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New methods for obtaining high-resolution NMR spectra of solid-phase synthesis resins, natural products, and solution-state combinatorial chemistry libraries

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Introduction

The primary goal of combinatorial chemistry is to produce more compounds, and a wider variety of compounds, in a shorter period of time. Recent developments in solid-phase synthesis, automation, information sciences and high-throughput screening have all contributed to speeding up the entire drug discovery process (1-6) so that the chemical analysis of the resulting samples is now a rate-limiting step. In addition, if solid-phase synthesis resins are used to produce compounds, the physical heterogeneity of a resin sample causes analysis difficulties that are well recognized (7-10). This is in contrast to a combinatorial chemistry library of solution-state samples, however, where the analysis difficulties are often largely due to just the very large numbers of samples being produced, especially when the term "combinatorial chemistry" is used to refer to a large library of pure compounds (as seen in multiple-parallel synthesis programs which are designed to produce collections of pure compounds). Although nuclear magnetic resonance (NMR) spectroscopy is often considered to be one of the most information-rich analytical techniques available, it has the reputation of requiring homogeneous samples, being too insensitive (or requiring large sample sizes), being too slow and requiring expensive deuterated solvents. This review will cover each of these issues in more detail to see how recent developments are now allowing NMR spectroscopy to play several new and different roles within a modern drug discovery program.

While combinatorial chemistry has embraced solid-phase synthesis (SPS) as one of its fundamental tools (11-13), it is unfortunate that (until recently) there have not been any analytical techniques that were useful for compounds still bound to SPS resins (7, 14-15). Conventional analytical techniques (*e.g.*, mass spectrometry [MS], HPLC and high-resolution [HR] NMR) normally require homogeneous samples, meaning that any samples needing analysis must first be cleaved from the SPS resin. This presents a significant limitation for any chemist needing to either monitor a reaction in progress or characterize an intermediate in a multistep synthesis; every cleavage reaction takes time, potentially alters the product, is probably irreversible and by definition lowers the yield of product. Several recent developments in NMR spectroscopy have eliminated this sample cleavage requirement, which means that high-resolution NMR spectra can now be obtained for samples still bound to SPS resins; these developments are the subject of the first part of this review.

The second half of this review will focus on some recently developed techniques which can now be used to more rapidly acquire NMR spectra of solution-state samples. While these tools were created originally for LC-NMR applications that may initially appear to be unrelated to combinatorial chemistry, these methods have recently been combined (in several different ways) to create tools which can acquire NMR spectra on combinatorial

chemistry samples in a rapid and automated fashion. This capability begs to be called "high-throughput spectroscopy" (HTS).

SPS resin analysis: gel-phase NMR

The NMR analysis of organic compounds is most effective only if the NMR data have good spectral resolution (narrow linewidths). This is typically obtained by analyzing solution-phase samples which are *physically* homogeneous and then "shimming" the static magnetic field (B_o) of the NMR magnet until it is *magnetically* homogeneous (to within ppb). All substances have a measurable physical property called "magnetic susceptibility" that measures the effect that substance has on surrounding magnetic fields. A uniform (homogeneous) substance will cause only a uniform change of the applied (static) magnetic field, an effect which can be easily "shimmed" out to generate a narrow linewidth and lineshape, especially if the sample forms a cylinder that appears to be infinitely long relative to the detection coil. A heterogeneous sample (*e.g.*, a slurry of a SPS resin), however, will contain regions of differing magnetic susceptibility throughout the sample (since the resin and the solvent will have different susceptibilities). The resulting variations (distortions) in the magnetic field will create linebroadening effects that are not correctable by shimming the field or by spinning the sample about the vertical (z) axis (16, 17).

Restricted molecular motions within a sample can also make the NMR lines broad; this is one of the reasons why solid samples exhibit broad resonances (18). Since significant amounts of motional freedom are required to generate narrow linewidths, all SPS resins are swollen in solvent (as much as possible) prior to acquiring NMR data. The technique of acquiring NMR data on these samples is called "gel-phase" NMR, since the resulting solvent-swollen slurries are neither fully solid nor fully liquid; unfortunately, the term gel-phase NMR has evolved to now refer only, and specifically, to the use of a conventional liquids probe (a probe in which the sample-spinning axis is aligned along the magnet bore [z] axis) to acquire the NMR data.

The ^{1}H NMR spectra of solvent-swollen resins obtained by this gel-phase NMR method (in which the sample is oriented along the z axis) typically exhibit very broad resonances, often 100-300 Hz or more (19, 20), as seen in Figures 1a and 2a. This linebroadening can arise from either limited motional freedom, chemical shift heterogeneity (*vide infra*), or magnetic susceptibility variations within the sample. Since the linebroadening effects of the magnetic susceptibility variations scale up with the NMR frequency, lower frequency nuclei (like ^{13}C) may produce linewidths of only 25-75 Hz. If the observed nucleus also happens to have a large chemical shift range (which ^{13}C does) the resulting spectra may be resolved well enough to allow reasonably easy structural assignments. Although this method has been used primarily for ^{13}C NMR (9, 21-30), the NMR spectra of other nuclei such as ^{19}F (25, 31, 32), ^{31}P (33-35) and ^{2}H (27) have also been reported.

While gel-phase ^{13}C NMR spectra do have a favorably large spectral dispersion, the spectra of natural abundance ^{13}C nuclei exhibit poor sensitivity; this has been compensated for by incorporating ^{13}C labels near the reaction site of interest. This method, called "FAST" ^{13}C NMR, has proven itself useful in monitoring the progress of reactions (24, 36-39).

Because of the low resolution of the gel-phase NMR spectra (especially ^{1}H NMR spectra), many users have adopted an alternative strategy called the "cleave-and-analyze" method (15), in which the compounds are simply cleaved from the resin prior to a conventional solution-state NMR analysis (22, 34, 36, 40-42). One drawback to this method is that the cleavage reaction itself can modify the compounds being monitored. This has encouraged research into alternative combinatorial chemistry strategies that might be able to take advantage of the ease of solution-state NMR; this has resulted in the development of the *soluble* polymeric supports like polyoxyethylene (POE) or polyethyleneglycol (PEG), which can be purified by ether precipitation (8, 43-44), as well as the development of the dendrimer supports (45), which can be purified by either size exclusion chromatography (SEC) or ultrafiltration. Fortunately, studies have indicated that the chemical shifts of compounds, regardless of whether they are bound to resins or in solution, are both virtually identical (9, 24, 32) and integratable (24, 34, 46). Since many SPS resins contain unbound materials which can be washed from the resin, solution-state ^{1}H NMR has also proven to be useful in monitoring both the quality and the nature of either the impurities which can be washed from a resin prior to its use (21) or of the leachable impurities generated by the resin during conditions of sample cleavage (47).

