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^{1}H NMR Characterization of the Product from Single Solid-Phase Resin Beads Using Capillary NMR Flow Probes

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A capillary NMR flow probe was designed to generate high-resolution ^{1}H NMR spectra at 600 MHz from the cleaved product of individual 160-μm Tentagel combinatorial chemistry beads. By injecting a dissolved sample sandwiched between an immiscible, perfluorinated organic liquid directly into the probe, NMR spectra of the product cleaved from single beads were acquired in just 1 h of spectrometer time without diffusional dilution. Sample handling efficiency on the single bead scale was comparable to that obtained with a bulk sample. Using the relative intensity of the DMSO-d_5H versus the analyte signals in a fully relaxed CPMG spectrum, the amount of product cleaved from a single bead was determined to be 540 $\pm$ 170 pmol in one of the samples. Following the NMR data collection, the samples were examined with electrospray ionization mass spectrometry to provide additional structural information. By coupling with microliter-volume fluidic capabilities, the capillary flow probe described here will enable multidimensional characterization of single solid-phase resin products in an online manner.

Key Words: **Tentagel beads; microcoil; nanoliter; mass spectrometry; nuclear magnetic resonance.**

INTRODUCTION

During the past decade, solid-phase synthesis techniques that were originally reported by Merrifield (*1*) have formed the foundation of the rapidly expanding field of combinatorial chemistry. Parallel synthetic methods have demonstrated the capability to generate enormously large numbers of compounds from relatively simple sets of reagents (*2*). The concomitant development of high-throughput screening assays has enabled rapid identification of potential leads for targets in fields such as pharmaceutical development, catalysis, and material science (*3–5*). Approaches to screening analytes from single solid-phase resin beads have a variety of advantages, not the least of which is the efficient application of split and pool synthesis methodology to generate "one bead, one compound" libraries (*6–8*). However, successful use of these types of libraries demands methods of chemical characterization that can provide detailed structural information from the minute amounts of product synthesized on an individual resin bead.

Within the past few years, various analytical techniques have been applied to the molecular characterization of single solid-phase synthesis (SPS) beads. While sensitivity remains the most fundamental requirement for analysis of these mass-limited samples, the structural information content provided by each method offers distinct advantages as well as limitations. For instance, Fourier transform infrared (FT-IR) spectroscopy has demonstrated utility in several examples of single-bead analysis for reaction monitoring (*9, 10*). However, since FT-IR provides data only about chemical functionality, complete structural elucidation of unknown compounds necessitates complementary techniques. Mass spectrometry (MS) has also shown significant success in the characterization of individual beads both as a stand-alone method (*11–13*) and as a detector for high-performance liquid chromatography (HPLC) (*14*). While high-resolution MS can provide important structural data such as molecular formulas and fragmentation products, additional information may be required for the unambiguous identification of combinatorial products that are frequently isomeric.

Nuclear magnetic resonance (NMR) spectroscopy can provide unparalleled data about molecular structure and dynamics in a nondestructive manner, with applications ranging from determination of three-dimensional protein conformations (*15*) to characterization of complex mixtures (*16*). However, NMR trails the other primary methods of molecular characterization (i.e., FT-IR and MS) by several orders of magnitude in terms of sensitivity (*17*). Typically, SPS resin beads range in diameter from 40 to 200 μm and contain between 0.1 and 1 nmol of material per bead. NMR experiments can be conducted either with the combinatorial product still bound to the resin or with the reaction product cleaved from the bead. Very few reports have utilized NMR for characterization of the product from individual beads because the sensitivity of most conventional systems is insufficient. For bulk on-bead analysis, one successful approach to obtaining high-resolution ^{1}H NMR spectra has

