, then spun at the magic angle at approximately 2 kHz. From Ref. 70.*

synthetically attached to the bead so that isotope-filtered experiments [1D and 2D $^1H\{^{13}C\}$ heteronuclear multiple-quantum coherence (HMQC)] could be used to detect selectively the protons bound to the ^{13}C-labeled groups. This single-bead detection capability could be used to decode a 'tag' or 'barcode' placed on the bead to record the synthetic-reaction history[74]. The 'tag' could be encoded by using different combinations of ^{13}C labeled nuclei on each bead (because each unique ^{13}C label would typically exhibit its own characteristic ^{13}C chemical shift). Both the regiochemistry of each ^{13}C label (as reported by the ^{13}C chemical shift) and the number of ^{13}C labels present on each bead could be used to encode information. For example, the presence or absence of any one of the three forms of ^{13}C-labeled acetate (C-1 mono-, C-2 mono- or C-1,2 di-labeled acetate) could encode three different bits of information, and this information would be quite distinct from the presence (or absence) of a labeled methoxy-ester carbon elsewhere on the bead. (It might even be possible to use the slightly different ^{13}C chemical shifts of any labeled acetate groups that are bound to different compounds – or to different locations within one compound – to encode information.) Other NMR-active nuclei (such as ^{19}F, ^{15}N, ^{31}P or ^2H) could also be used to encode information (see below). Although most 'tags' are normally located in separate structures on the beads, an isotope label also allows the information to be encoded either within the linker or within the compound itself. Uniquely labeled linkers could be used to identify different starting scaffolds or batches of resin, or the compounds themselves could incorporate uniquely ^{13}C-labeled compounds at different stages of the reaction history; isotopic labels like this represent the most chemically inert tags available, and NMR analysis (being nondestructive) would leave the bead fully intact (this also avoids the need for a parallel synthesis of a separate coding structure on each resin). The demonstration that 2D $^{1}\mathrm{H}\{^{13}\mathrm{C}\}$ HMQC data can also be obtained shows that *mixtures* of different resin beads could be easily deconvoluted. Data recently obtained on a newer-design Nanoprobe suggest that it will soon be possible to acquire similar 2D $^{1}\mathrm{H}\{^{13}\mathrm{C}\}$ HMQC datasets on this same single-bead sample in about 15 minutes (data unpublished).

Single bead detection has also been accomplished on large (400–750 μm) resins using both conventional liquids and MAS NMR probes[63]. These large ('microreactor') resins, however, are designed to contain 1,000 times more sample per bead than a 100 μm resin, and are not in routine use.

Advanced, multidimensional and multinuclear NMR methods

The utility of advanced one-dimensional and two-dimensional NMR experiments has also been recognized. Proton-detected homonuclear (COSY, DQFCOSY, NOESY and TOCSY) and heteronuclear ($^{1}\mathrm{H}\{^{13}\mathrm{C}\}$ HMQC) 2D NMR data have all been reported, as have ^{13}C-detected APT, DEPT and HETCOR (^{1}H-^{13}C heteronuclear correlation) spectra (Box 1), all on resins in either true high-resolution MAS probes[12,57,72,75] or in standard- to moderate- to 'high'-resolution MAS probes[61–65,76,77].

Resin spectra often are complicated by broad signals arising from the polymer support itself. Although either the linewidths or the chemical shifts of the desired components may sometimes be similar to those of the polymer support (which complicates the suppression of the polymer signals), in many favorable cases the 'background' signals from the polymer can be removed by selective presaturation[12], spin echoes[75,76], or both[57]. Presaturation is particularly effective because it also removes spinning-sideband artifacts (Figures 1a and 2b), although it emphasizes the mis-shapen lineshapes of any 'high-resolution' MAS probes for which magnetic-susceptibility is not properly matched. Spin echoes and backwards-linear-prediction processing can also remove broad resonances, but their usefulness is minimal because resin spectra often exhibit a continuous range of linewidths.