SPS resin analysis: high-resolution NMR by MAS

Hardware requirements

It has been known for almost 30 years now that the magnetic susceptibility-induced linebroadenings that can be observed in the NMR of heterogeneous samples (48) can be removed by using magic angle spinning (MAS) NMR (49). This has been demonstrated on a variety of physically heterogeneous systems, including solid-liquid mixtures (49, 50), powdered solids (51), compartmentalized liquid samples (52), membranes (53), seeds (54), leaves (16) and emulsions (55). Magic angle spinning (MAS) has also been shown to provide substantial linenarrowing in one-dimensional ^{13}C and ^{1}H NMR spectra of solvent-swollen polymer gels (56-61) (where MAS was assumed to remove either susceptibility broadenings or homonuclear dipolar couplings, or both), but these studies all used "conventional" MAS probes which are made

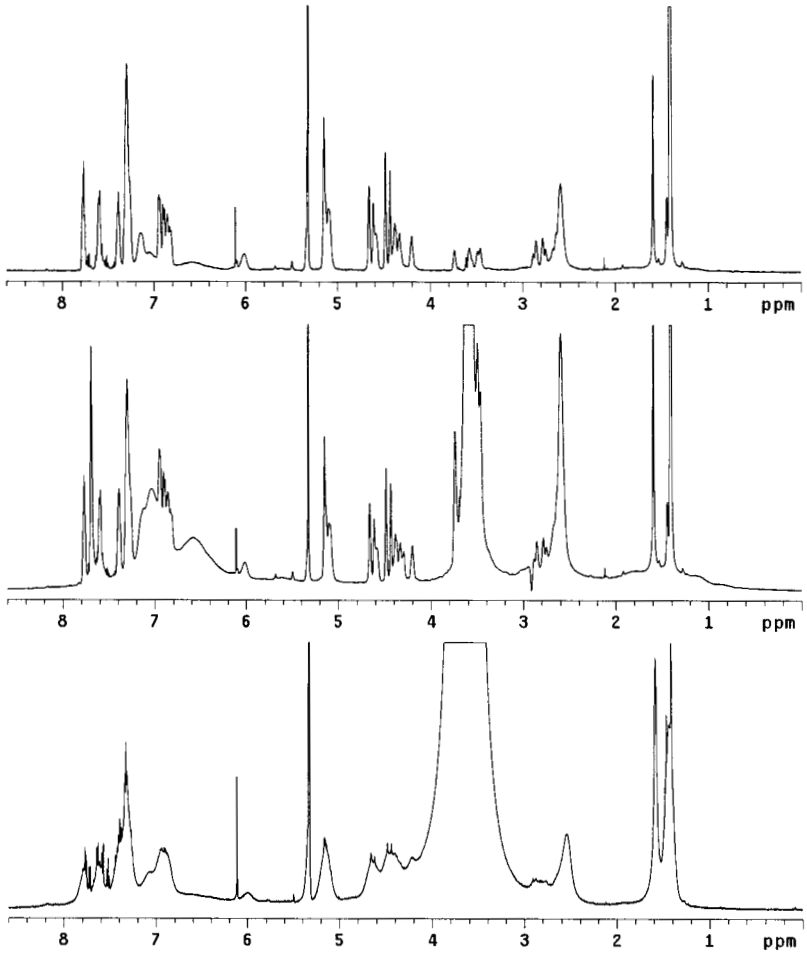

Fig. 1. Three 500-MHz proton NMR spectra obtained on an aspartic acid derivative bound to a Tentagel resin (as an example of a resin which provides "good" ^{1}H NMR data). Fig. 1a (bottom) was acquired with a "conventional" 5-mm high-resolution liquids NMR probe, while Fig. 1b (middle) and 1c (top) were acquired with a ^{1}H Nanoprobe (Varian NMR Instruments). Fig. 1a and 1b were obtained by using a simple "one-pulse" (Bloch decay) experiment, while Fig. 1c included presaturation of the resonance at 3.6 ppm. Notice that the presaturation suppresses not only the 3.6 ppm polyethylene-oxide resonances but also most of the broader polystyrene resonances located at 1.5, 2.8, 6.6 and 7.0 ppm, undoubtedly by a spin diffusion process. Presaturation can clearly be an effective tool for acquiring ^{1}H NMR spectra of all "Tentagel-style" resins; however, if the remaining signals from the line being irradiated look unusual, broad or appear as a large asymmetric lump in the baseline, the magnetic susceptibility mismatches within the probe may not have been properly minimized (this is a characteristic of the probe's design and construction). In such cases the less discriminating spin-echo type experiments may be the next best – or the only – alternative. All spectra were obtained on Fmoc-Asp(OtBu)-NovaSyn TGA resin (Novabiochem) slurried in CD_2Cl_2. Fig. 1a was run nonspin, while the Nanoprobe spectra used an MAS spin rate of approximately 2.0 KHz. The presaturation experiment used a field strength of 114 Hz for 2.0 sec. Data were acquired in 8, 64 and 32 scans on 70, 3 and 3 mg, respectively, and were processed using 0.1 Hz linebroadening. (see ref. 63 and 73)

to provide, by solution-state NMR standards, only moderate resolution. It was not until MAS spinning and proper probe construction (using magnetic susceptibility matching probe technology) were combined within one NMR probe that *high-resolution* MAS spectra, especially high-field ^{1}H spectra, were obtained. The resulting probe, called a Nano·nmr™ probe (Varian NMR Instruments) was used by Fitch and coworkers (62) to make the first observation of ^{1}H linewidths as narrow as 8 Hz in the NMR spectra of organic compounds still bound to SPS resins (at 500 MHz). Since then, Nano·nmr probes have been used to observe ^{1}H linewidths that were as narrow as 4 Hz (63) and 1 Hz (64) for resin-bound samples (at 500 MHz).

The Nano·nmr probe achieves the highest possible detection sensitivity by placing the entire sample within the active region of the receiver coil. By itself, this sample geometry would generate bad lineshapes (which would be both unsymmetric and unshimmable) caused by the magnetic susceptibility discontinuities at the edges of the sample (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 the magnetic susceptibility terms in the linebroadening equations to go to zero (65, 66) so the probe can now