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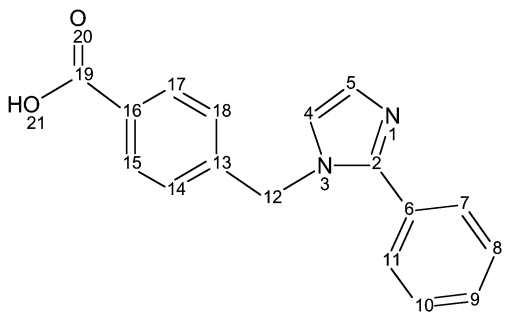
averaged the magnetic susceptibility-induced line broadening to zero by spinning the sample at high speed at the magic angle (*18*). The availability of commercial magic angle spinning (MAS) NMR microprobes with increased sensitivity has facilitated such measurements (*19*). MAS-NMR combined with the use of larger (400–750 μm) resin beads that offer higher loading capacities has enabled single-bead NMR analysis (*20, 21*). The incorporation of a ^{13}C-labeled moiety in the analyte has also proven useful in overcoming background impurity interferences for isotope-filtered NMR experiments on individual beads of approximately 100 μm in diameter (*22*).

While NMR data collection from analytes still bound to beads provides important benefits, such as the ability to monitor reaction progress without interrupting the synthesis, several advantages of characterizing the cleaved product from single beads merit its exploration. For example, peptides in solution may adopt different conformations that are not evident from on-bead NMR spectra (*21*). Furthermore, spinning sidebands in MAS-NMR may interfere with analyte resonances and require data collection at several spinning speeds, thus lowering the overall efficiency of the method. In addition, on-bead quantitation by NMR has proven difficult. Although strategies have been presented to circumvent this problem by cleaving the analyte from the bead after NMR data collection and using a fluorescent tag as an intramolecular standard (*21*) or using the ^{13}C resonances of the resin polymer itself (23), the broader applicability of this approach to quantitation has not yet been demonstrated. Finally, while combinatorial chemistry originated from a foundation of solid-phase synthesis, solution-phase combinatorial approaches have been receiving increasing attention (*24, 25*). To our knowledge, only one example of the NMR analysis of the cleaved product from a single combinatorial chemistry bead has appeared in the literature (*26*). In this study, relatively large, high-capacity resin beads were employed such that each bead contained approximately 32 nmol of material. Although conventional liquid-phase NMR probes can be used with such a quantity, these types of resins do not currently enjoy widespread applicability.

While increased magnetic field strengths have yielded significant gains in NMR sensitivity over the years, escalating costs and formidable technical obstacles have motivated additional strategies such as polarization transfer techniques, cryogenic probes, and reduced-diameter radiofrequency (RF) coils (*17, 27–30*). The cumulative effects of these advances have greatly extended the range of mass-limited NMR experiments. Solenoidal microcoil probes, which have observe volumes (V_{obs}) of 5 nL–1 μL, have shown even further improvements in mass sensitivity (*31, 32*). We report here the use of a custom-built NMR microprobe to generate high-resolution ^{1}H NMR spectra from the cleaved product of individual 160-μm Tentagel beads. By injecting a dissolved sample sandwiched between an immiscible, perfluorinated organic liquid directly into a capillary microcoil flow probe, NMR spectra of the product cleaved from single beads were acquired in just 1 h of NMR spectrometer time. The volume of the flow cell is sufficiently well matched to the sample quantity so that the analyte is presented efficiently to the detector. This experimental arrangement has overcome the traditional drawbacks in the detection of small quantities by NMR, of solvent impurities significantly contributing to the detected signal and prohibitive acquisition times to build up adequate signal-to-noise (*S/N*).

This work focused on one compound discovered in a series published on serine protease inhibitors (*33*), with leukotriene B4 (LTB4) receptor binding affinity. The key compound in this study has the structure **1**:



To demonstrate the nondestructive nature of the NMR analysis, MS data also were collected from the samples after the NMR experiments were completed. Although not demonstrated here, the flow probe configuration facilitates coupling to other analytical systems such as UV-Vis spectroscopy, FT-IR, MS, etc., in an online manner. As a result, the cleaved product from single beads can now be subjected to a plethora of characterization techniques so that complete structural elucidation of single bead products is feasible.