Although almost all resin spectra published to date have used ^{1}H and ^{13}C NMR, multinuclear NMR is also possible. While multinuclear NMR studies of resins that did not use MAS are well known (see above), only one report of MAS multinuclear NMR (using ^{19}F) has been published[78]. My recent experiences with ^{19}F, ^{2}H, ^{29}Si, and ^{31}P MAS-NMR of SPS resins, however, indicate that there is a significant amount of unrecognized potential in multinuclear NMR.

Box 1. Abbreviations used in the text

APT	Attached proton test
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DQFCOSY	Double-quantum filtered correlation spectroscopy
HETCOR	$^{13}\mathrm{C}\{^{1}\mathrm{H}\}$-Heteronuclear correlation
HMQC	Heteronuclear multiple-quantum coherence
LC-NMR	Liquid chromatography nuclear magnetic resonance
MAS	Magic angle spinning
NOESY	Nuclear Overhauser effect spectroscopy
ppb	Parts per billion
PFG	Pulsed field gradient
TOCSY	Total correlation spectroscopy
VAST	Versatile automated sample transport
WET	Water suppression enhanced through T_1 effects

Solution-phase NMR: high-throughput NMR spectroscopy

In most drug discovery programs, the bioassays are conducted on compounds in solution rather than bound to a solid support. All compounds will therefore eventually be cleaved into solution, regardless of whether they were originally synthesized by solution- or solid-phase (SPS resin) synthetic methods. Thus, although the NMR analysis of the solid-phase (resin-bound) compounds will be important during the initial stages of a drug discovery program (to monitor the progress of SPS reactions) the confirmation (or determination) of the structure of the compounds actually being assayed can be accomplished by using a more traditional solution-phase NMR method.

With a lead-optimization library, it is probably desirable to characterize every compound being bioassayed, whereas in a lead-generation library, it is likely that only the bioactive 'hits' will need to be characterized. Traditional solution-phase NMR could play a role in either case, although, in practice, few drug discovery programs currently obtain NMR data at the bioassay stage (even though NMR is used during the initial synthetic method development stages and the subsequent scale-up production stages). It is typically assumed that the acquisition of NMR data is difficult at the bioassay stage for the following reasons:

- the samples are normally dissolved in protonated solvents, which generate large background signals that complicate the NMR spectra;
- the volumes of solution are often too small for conventional NMR spectroscopy (<500 μl);
- the samples are often stored in containers that an NMR probe cannot use (i.e. 96-well microtiter plates); and
- conventional NMR analysis is too slow, especially because the number of samples is very large.

These problems have now been solved by some recent developments in LC-NMR, which are allowing high-throughput NMR spectroscopy (especially as is needed for combinatorial chemistry) to be developed[79].

Solvent suppression

The first problem (background signals arising from protonated solvents) has long been an issue in LC-NMR (Ref. 80). It has been addressed either by using presaturation solvent suppression[81] or fully deuterated solvents[82,83], but a much more effective and general solution – 'WET' solvent-suppression – has been invented[84]. WET uses a series of four frequency-selective (shaped rf) pulses, each having simultaneous ^{13}C decoupling and each followed by a pulsed field gradient (PFG); the resulting four-element pattern has been optimized to create a highly-effective solvent-suppression pulse sequence element that has been incorporated into a number of 1D- and 2D-NMR sequences[84]. The advantages of WET are as follows:

- it provides high-quality solvent suppression for even protonated solvents;
- it routinely uses multifrequency shifted-laminar pulse (SLP; or 'phase-ramped') pulses[85], so it is capable of suppressing multiple solvent resonances (with only *one* NMR transmitter);
- it selectively eliminates the ^{13}C satellites of solvent resonances (like CH_3OH and CD_3CN) without bleaching or otherwise affecting coincident resonances;
- it is 'robust' because it self-compensates for miscalibrated pulsewidths, mistuned probes, or flowing samples;
- its suppression is fast (<100 ms) so it generates the highest sensitivity (most scans) and best digital resolution (longest acquisition time) per unit time; and
- it provides the highest-quality solvent suppression under both stopped-flow and flowing conditions, all without optimization.