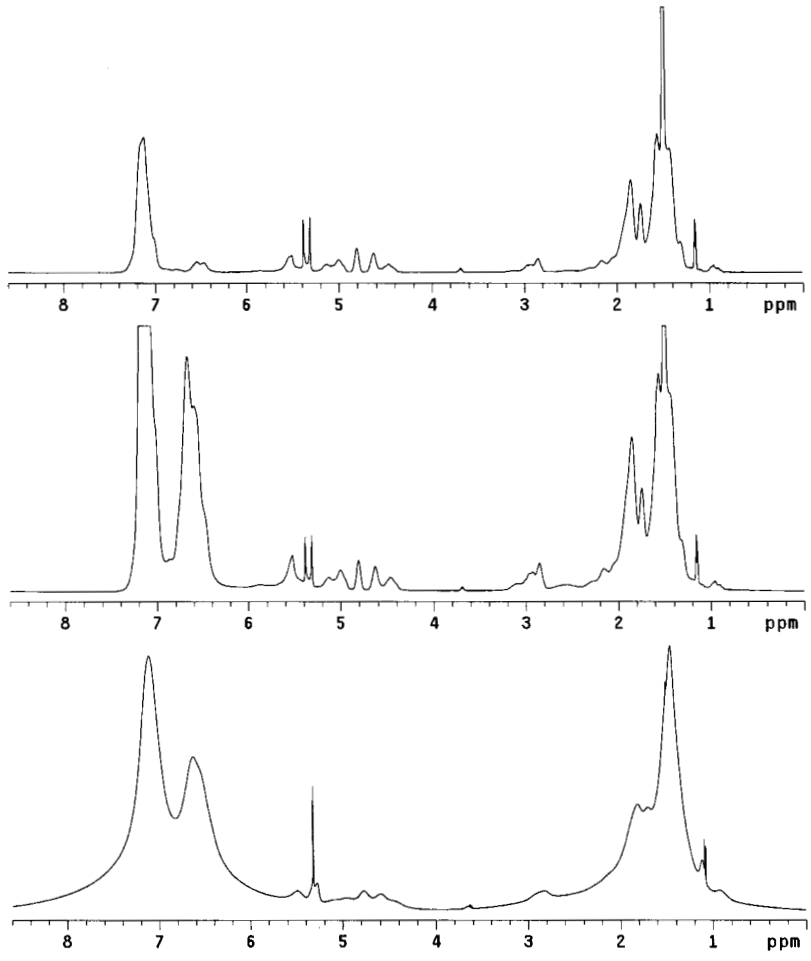

Fig. 2. Three proton spectra obtained on a Merrifield resin (as an example of a resin which provides "poor" ^{1}H NMR data, compared to the Tentagel-resin spectra in Fig. 1). Fig. 2a (bottom) was acquired with a "conventional" 5-mm high-resolution liquids NMR probe, while Fig. 2b (middle) and 2c (top) were acquired with a ^{1}H Nanoprobe (Varian NMR Instruments). Fig. 2a and 2b were obtained by using a simple "one-pulse" (Bloch decay) experiment, while Fig. 2c included presaturation of a polystyrene resonance at 6.6 ppm. In Merrifield style resins, presaturation is typically much less effective at suppressing other polymer resonances in the spectrum; hence, the current interest in developing alternative signal suppression methods (see text). In addition, broader linewidths are typically observed for compounds bound to Merrifield resins, presumably due to the absence of a "tether" structure. Without a tether, the compounds bound to the resin are less mobile, and therefore relax faster (shorter T_2); this means that the ^{1}H spectra of any Merrifield resin will exhibit mostly broad resonances, regardless of the quality of MAS probe used. The sharp resonances at 5.3 ppm are from the CD_2Cl_2 solvent. (All spectra were obtained on BOC-Asp(OcHx)-Merrifield resin (Novabiochem) slurried in excess CD_2Cl_2. Fig. 2a was run nonspin, while Fig. 2b and 2c (the Nanoprobe spectra) used a 2.0 KHz MAS spin rate. The presaturation experiment used a field strength of 114 Hz for 2.0 sec. Data were acquired in 8, 64 and 32 scans on 60, 3 and 3 mg, respectively, and were processed using 0.1 Hz linebroadening. (see ref. 63 and 73)

generate narrow lineshapes; hence, this Nanoprobe can properly be thought of as a normal high-resolution NMR probe that *just happens* to use MAS (a specialized, yet simplified, MAS probe). Conventional high-resolution probes use long vertical sample tubes to create a sample geometry which is long compared to the receiver coil (the so-called "infinite-cylinder approximation"). This facilitates good (narrow) lineshapes by moving all nonsymmetric susceptibility interfaces (*i.e.*, the top and the bottom of the sample) far away from the active volume of the receiver coil; however, since only the middle third of the resulting sample is detected by the receiver coil, this strategy limits the NMR detection efficiency (the sensitivity). The idea of using MAS to overcome these susceptibility-induced lineshape distortions for very small *solution-state* samples is unique to the Nanoprobe (and to Varian), although this application nicely complements the use of MAS to overcome the lineshape distortions (linebroadenings) seen in the NMR spectra of SPS resins. In resin spectra, the linebroadenings occur because the SPS-resin slurry is a heterogeneous sample, and hence has magnetic susceptibility discontinuities (variations) *within* the sample; MAS is the *only* effective tool for removing this source of linebroadening.

While 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 only attempt to *minimize* the effect of the discontinuities, and only those discontinuities *surrounding* the sample, and are hence of no utility in acquiring SPS resin spectra. This is in contrast to MAS which completely *eliminates* the effects of all discontinuities both around *and within* the sample – the ability to eliminate these effects becomes increasingly important at higher field strengths and with higher frequency nuclei (*e.g.*, ^{1}H) since in those cases the linebroadening becomes more severe and the chemical shift dispersion is typically small (16, 17). Note that the susceptibility broadening is proportional to both the observation frequency (^{1}H linewidths may be up to 4 times broader than ^{13}C linewidths) and the field strength (600 MHz ^{1}H linewidths may be up to 2 times broader than 300 MHz ^{1}H linewidths) – assuming that the linewidth is determined primarily by magnetic susceptibility contributions and not by restricted motions, homonuclear dipolar couplings or chemical shift heterogeneities (35, 61). Studies finding that "13-Hz wide ^{13}C lines" obtained on a 300 MHz spectrometer are "entirely satisfactory" (67) contrast with concerns that this 13 Hz wide carbon resonance has less than a quarter of the signal intensity of a 3 Hz wide carbon resonance; the corresponding 600-MHz proton signal could be as much as 8 times wider.

In addition to magnetic susceptibility discontinuities *within the sample*, the narrowest NMR linewidths can only be obtained if all the materials used *within the probe* (which don't undergo MAS) are also designed and constructed so as to minimize magnetic susceptibility discontinuities (68, 69). While this has long been a critical consideration in building modern high-resolution probes for solution-state NMR (as documented in Fig. 3), it has historically not been a consideration in building MAS probes since their typical application (*i.e.*, solid-state NMR) is primarily concerned with high spinning speeds, high-power handling and wide variable temperature ranges. The recent interest in resin NMR, however, is causing some conventional design MAS probes to be redesigned to incorporate various degrees of susceptibility matching. High spin rates don't appear to be necessary for acquiring SPS resin spectra (19); a threshold of ≥1 KHz is typically sufficient (since the spin rate really only needs to be greater than the nonspin linewidth), although spin rates of 2-3 KHz are often used (49). The intensity of spinning sidebands is low (< 1%), but because they may still be visible in ^{1}H NMR spectra, a user may occasionally want to adjust the spin rate to move the spinning sidebands of the large peaks away from any small peaks of interest. The spin rate in ^{13}C NMR spectra is less critical because the spinning sidebands are not normally visible (19). Sample cells made of transparent material are preferred as they facilitate sample handling; since the cells will also be spun at the magic angle they do not need to be constructed of susceptibility matched material (4-mm clear glass rotors are known to handle spin rates up to 5 KHz).

In summary, the observed lineshapes in high-resolution NMR spectra of SPS resins are the sum of linebroadenings arising from: 1) magnetic susceptibility mismatches within the probe; 2) susceptibility discontinuities around the surfaces and edges of small (noninfinite cylinder geometry) samples; 3) susceptibility variations within the (heterogeneous) sample itself; 4) restricted molecular motions; and 5) chemical shift variabilities within heterogeneous samples. Magic angle spinning is the only viable solution to #3, and is a novel (and probably the most useful) solution to #2. MAS will also remove those parts of #1 which are due to the sample container (which rotates during MAS), but most of #1 must be addressed by the materials, construction and design of the probe hardware. Molecular motions (#4) are influenced by the resin's structure and rigidity and by the properties of the solvent used to swell the resin (discussed below). Chemical shift variability, or heterogeneity (#5) causes an apparent "linebroadening", which is actually the net result of many different nuclei precessing at similar *but not quite identical* frequencies, all contributing to a "single" resonance; the observed lineshape is then a measure of the range and distribution of microenvironments within that sample. This effect has been observed in our laboratory in SPS resin spectra (unpublished data).

Resin and solvent requirements

The quality of an NMR spectrum of a resin-bound compound is best evaluated by measuring the minimum achieved linewidths (and lineshapes). The first high-resolution ^{1}H studies of resin-bound compounds, in which linewidths as narrow as 8 Hz were obtained (at 500 MHz), used compounds bound to 5-mg samples of Tentagel resin (Rapp Polymere) (35, 46, 70) slurried in DMSO-d_6 (62). Subsequently, ^{1}H linewidths of < 6 Hz and ^{13}C linewidths of < 3 Hz were obtained on a 2-mg sample of Fmoc-Cys(Trt)-Tentagel S PHB in CD_2Cl_2 (Novabiochem) (unpublished data). More recently, linewidths as narrow as 4 Hz (63) and even 1 Hz (64) have been observed for resin-bound samples in Nanoprobes at 500 MHz.

The quality of the NMR spectra acquired on any given probe can be quite variable, depending upon the resin and solvent used. It is well known that different resins swell to different degrees in various solvents (25, 27, 70-72), and it is easy to assume that a solvent that causes more swelling will produce narrower linewidths due to increased motion within the swollen resin (35). Systematic studies of the influence of different solvents upon the ^{1}H NMR spectra of different resins have demonstrated, however, that this generalization is far too simplistic to account for the observed linewidths (63, 73-75).