EXPERIMENTAL

Reagents. Deuterium oxide (D_2O, 99.9% D) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.9% D) were from Cambridge Isotope Labs (Andover, MA). Deuterium chloride (DCl, 99.5% D, 37 wt% in D_2O), deuterated methanol (99.5% D), and imidazole (99%) were obtained from Aldrich (Milwaukee, WI). Trifluoroacetic acid (TFA) and the dipeptide alanine–aspartic acid (Ala-Asp) were purchased from Sigma (St. Louis, MO). Potassium hydrogen phthalate (KHP) and hydrofluoric acid (HF, 49%) were obtained from Fisher Scientific (Pittsburgh, PA). Tentagel beads (160 μm) from Rapp Polymere (Tubingen, Germany) were used in the solid-phase synthesis with 4-(4′-hydroxy-3-methoxyphenoxy)-butyric acid (Senn Chemicals, Dielsdorf, Switzerland) as the linker. The SPS reagents were 4-formylbenzoic acid and 2-phenylimidazole (Sigma-Aldrich, Gillingham, Dorset, UK). All reagents were used as received. H_2O was dispensed from a Milli-Q water purification system (Millipore, Bedford, MA).

Capillary NMR probe. A bubble-type NMR flow cell was made by localized etching of a capillary according to previously

reported procedures (*34*). Briefly, an ~10-cm-long segment of 75-μm inner diameter (i.d.)/800-μm outer diameter (o.d.) uncoated quartz capillary (Polymicro Technologies, Phoenix, AZ) was threaded through the center of a 1-cm-long solenoid made from Nichrome wire. As the wire was heated resistively, fresh HF solution (10% HF in H_2O) was flushed through the capillary with a syringe. The localized heating produced an enlarged region with a bubble-type geometry (as illustrated in Fig. 1).

A solenoidal RF coil was constructed from copper wire (California Fine Wire Co., Grover Beach, CA) to produce a V_{obs} of ~800 nL as determined by volume displacement, injecting a visible dye under a microscope. To fit within the bore of the magnet, the capillary flow cell was bent into a "U" shape with an acetylene/oxygen torch. The solenoidal coil was connected electrically to the proper impedance matching circuitry for a proton observation channel (600 MHz) with a deuterium lock channel (92 MHz). For magnetic susceptibility matching purposes, the microcoil was enclosed by a 10-mL polyethylene bottle filled with a perfluorinated organic liquid (MF-1, MRM Corp., Savoy, IL). Each end of the flow cell capillary (75-μm i.d./800-μm o.d.) was attached to approximately 65 cm of 75 μm/360 μm polyimide-coated fused silica capillary via a custom-machined PEEK union. The static linewidth at half-maximum for this probe was 1.4 Hz. The 90° pulse width was 7.5 μs for a transmitter attenuation of 36 dB on a Varian Inova console.

Solid-phase synthesis. The samples were synthesized using Tentagel beads (160 μm from Rapp Polymere) and 4-(4′-hydroxy-3-methoxyphenoxy)-butyric acid linker. The 4-formylbenzoic acid was attached to the linker, activated, and the 2-phenylimidazole added as described (*35*).