In addition, a routine called the 'SCOUT-Scan' method has been developed to fully automate the setup of WET (the solvent suppression conditions and NMR parameters); the SCOUT-Scan method reduces the setup of WET to a quick one-button operation[84].

On-flow probes for small-volume samples

The second problem (small sample volumes) has been addressed by the development of high-sensitivity 'on-flow' (LC-NMR) probes designed for small-volume samples (i.e. HPLC peaks). These NMR probes are then directly connected to the HPLC hardware so that the samples flow through narrow-bore tubing directly into the NMR coil; these probes do not use conventional sample tubes. These 'on-flow' probes have characteristics that are uniquely suited to high-throughput NMR spectroscopy, namely:

- their high sensitivity enables them to detect small samples in quantities from micrograms down into the hundreds-of-nanograms range (from 1–100 nanomoles or less);
- their fixed sample geometry minimizes the need to re-shim each sample;
- their PFG coils allow very fast automatic ^1H or ^2H gradient shimming of the sample (if necessary); and
- expensive sample tubes never need to be bought, filled, inserted, broken, changed or cleaned.

These developments have now elevated LC-NMR into a high-performance technique for the analysis of samples needing (typically reversed-phase) chromatography. A typical system configuration is: [HPLC pump]–[injector]–[HPLC column]–[optional UV detector]–[NMR probe] (Method 1). The performance capabilities of a modern-generation LC-NMR are especially made more powerful if the system is equipped with HPLC-to-NMR communication and control, WET solvent suppression and SCOUT-Scan automation (to set up the solvent-suppression conditions and parameters for the NMR automatically). Although it is referred to as 'on-flow' NMR, the solvent flow may always be temporarily paused to trap the sample in the detector coil during acquisition (to allow time averaging to be used to improve the sensitivity). The critical aspect is the concept of delivering a sample to the detector (the NMR coil) by using a flowing liquid (the mobile phase or the solvent).

The problems of microtiter-plate formats and limited throughput can be solved by incorporating a full-function HPLC autosampler at the front end of this LC-NMR. The Gilson Model 215 Liquid Handler (Gilson, Middleton, WI, USA) is capable of autosampling from microtiter plates (or a wide variety of other vials or tubes) with a variable-sized syringe into a variable-sized injector loop – all under computer control. This creates an 'autosampled LC-NMR' in which the autosampler, the LC pump, the LC detector and the NMR are all interconnected and suitably programmed for coordinated communication such that any series of chromatographic peaks can be automatically stopped in the NMR coil for additional signal averaging (Method 2).

'Columnless' LC-NMR

The system can then be simplified by removing the HPLC column; when the autosampler injects an aliquot of sample from a microtiter plate, the LC mobile phase will sweep it through the tubing and into the NMR probe. As before, the solvent flow can be automatically paused to retain the plug of injected sample in the NMR coil until the signal averaging is complete; either by using an optional LC detector to detect the sample plug and stop the pump (after a precalibrated lag time) or by simply pumping for a known solvent volume (the dead volume of the tubing that connects the injector to the probe). This process, when combined with the SCOUT-Scan technique and optional locking and shimming, is quite fast; if a ^{1}H NMR spectrum requires one minute of signal averaging (e.g. 32 scans), the entire 'deliver/acquire/flush-out' process for each sample may take as little as 3 min. An example of the attainable sensitivity and spectral quality of this 'columnless LC-NMR' (Method 3) is shown in Figure 3c.