The achievable linewidths for the bound compounds are primarily determined by the choice of the resin, although this choice is usually made on the basis of the resin's synthetic utility rather than its NMR properties. Resins which offer the bound compounds the greatest mobility, particularly those resins which contain long PEG tethers, have been shown to produce the narrowest ^{1}H NMR linewidths (63, 73, 74) (compare Fig. 1b and 1c against Fig. 2b and 2c). The choice of solvent then

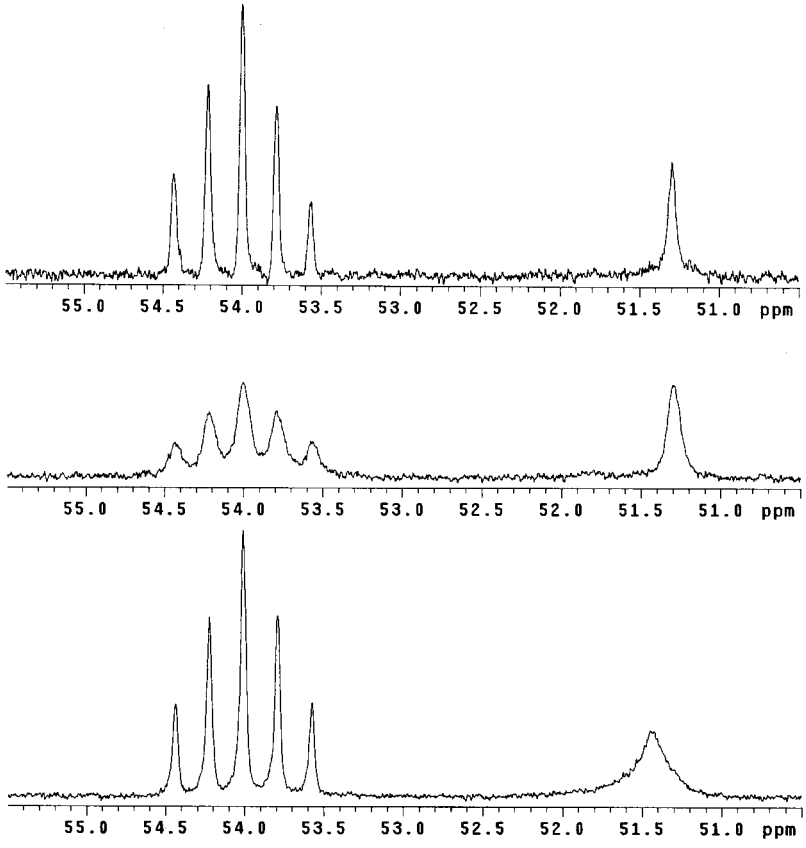

Fig. 3. Expansions of the ^{13}C NMR spectra acquired by using three very different probes on the same SPS resin (Fmoc-Asp(OtBu)-Wang resin [Novabiochem] slurried in excess CD_2Cl_2). Fig. 3a (bottom) was acquired with a conventional 5-mm high-resolution liquids NMR probe, Fig. 3b (middle) was acquired on a "conventional" CP/MAS probe (built without magnetic susceptibility-matching technology) and Fig. 3c (top) was acquired on a ^{13}C Nanoprobe. The right-most resonance arises from a nucleus bound to the resin, while the left-most resonances (at 54 ppm) are from the deuterated solvent (CD_2Cl_2). The solvent resonances are narrow in both the conventional 5-mm probe and the Nanoprobe data (bottom and top spectra, respectively) but are much broader in the middle spectrum because of the magnetic susceptibility mismatches present in a conventional CP/MAS probe. (The solvent resonances in the Nanoprobe spectrum [top] are actually a bit narrower than in the data acquired without MAS [bottom], presumably because the observed resonances are a superposition of polymer entrained solvent signals upon the freely mobile solvent signals; the linewidths of the former signals can be narrowed by MAS.) In contrast, the "resin resonance" at 51.3 ppm exhibits susceptibility mismatch linebroadening from two different sources (first, the sample and second, the probe). The signal is broad in Fig. 3a because of susceptibility mismatches within the heterogeneous sample (although the probe hardware is susceptibility matched), while the signal in Fig. 3b is narrower (than in Fig. 3a) because the linebroadenings caused by susceptibility mismatches *within the sample* were eliminated by MAS. Unfortunately, this signal (in Fig. 3b) is not as narrow as in Fig. 3c because of the normally large mismatches *within the probe*. The signal is narrowest in Fig. 3c because the Nanoprobe uses both MAS *and* susceptibility matched probe materials and designs. (All spectra were obtained using 8000 scans of a "one-pulse" experiment with simultaneous WALTZ decoupling on ^1H. Fig. 3a was acquired in a nonspinning vertical sample tube, while Fig. 3b and 3c used spin rates of approximately 3.8 and 2.0 KHz, respectively. Data were acquired on 91, 20 and 5 mg samples of resin, respectively; all spectra were processed with 0.5 Hz linebroadening and zero filling. (see ref. 19)

becomes a secondary issue, and this choice 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 contain the narrow resonances (1-25 Hz) for the bound compounds of interest, superimposed upon the broader resonances (50-300 Hz) arising from the cross-linked polymer core.

Narrow NMR resonances will be obtained only if both the compound and its supporting structures (the cross-linked polymer and any tethers) are well solvated. If the polymer is hydrophobic but the compound is hydrophilic, although any solvent that swells the resin bead is undoubtedly solvating the large internal core of the resin (the polystyrene), this does not necessarily correspond to increased solvation (or motion) of the compounds *bound* to the resin core, especially if the compounds are hydrophilic and they are bound to the core of the resin with a long hydrophilic polyethyleneglycol [PEG] tether (as in the Tentagel resins). If only one pure solvent is used to slurry the resin, that solvent must solvate all parts

of the resin; this suggests that the solvent of choice will depend upon the structure of both the compound and the resin. Dichloromethane-d_2 is a good first choice of solvent due to the narrow resin linewidths it usually produces, its swelling properties, the unobtrusive location of the residual solvent resonances (^{1}H triplet at 5.32 ppm, ^{13}C quintuplet at 53.8 ppm), and (for the spectroscopist doing sample preparation) its ease of evaporative removal. DMF or $CDCl_3$ are good second choices, while DMSO produces variable results.

A second set of (slightly broader) solvent resonances can often be observed in the NMR spectra of solvent-swollen resins. These extra resonances are known to arise from solvent molecules entrained in different environments within the solvent-swollen polymer bead (31, 63, 76). Since the entrained solvent often resonates at a slightly different chemical shift, this means that all resin slurry samples should be made quite "wet" (with an excess of "free" deuterated solvent) to ensure that the spectrometer's ^{2}H lock is set on the sharper and more chemical shift-constant resonances of the free solvent. Despite some published concerns that MAS of a resin slurry which has excess solvent could result in "phase separations" that might broaden lines (67), experiments have shown that the opposite is true; that is, a significant excess of free (deuterated) solvent provides the sharpest, strongest and most reproducible lock signal and actually appears to produce the best spectra (19, 77, and unpublished work).