Sample preparation for bead analysis. To remove noncovalently bound synthesis impurities, the solid-phase resin beads were rinsed with deuterated methanol and dried prior to cleavage and analysis. Individual beads were isolated using a finely drawn capillary tip under a microscope. The beads were placed into the bottom of conical, low-volume vials (Total Recovery Vials, Waters Corp., Milford, MA) that had been prerinsed with H_2O and dried in an oven (~110°C) for >1.5 h. For the single-bead cleavage reactions, 2.5 μL of freshly prepared 90% TFA/10% D_2O was added to each vial. After 3 h, the TFA/D_2O solution was transferred into a separate clean vial and blown dry under nitrogen. An additional 3 μL of freshly prepared 90% TFA/10% D_2O was added to each bead-containing vial. After 2.5 h, the remaining solution was transferred to the corresponding vial containing the dried product from the first cleavage. The samples were blown to dryness under nitrogen and placed in a vacuum desiccator overnight. For one set of single-bead NMR studies, 2.5 μL of DMSO-d_6 was added to the cleaved product in the vials. In a separate set of single-bead NMR analyses, 2.5 μL of DMSO-d_6 acidified with 0.1 vol% DCl was added to the cleaved product in the vials. In both cases, the resulting analyte solution was loaded into a 10-μL syringe with a fused silica needle (Hamilton, Reno, NV), bracketed on both sides with an immiscible perfluorinated organic liquid (Fluorinert, FC-43, 3M, St. Paul, MN), and injected into the microcoil NMR probe. The probe was initially shimmed and locked on DMSO-d_6. When the FC-43 entered the V_{obs}, the lock signal was lost. As the solution continued to flow, the lock level approached its initial value, which indicated that the sample entered the detection region of the flow cell.

For the multibead experiment, an approximately 9-mg quantity of beads containing the desired product **1** was collectively cleaved using 1 mL of 90% TFA/10% D_2O in a 1.50-mL polyethylene centrifuge vial, using the same procedure as mentioned above for the single beads. After evaporation to dryness, the resultant fine film was redissolved in 600 μL of DMSO-d_6.

NMR spectroscopy. A ^{1}H NMR spectrum of the multibead sample was measured at 400 MHz on a Bruker DPX400 (9.4 T) spectrometer at room temperature (~295 K) with a 30° pulse flip angle. A total of 16 transients with a pulse repetition of 3.0 s and a SW of 8223 Hz (20.5 ppm) were collected into 32,768 data points. A line broadening apodization of

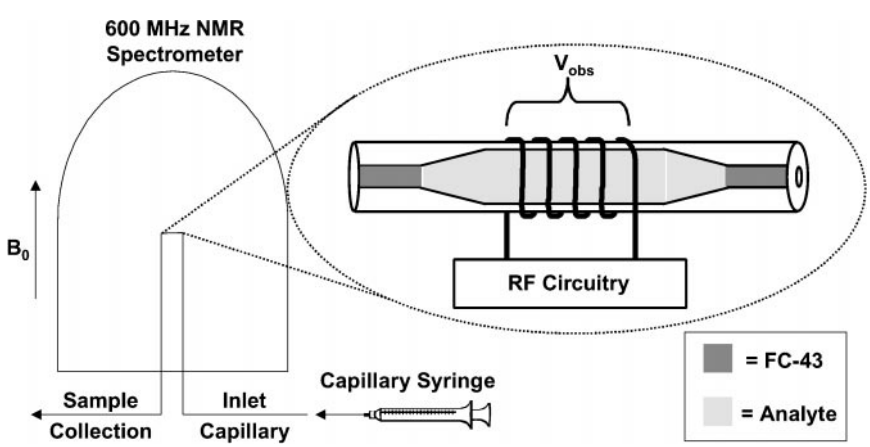


FIG. 1. Schematic of the capillary NMR flow probe used for the single-bead analyses by NMR. The inset illustrates the bubble-type geometry of the flow cell.