Methods 1 and 2 (LC-NMR and autosampled LC-NMR) have the advantage of being able to provide chromatographic separation for mixtures, but only at a disadvantageous increase in analysis time. Method 3 (and Method 4 below) use the opposite approach – no chromatographic separation is necessary (or even desired), so the analysis time is fast. Methods 3 and 4 are designed to evaluate the entire intact sample and allow either the sample's identity or its purity to be rapidly confirmed and documented. Methods 1 and 2 parallel the capabilities of LC-MS, whereas Method 4 (below) parallels direct-injection mass spectrometry; columnless LC-NMR (Method 3) blends the characteristics of both. Although columnless LC-NMR is expected to be less useful than Method 4, it may be better for the analysis of samples dissolved in aggressive solvents, or samples containing reactive reagents that need to be quenched or hydrolyzed. It could also allow every sample to be analyzed at a controlled (buffered) pH, or, with the aid of a few theoretical plates of chromatographic or ion-exchange media, could separate the solutes of interest away from associated salts or NMR-unfriendly solvents.

High-throughput NMR spectroscopy

This system can be simplified even further by removing the HPLC pump, the detector, and the mobile phase, and replumbing the system to create an [Autosampler]–['injector']–[NMR probe] system. In this direct injection system (Method 4), the autosampler takes a sample aliquot from a microtiter plate (typically 100–300 μl of a 'one-well, one-compound' sample) and, by using the same 'injector', loads it directly into the detector cell of the NMR probe – without the use of any mobile phase or dilution. The NMR spectrum can be taken immediately (see Figures 3a and 3b). After completion, the process is reversed to return the intact sample directly back into the microtiter plate (or the sample can be flushed to a waste container). Because of the generic utility of this system, it has become known as versatile automated sample transport (VAST) NMR.

Because the throughput of this system can be less than 3 min per sample (including rinses of the probe and syringe), it constitutes high-throughput NMR spectroscopy. VAST refers specifically to the use of SCOUT-Scan automated setup and WET solvent suppression, combined with a bottom-loading NMR on-flow probe. It produces higher sensitivity than 'columnless LC-NMR' because there is no dilution; this together with the simpler plumbing also allow the sample to be recovered intact. The ability of VAST-NMR to acquire one-dimensional proton spectra of every sample in a 96-well microtiter plate within a few hours suggests that it could play a significant role in analyzing drug discovery libraries.

Future potentials for VAST

VAST is a specific implementation of direct-injection NMR that uses an innovative combination of technologies and can clearly be used to acquire high-quality NMR spectra in a rapid and automated fashion for samples dissolved (1 mg/ml) in mixtures of either CH_3CN or CH_3OH and either