Sensitivity limitations

Another challenge in combinatorial chemistry is the analysis of a "one-bead, one-compound" library (10, 78); the ultimate test of sensitivity in SPS resin NMR is the ability to detect signals arising from only a single bead of resin. This has been accomplished in a recent communication that demonstrated that the ^{1}H resonances of only one 90 μm bead (and the ^{13}C resonances of its ^{13}C-labeled nuclei) could be detected using Nano·nmr probes (by using 1D ^{1}H and ^{13}C{^{1}H} direct detection as well as 1D and 2D ^{1}H{^{13}C} indirect detection) (77). The samples contained an estimated ≤ 500 picomoles of bound compound. Since fingerprints and solvent contaminants can overwhelm the desired signal intensity from a sample this small, an isotope-filtered experiment (1D-HMQC) was used to selectively detect the signals arising from some ^{13}C-labeled methoxy groups which had been selectively placed on the resin. Although the demonstrated sensitivity is not yet sufficient to allow a complete *de novo* structure elucidation, these spectra could easily be used to decode a binary "tag" or "barcode" on a bead that is composed of different isotopic labels. This is in contrast to the more well-known methods of "tagging" that use halogen atoms or hydrocarbon chain lengths to record the synthetic reaction history of the bead (79, 80). While such tags are normally incorporated into a separate structure on the bead, an isotopic label could actually be incorporated into either the linker or the compound itself. Linkers containing unique ^{13}C labels could be used to identify either different starting scaffolds or different batches of resin; or the compounds themselves could incorporate unique ^{13}C labels during different stages of the synthesis to document its reaction history. An isotopic label is the most chemically inert tag available and, since the NMR analysis is nondestructive, it would leave the bead fully intact. This would also avoid the need for the parallel synthesis of a separate coding structure on each resin. Two-dimensional HMQC spectra of single-bead samples have also been acquired; these can be used to deconvolute mixtures of uniquely tagged beads (77).

While it can be claimed that single-bead detection has also been performed on conventional liquids and MAS probes, this was only accomplished by using large (400-750 μm) "microreactor" resins that contain 1000 times more sample per bead than a 90 μm resin (70, 81). Similarly, the entire "crown" of a "multipin" structure can also be placed intact within an MAS rotor to generate either ^{1}H (82) or ^{13}C (83) NMR spectra.

Advanced, multidimensional and multinuclear NMR methods

As expected, advanced one-dimensional and two-dimensional NMR experiments are also proving to be useful in analyzing SPS resins. Proton-detected homonuclear (COSY, DQFCOSY, TOCSY and NOESY) and heteronuclear (^{1}H-^{13}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, all on either high-resolution MAS (19, 74, 77, 84) or moderate/standard-resolution MAS probes (67, 75, 81, 85-87).

The interpretation of these resin spectra is often complicated by broader signals arising from the polymer support itself. Frequency selective methods of suppressing these signals might initially appear to be useless because they often have the same chemical shifts as the signals from the bound compounds of interest. It has been noticed, however, that selective presaturation of a resonance from the tether can also sometimes reduce the signal intensities of other polymer resonances at totally different chemical shifts (19). The effectiveness of this method depends heavily upon the resin's structure: if it contains a PEG tether it may work quite well (Fig. 1c), but if no tether exists it is much less useful (Fig. 2c). (The signal saturation is presumably spread by a spin-diffusion process, and the effectiveness of this process is undoubtedly affected by the motional differences between the two resins.) Spin echoes have also been used to suppress broad polymer signals in resins that do not contain tethers (60, 84), as has the combined use of both presaturation and spin echoes (74); the spin echoes, however, cause distortions in both the lineshapes and amplitudes of the resulting signals and this renders the resulting (magnitude mode) spectra nonquantitative. (On the other hand, presaturation is particularly effective because it

also selectively removes the spinning sideband artifacts of the irradiated lines.) It has been proposed to use the untilted projections of homonuclear J-spectra to remove the background signals (88), but, since this is also a spin-echo experiment, it still creates distortions in the coupling constants and peak amplitudes. Freeman's methods for generating proton chemical shift spectra might prove to be a better way to do this (89). Another promising approach is to use chemical shift scaled E.COSY spectra to extract signal multiplicities and coupling constants (90). Although backwards linear prediction processing can also be used to selectively remove broad resonances, it appears to be of a limited utility, since resin spectra often exhibit a continuous range of linewidths.

Although the vast majority of published resin data consist of ^{1}H and ^{13}C NMR, multinuclear NMR is also possible. Examples of non-MAS multinuclear data are described above, and ^{19}F spectra have been acquired by using MAS (91). This author's recent experiences with ^{19}F, ^{2}H, ^{29}Si and ^{31}P resin NMR indicates that there is still a significant amount of unrecognized potential in multinuclear MAS NMR of SPS resins.

Competitive issues

The demonstrated utility of the Nanoprobe (from Varian) has recently prompted other manufacturers to also develop NMR probes for the analysis of solid-phase synthesis resins. Bruker has modified a traditional MAS probe into a "combinatorial chemistry accessory" (even though not all SPS resin users do combinatorial chemistry and *vice versa*). Its advantage lies in its use of a familiar top-loading "tube" to introduce samples into the probe, but its disadvantages lie in the resulting performance compromises: it has a significantly broader lineshape (at 50, 0.55 and 0.11% of the peak height) and a wider linewidth (at 50%), lower sensitivity, a higher sample-cell cost and, since it cannot handle solution-state samples nor run CRAMPS- or CP/MAS-style experiments, it really only supports a limited range of applications (67, 86). Although the *linewidth* (the resolution) of a Bruker CCA probe is narrower than that of a conventional MAS probe, its *lineshape* (which specifies the shape and width of the entire line, including its base) is much broader than that obtained in a Nanoprobe (67, 86); this inhibits its use in studies of any samples having tall or narrow solute or solvent resonances (like Tentagel or water) and it precludes the use of presaturation. It is now apparent that any probe which is called a "high-resolution probe" must be evaluated by specifying *both the linewidth and the lineshape*. (The linewidth alone is insufficient, since many probes that can be shimmed to a narrow *linewidth* will not produce a good *lineshape*. If a probe can only produce a broad and asymmetric *lineshape* – like you get when you try to shim a 200 μl sample in a normal 5-mm "vertical-tube" probe – it suggests that the materials used in the probe have not yet been properly susceptibility matched.)

The less efficient detection sensitivity of the CCA probe requires (81) – but then also allows – larger sample volumes to be used (*i.e.*, 7-mm rotors containing 75 mg of resin) (75, 87) as compared to a Nanoprobe, but this inhibits the analysis of very small samples (especially single beads). Pulsed-field gradient capabilities are also being investigated (92). Doty Scientific is introducing a line of probes (called "XC-5") that promise to support a wide range of applications, including solid-state NMR (by providing high-power handling and fast spin rates) and some solution-state NMR (by providing nonleaking rotors, good lineshapes and reasonable detection efficiency/sensitivity), albeit without any automation. The Varian Nanoprobe supports a similarly wide range of applications, but with an emphasis on the "solution-state" and "highest resolution" end of the applications continuum; its power handling is efficient enough to allow CRAMPS to be obtained (< 2 μsec 90° pulse widths) but its spin rates (< 5 KHz) are too low for typical solid-state applications. An automated sample changer for the Nanoprobes has recently been introduced; it also includes a bottom loading probe elevator that can be used to automate the changing of any probe in a narrow bore magnet.

Varian's "combinatorial chemistry accessory" would actually be its high-throughput NMR analyzer called VAST (Versatile Automated Sample Transport) which has been specifically designed for the analysis of libraries of solution-state samples, even microtiter-plate based libraries. The "combinatorial chemistry accessory" moniker emphasizes the point that all combinatorial chemistry projects ultimately result in libraries of solution-state samples, especially if bioassays are being used, whereas the use of solid-phase synthesis resins is neither exclusively limited to, nor directly required for, combinatorial chemistry. Although it is actually a more general purpose tool, only the combinatorial chemistry applications of VAST NMR will be covered in the review (in the last section).

Small-volume solution-state samples: high-resolution MAS

While the advantages of using a Nano·nmr probe for SPS resin NMR are documented above, and are now well recognized (74, 93-95), the Nanoprobes have also been specifically designed to handle small-volume (≤ 40 μl) liquid samples. To analyze ever smaller volumes of solution-state samples, the temptation is to place it in a standard NMR tube, where it will form a shorter than normal column of liquid; if this sample volume is too small to properly fill the receiver coil, however, the resulting magnetic susceptibility discontinuities at the ends of the sample (the "end effects") will make the sample hard to shim and will generate distorted lineshapes. (To properly simulate an infinitely long cylinder in a 5-mm probe, the sample needs to be 20-30 mm longer than the receiver coil). One way to address this problem is to place the sample in a smaller diameter tube (a 1-3 mm capillary or microprobe

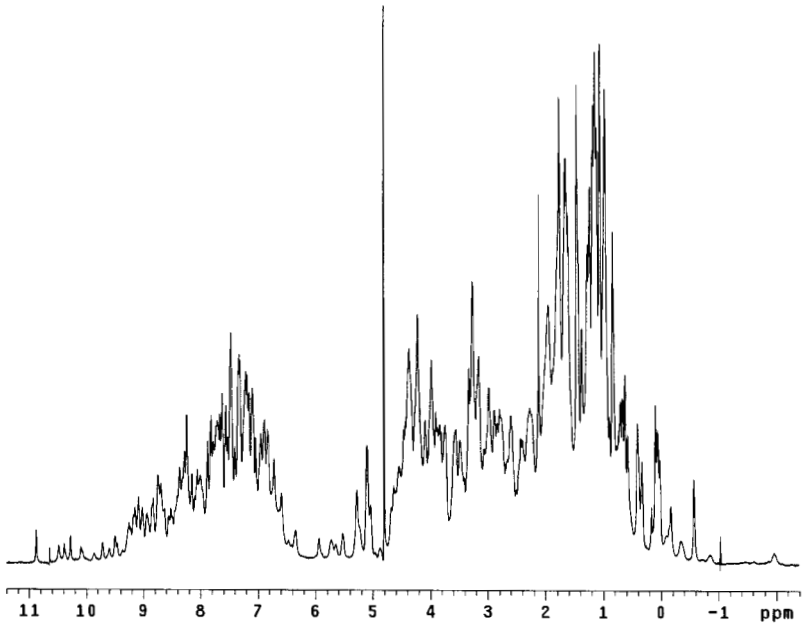

Fig. 4. A solution-state NMR spectrum acquired with a ^{1}H Nanoprobe on ≤ 40 μl of 1 mM lysozyme in H_2O:D_2O (90:10) using presaturation for solvent suppression. This spectrum uses MAS (2920 Hz) to remove the susceptibility linebroadenings caused by both the small sample volume (which creates a noninfinite cylinder sample geometry) and the effects of any air bubbles or particulate matter in the sample. The residual water signal in the resulting spectrum is phenomenally narrow after only minimal shimming (approximately 10 Hz near the base [at 4.8 ppm] after approximately 1 minute of shimming); in addition, with a Nanoprobe there is no such thing as a sample that is "too small to shim" – a 2 μl sample will use essentially the same shim corrections as a 40 μl sample. This spectrum was obtained by using a 0.547 sec acquisition time, a 2 sec presaturation pulse (50 Hz field strength) and 256 scans. The data were processed with 1 Hz of linebroadening and zero filling; no other data massaging or signal subtraction (of any kind) was used. (see also ref. 99)

tube) where it will form a longer length cylinder (although very small samples may still not meet the infinite cylinder condition without being diluted). The use of these smaller diameter tubes was popularized by J.N. Shoolery (Varian) in the 1970s (96), who recognized that real-life chemical and biological problems will always create ever smaller samples that need to be analyzed with ever increasing sensitivity. Unfortunately, attempts to make NMR probes for sample tubes of < 1-mm diameter have historically not been too successful. (It is hard to build good NMR probes at such a small scale, and minor imperfections in the coil materials still result in broad, distorted and "unshimmable" NMR lineshapes.) An alternative strategy, also developed by Shoolery, is to exploit the $(1\text{-}3\cos^2\theta$ dependence of the susceptibility broadening term in the NMR Hamiltonians; by spinning the sample at the magic angle ($\theta = 54.7°$ relative to the z axis) the entire term reduces to zero and the effect disappears. This justifies the development of a high-resolution probe that uses MAS to eliminate susceptibility mismatch linebroadening effects for small-volume liquid samples – the Nanoprobe. Because MAS will allow the entire sample to be confined within the (selenoidal) receiver coil, the Nanoprobe is the highest sensitivity per nucleus NMR probe commercially available (with the possible exception of NMR probes using high-temperature superconductor [HTS] coils), yet its lineshape and resolution are comparable to a conventional liquids probe. Because it uses MAS to eliminate susceptibility mismatch linebroadening effects, it shims equally well for both 40 μl and 4 μl samples, and it has the unique capability of producing a *very* narrow water lineshape (at the base) for samples in H_2O (Fig. 4). Some projects in which a Nanoprobe has proven uniquely useful include:

1) A ^{1}H Nanoprobe was used to analyze several 10-20 μg samples of purified human melanoma cell products. The samples were shown to be various mixtures of carbohydrates, having masses up to MW 1011, by 1D and 2D ^{1}H MAS NMR spectroscopy (97) (Fig. 5).

2) A ^{13}C Nanoprobe was used to acquire ^{13}C-detected INADEQUATE data on 10 mg of a natural product ($C_{21}H_{32}O_3$). The data determined the complete carbon skeleton of the unknown molecule, and did so more rigorously than any indirect detection method (like HMBC) could; the "FRED" analysis software was used to facilitate the assignments (98).

3) A ^{1}H Nanoprobe was used to acquire ^{1}H NMR spectra of a 35-amino acid peptide dissolved in 90:10 H_2O:D_2O. The structure was determined primarily by using 2D-NOESY and TOCSY data; all spectra were acquired using presaturation for water suppression (99).

The use of MAS during the acquisition of solution-state data does generate some experimental limitations. Because the sample is spinning during the entire experiment, spinning sidebands may be observed in all spectra, including all dimensions of multidimensional spectra. The sample spinning also decreases the stability of the NMR

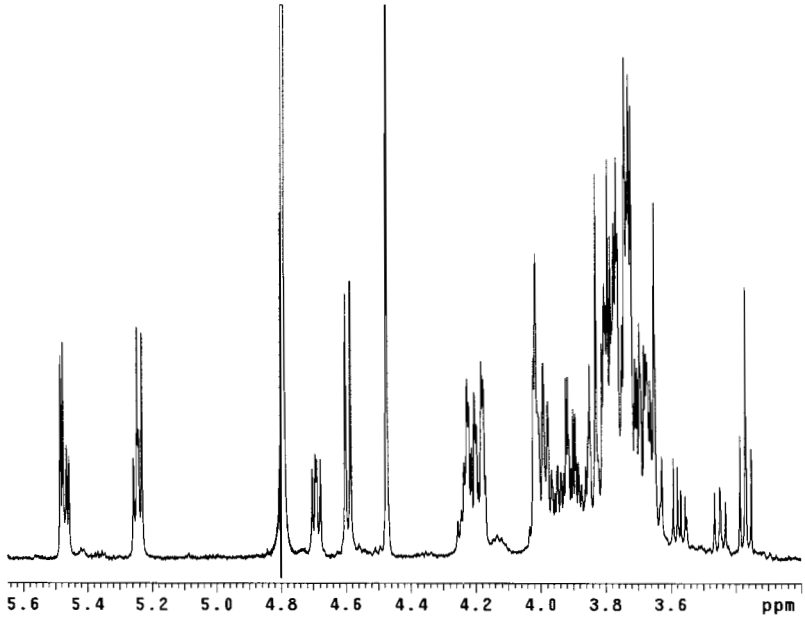

Fig. 5. A solution-state NMR spectrum acquired by using a ^{1}H Nanoprobe on ²40 mcl of a solution containing 25 mcg of an unknown xyloside (MW = 1011 by FAB-MS) dissolved in a mixture of D_2O and H_2O. The sample, which was isolated from human melanoma cells, is an unequal mixture of two compounds, each present in two anomeric forms (making this a four-component mixture; see ref. 97). The experiment used presaturation for solvent suppression and MAS (2240 Hz) to eliminate linebroadenings caused by susceptibility mismatches between the sample and its container. NMR studies of any molecule which has resonances near 4.8 ppm (like carbohydrates and proteins) would be facilitated by this (typically) narrow residual water signal. This spectrum was obtained in 360 scans, using a 1.5 sec of presaturation pulse (50 Hz field strength) and a 2 sec acquisition time; it was processed with 0.2 Hz of linebroadening.