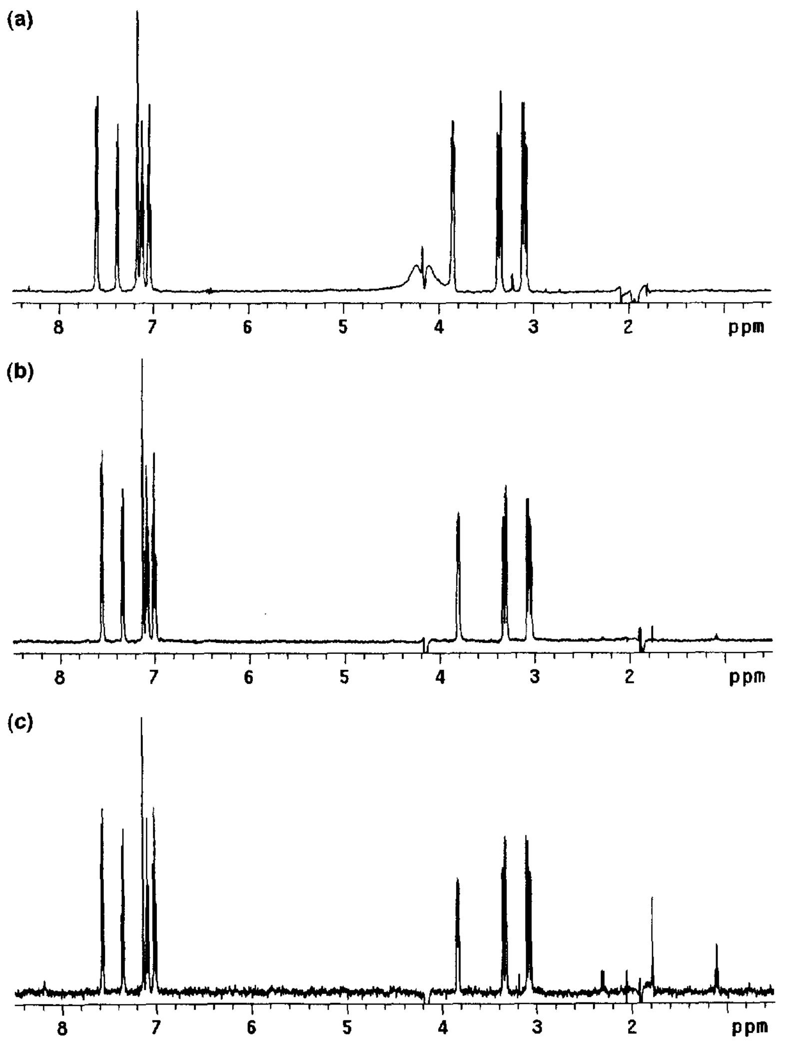

Figure 3. *Direct-injection and 'on-flow' ^1H spectra acquired on a tryptophan solution (1 mg/mL). WET solvent suppression was used for both the CH_3CN resonance (at 1.95 ppm) and for the D_2O / HOD / H_2O resonance (at 4.8 ppm); WET also selectively suppressed the ^{13}C satellites of acetonitrile. The top spectrum (a) was acquired with VAST NMR, with the tryptophan dissolved in a fully protonated solvent mixture (1 mg/ml tryptophan dissolved in 50:50 CH_3CN:H_2O). This is possible because WET suppression does not require the NMR to maintain a ^2H 'lock' for stability of the magnetic field (so a deuterated solvent is not required) and WET is fully capable of high-quality H_2O-signal suppression. The middle spectrum (b) was also acquired by using VAST NMR to inject a tryptophan solution directly into the probe (and return it directly to the sample container when finished), but a different tryptophan solution (1 mg/ml in 50:50 CH_3CN:D_2O) was used. The bottom spectrum (c) was acquired using 'columnless' LC-NMR, whereby 100 µl of a solution of tryptophan (1 mg/ml in 50:50 CH_3CN:D_2O) was injected into a flowing mobile phase – also composed of 50:50 CH_3CN:D_2O (in 'columnless' LC-NMR the mobile-phase sweeps the sample from the injector loop into the NMR probe for detection, then rinses out the NMR probe at the end of signal averaging; analogous to a 'stopped-flow' version of a 'carrier gas' in GC). All spectra were acquired at 500 MHz, using 1 min acquisitions (32 scans each), without any form of chromatographic separation.*

H_2O (Figure 3a) or D_2O (Figures 3b and 3c). Extensions to other ^{1}H-NMR-friendly solvents (for which proton resonances are singlets) such as $CHCl_3$ or DMSO are forthcoming.

Although the interpretation of all these spectra remains a bottleneck, progress in the development of analysis tools is being made. Specific details are beyond the scope of this review, but recent developments in the calculations of NMR spectra from chemical structures, as well as in the spectral analyses of experimental data[86] offer the future potential of a fully automated microtiter-plate NMR-analysis system for combinatorial libraries. These tools will become even more important as solution-state combinatorial synthesis methods (e.g. dendrimer methods[38]) supplant the now-common SPS methods, and as ever-more powerful hyphenated techniques such as LC-NMR-MS (Ref. 87) evolve.

There are two reasons to focus on the analysis of solution-phase samples. First, most drug discovery programs perform the all-important bioassays on the cleaved (solution-state) compounds. Second, the automated manipulation of a homogeneous liquid is easier and more robust than the automated manipulation of a heterogeneous SPS-resin slurry. The direct structural characterizations of the compounds that are actually being bioassayed, regardless of whether those compounds are synthesized by solution- or solid-phase (resin) methods, are probably the most important analyses in the entire drug discovery process.

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