signal, which can increase the level of t_1- noise in experiments that use phase-cycled cancellation of large signals (like indirect detection). This suggests that if you need HMBC on a tiny amount of a homogeneous liquid, you might be better off using a (nonspinning) microprobe; however, if you need HMBC data on a heterogeneous sample (like a SPS resin slurry), you should be better off using an MAS probe since the greatly improved spectral resolution will far outweigh any increase in the t_1-noise level. Only when trying to obtain NMR data on small quantities of homogeneous (solution-state samples) does one have a significant number of hardware alternatives, which include: 1) spherical microcells or susceptibility plugs for conventional (vertical) NMR sample tubes; 2) superconducting probes (same sample and same signal, just less noise); 3) microprobes, which use smaller diameter vertical sample tubes in conventional geometry probes; 4) Nanoprobes (the highest resolution MAS probe by solution-state NMR definitions); 5) flow probes (one can use HPLC to fractionate and consolidate a desired compound from a complex mixture into a single chromatographic peak, for either better sensitivity or cleaner spectra; another application of flow NMR is discussed below); 6) "microcoil" probes, which contain nanoliter to microliter volume capillaries for on-flow or static NMR analysis; these probes are awaiting development of novel coil-design methods (100).

Solution-state library analysis:

high-throughput spectroscopy

On-flow ("tubeless") microtiter plate-based NMR

Most drug discovery programs conduct their bioassays on compounds in solution (*versus* bound to a solid support). This means that, even if SPS resins were used to *make* the compounds, the compounds must be cleaved from the resin prior to running the bioassays; this should allow conventional solution-state NMR techniques to be used to characterize the final products. In practice, however, many drug discovery programs do not obtain NMR data at this stage (even if NMR is used during either the initial synthetic methods development stage or the subsequent scale-up production stages). The acquisition of NMR data at this bioassay stage is typically neglected and assumed to be difficult because: 1) the samples are normally dissolved in protonated solvents (which generate large background signals that complicate the NMR analysis); 2) the solution volumes are often too small to allow conventional NMR samples to be taken (< 500 μl); 3) the samples are often stored in containers which an NMR probe cannot use (*i.e.*, 96-well microtiter plates); and 4) the throughput of conventional NMR spectroscopy is insufficient to match the analysis needs (since the number of samples is too large). These problems have been solved by some recent developments in LC-NMR, allowing a high-throughput spectroscopy (HTS) technique

to be developed for combinatorial chemistry.

LC-NMR

The first problem (background signals arising from solvents) has been an issue in LC-NMR for years (101, 102). It has been addressed by either using presaturation for solvent suppression (103) or by using fully deuterated solvents (104, 105), but a more effective and general solution was developed recently with "WET" solvent suppression (106). WET is composed of a series of four frequency-selective shaped rf pulses, each followed by a field-gradient (PFG) pulse (to dephase the excited magnetization); ^{13}C decoupling is applied during the rf pulses if selective ^{13}C satellite suppression is desired. The advantages of WET are that it provides high-quality solvent suppression for even fully protonated solvents, it will easily suppress multiple solvent resonances (if desired) by using SLP pulses (107), it suppresses the ^{13}C satellites of solvent resonances like CH_3OH and CD_3CN, it is self-compensating for miscalibrated pulse widths or probe mistunings, its suppression is fast (<100 msec) which means that it provides the most scans (the highest sensitivity) and the best digital resolution possible per unit time, and it provides the highest quality solvent suppression equally well in both stopped flow *and flowing* conditions, all without optimization. In addition, the setup of the WET solvent suppression has been fully automated to create what is called the "SCOUT-Scan" method (106).

The second problem (small sample volumes) has been addressed by the development of high sensitivity "flow" probes which have been designed for small-volume samples (*i.e.*, HPLC peaks). To allow these probes to be directly connected to HPLC columns the sample flows through narrow bore tubing into the NMR coil; hence, these probes do not need to use any special sample tubes. These modern flow probes have characteristics which are uniquely suited to high-throughput NMR spectroscopy, namely, their high sensitivity enables them to detect small samples down into the micrograms to hundreds of nanograms range (1-100 nanomoles or less); their sample geometry is fixed (and approximates an infinite cylinder) which eliminates the need to reshim each sample; their built-in pulsed-field gradient (PFG) coils allow (very fast) ^1H or ^2H gradient shimming of the B_o field (if ever necessary); and expensive sample tubes never need to be bought, filled, inserted, broken, changed or cleaned.

These developments have been listed to document how LC-NMR has evolved into what is now a high-performance technique for the analysis of samples needing (typically reversed-phase) chromatography. Hopefully, the reader can begin to appreciate the performance capabilities of a modern generation LC-NMR, which is typically composed of an [HPLC pump]-[injector]-[HPLC column]-[optional UV detector]-[NMR probe] system, especially when the system is equipped with WET solvent suppression, SCOUT-Scan and HPLC-to-NMR communication and control.

We recognized that the third and fourth problems (microtiter plate formats and limited throughput) could be solved by adding a full function HPLC autosampler to the front end of this LC-NMR. We chose a programmable autosampler (Model 215 Liquid Handler, Gilson, Middleton, WI, USA) which is capable of sampling from microtiter plates (and a wide variety of other vials or tubes), with a variable sized syringe, into a variable sized injector loop, and do all of this under computer control. At this stage one now has an autosampled LC-NMR capable of "auto-NMR setup" (using WET and SCOUT-Scan) in which the LC pump, the LC detector and the NMR all have coordinated communication so that any series of chromatographic peaks can be automatically stopped in the NMR coil for additional signal averaging.

"Columnless" LC-NMR (flow-injection analysis NMR)

If we now simplify this system by removing the HPLC column, when the Gilson injects an aliquot of sample from a microtiter plate, that sample will simply be swept through the tubing and the NMR probe by the pumping LC mobile phase. As before, the solvent flow can be automatically paused in a way that places 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). This process, when combined with automated WET solvent suppression setup (and optional locking and shimming), can be completed in only a of couple minutes; if a ^1H NMR spectrum requires 1 minute of signal averaging (*e.g.*, 32 scans), the entire cycle time for each sample could take as little as 3 minutes. An example of the attainable sensitivity and spectral quality of this "columnless LC-NMR" is shown in Figure 6a (on 100 µl of a 1 mg/ml solution of tryptophan).

Sensitivity optimized VAST (direct-injection NMR)

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] direct injection flow NMR system. In this case, the autosampler's syringe transfers a sample aliquot (typically 150-300 µl of a 1-well, 1-compound sample) from a microtiter plate well directly into the NMR detector cell without the use of any mobile phase (therefore no dilution). The NMR spectrum can be taken immediately; examples of the resulting NMR spectra (using 300 µl of a 1 mg/ml solution of tryptophan) are shown in Figure 6b and 6c. After completion, the process can be reversed to return the sample to the microtiter plate intact (or the sample can be flushed to a waste container). Because the throughput rate of this system can be less than 3 minutes per sample (including probe and syringe

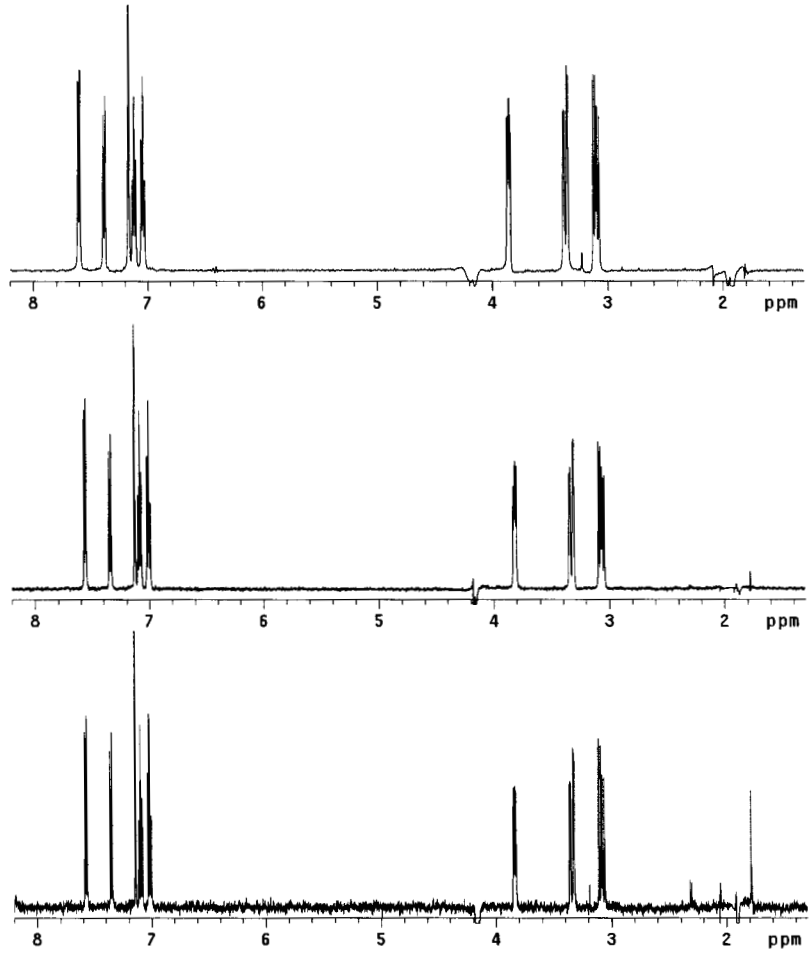

Fig. 6. Solution-state ^{1}H NMR spectra acquired using either "on-flow" (Fig. 6a) or "direct injection" (Fig. 6b and 6c) techniques to analyze a 1 mg/ml solution of tryptophan dissolved in either $CH_3CN:D_2O$ or $CH_3CN:H_2O$. The bottom spectrum (Fig. 6a) was acquired using "columnless" LC-NMR (see text). In this technique, 100 µl of a tryptophan solution (1 mg/ml in 50:50 $CH_3CN:D_2O$) was injected into a flowing mobile phase (50:50 $CH_3CN:D_2O$) which swept the sample from the injector loop, through a UV detector, and into the NMR probe for detection. The mobile phase was automatically stopped (by a signal from the UV detector) so as to position the injected sample plug in the center of the NMR probe's receiver coil for signal averaging; at the end of signal averaging the flow was resumed, to both carry the sample to waste and to rinse out the NMR probe (analogous to a "carrier gas" in GC but operated in a "stopped-flow" manner). The middle spectrum (Fig. 6b) was acquired by using VAST NMR to inject the same tryptophan solution directly into the probe, acquire an NMR spectrum on the now static sample, and return of the sample directly to its original container (if desired). The top spectrum (Fig. 6c) was acquired in the same manner (by using VAST NMR), but now on a sample dissolved in fully protonated solvents (tryptophan dissolved 1 mg/ml in 50:50 $CH_3CN:H_2O$); this is possible because VAST NMR can easily suppress multiple proton resonances, and VAST does not require a ^{2}H lock signal. All spectra were acquired at 500 MHz, without any form of chromatographic separation, using 1-minute experiments (32 scans each); the data were processed with 1 Hz linebroadening and a solvent suppression high-pass DSP filter. Dual frequency solvent suppression was accomplished by using the WET sequence to suppress both the CH_3CN resonances (the central resonance at 1.95 ppm as well as its ^{13}C satellites) and the $D_2O/HOD/H_2O$ resonance (at 4.8 ppm). The setup of the solvent suppression conditions was fully automated by using the SCOUT scan method on every dataset (see text).

rinsing), this system is a "high-throughput spectroscopy" (HTS) NMR system which we call the "Versatile Automated Sample Transport" (VAST) NMR. (The versatility of VAST allows it to serve as an automatic sample changer for a wide range of other NMR applications). It produces higher sensitivity than columnless NMR because there is no sample dilution; this difference, as well as the simpler plumbing, allows the sample to be recovered intact. The ability of VAST NMR to acquire 1D proton spectra of every sample in a 96-well microtiter plate within a matter of hours suggests that it could play a significant role in analyzing drug discovery libraries.

Future potentials for VAST

It is now clearly possible 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 D_2O (Figs. 6a and 6b) or H_2O (Fig. 6c). The use of other ^1H-NMR-friendly solvents such as $CHCl_3$ or DMSO (in which the proton resonances are singlets) is straightforward. Sample mixtures can be edited by using DOSY to sort compounds on the basis of their diffusion rates (108-110). The interpretation of the resulting spectra is still a bottleneck, although recently developed NMR techniques such as proton chemical shift spectra from z-filtered J-spectra (89, 111), reference deconvolution (112, 113), spectral calculations and automated spectral analysis (*i.e.*, Bayesian analysis [114]), offer the potential that a fully automated real-time microtiter plate NMR analysis system for combinatorial chemistry libraries might some day be developed. These tools will become even more important if solution-state combinatorial synthesis methods (like the dendrimer methods [45]) supplant the now common SPS methods, and if ever more powerful hyphenated techniques such as LC-NMR-MS (115) continue to be developed. While VAST (which is an innovation that was conceived and developed within Varian) is obviously useful for the analysis of combinatorial chemistry libraries, it is also proving to be a general and routine technique which is applicable to many other kinds of NMR problems. Recently Fesik and coworkers described a promising new "SAR by NMR" method (116) which facilitates the discovery of high-affinity ligands, especially in target-directed drug research programs; VAST has now also been adopted to automate this technique.

There are two reasons to focus on the analysis of solution-phase samples: first, most drug discovery programs perform the critical bioassay studies on solution-state (cleaved) compounds, and second, the automated manipulation of liquid samples is much easier (and more reliable) than the automated manipulation of heterogeneous SPS resin slurries. The ability to characterize the actual samples being bioassayed, regardless of whether these compounds were synthesized by solution-phase or solid-phase (resin) synthetic methods, allows NMR to perform what is probably the most important chemical analysis in the entire drug discovery process.

Conclusions

This review has shown how several recent developments in NMR spectroscopy have evolved to create two new methods of NMR analysis for drug discovery processes. These complimentary methods are proving to be especially useful in combinatorial chemistry applications. The first method includes the ability to acquire high-resolution NMR spectra (in particular ^1H spectra) of compounds still bound to solid-phase synthesis (SPS) resins. The resulting spectral quality (as measured by the attainable linewidths) is influenced by (in order): the NMR probe, the SPS resin structure and the solvent used to swell the resin. The second method includes an automated high-throughput (NMR) spectroscopy (HTS) technique called VAST, which is capable of rapidly acquiring high-resolution NMR spectra for libraries of solution-state samples. Samples can be rapidly introduced to (and removed from) the NMR magnet in a "tubeless" fashion, directly from a microtiter plate (if desired), in a nondestructive fashion (if desired), and can produce high-quality spectra, even without the use of expensive deuterated solvents. This VAST technique appears to be a general purpose tool and it is proving itself to be useful for many kinds of repetitive NMR analyses.

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