0.5 Hz was used prior to Fourier transform, phasing, and integration.

All single-bead NMR experiments were conducted at room temperature (~295 K) using a 600-MHz (14.1 T) spectrometer with a 51-mm bore. The shims, probe tuning, and matching were checked for each sample. For the standard one-dimensional single-pulse and the Carr Purcell Meiboom Gill (CPMG) experiments (*36, 37*), 1216 transients (NT) were collected with a 60° flip angle, an acquisition time (AT) of 3 s per transient with no additional relaxation delay (d1), a spectral width (SW) of 10,000 Hz, and 60,000 complex points (NP). The CPMG experiments employed a total transverse relaxation time (τ) of 2 ms, using a delay of 0.5 ms between the 180° pulses. The total NMR experimental time per spectrum was 1 h. For processing purposes, data were zero-filled to 131,072 points and exponentially multiplied by a line broadening (LB) value of 2 Hz. The baselines of the CPMG spectra were corrected by a spline fit of selected spectral regions. To determine the quantity of analyte produced by cleavage of a single bead, a fully relaxed CPMG spectrum was measured for one sample dissolved in neat DMSO-d_6, using parameters similar to those described previously (except for NT = 2500 and d1 = 10 s). The total NMR experimental time for this spectrum was 9 h. The DMSO-d_5H T_1 relaxation time was reduced to less than 2 s due to the high water content of the single-bead sample. Data were processed in the same manner as above. To aid in the resonance assignments and to evaluate the effects of pH on a distinct yet structurally similar molecule, spectra were also acquired from a sample of imidazole in DMSO-d_6 under both acidic and basic conditions. The residual protonated signal from DMSO-d_5H was used as an internal chemical shift reference at 2.49 ppm.

For quantitation purposes, a solution of 23.4 mM KHP and 18.5 mM Ala-Asp was prepared in 60% DMSO-d_6/40% D_2O. Single-pulse and CPMG spectra of this solution were acquired with a 90° excitation pulse, NT = 16, d1 = 15 s, and $\tau = 0.002$ s (for the CPMG experiment). The apparent DMSO-d_6 concentration in the sample was determined based upon the relative integrated signal intensity of the KHP aromatic signals with respect to the residual protonated DMSO-d_5H in the sample. The DMSO-d_5H concentration in the standard solution was corrected for incomplete T_1 relaxation (*38*) and extrapolated to a solvent composition of 100% DMSO-d_6. The T_1 relaxation times of the KHP and DMSO-d_5H protons in this sample were determined to be 3.1, 2.3, and 10.5 s, respectively, in separate inversion-recovery experiments using relaxation times more than six times greater than T_1. It should be noted that the T_1 relaxation times measured in the microcoil NMR probe are consistently shorter than those obtained for similar samples measured in 5-mm NMR tubes at the same field. While a variety of factors may contribute to this phenomenon, we attribute the shorter T_1 times measured with the microprobe at least in part to enhanced O_2 dissolution as the sample is drawn into a microliter syringe and loaded into the probe. The integrated NMR signal intensities from the cleaved bead samples were calculated and corrected for the 60° excitation pulse. No correction for transverse (T_2) relaxation was required due to the short 180° pulse train used.

Mass spectrometry. Cleaved bead samples were collected in separate vials after the NMR measurements, blown dry under nitrogen, and then dried overnight *in vacuo*. The recovered product was acidified with formic acid for electrospray ionization in positive ion mode. The samples were analyzed using a quadrupole–hexapole–quadrupole mass spectrometer (Quattro I, Micromass, Manchester, UK). Data acquisition and processing were controlled by the Micromass MassLynx NT data system.

RESULTS AND DISCUSSION

The cleavage products from SPS beads examined in these experiments were part of a series synthesized as trypsin-like serine protease inhibitors (*33*). These analytes were used as model compounds to demonstrate the utility of the experimental approach reported here. Figure 2 shows the 400-MHz ^{1}H NMR spectrum of the material cleaved from approximately 9 mg of resin containing many thousands of beads. This reference spectrum provided a measure of comparison between the experiments performed with bulk samples and those performed with individual SPS beads in terms of both sample handling and NMR probe sensitivity.

Optimal sample handling procedures are paramount in any attempt to generate NMR spectra from single SPS beads with acceptable S/N in a reasonable amount of time. Highly sensitive NMR probes must be combined with efficient sample loading so that the maximal amount of analyte is present within the V_{obs} of the probe. Consequently, the experimental strategy reported here uses an immiscible, perfluorinated liquid to prevent diffusional dilution during the measurement and a bubble-type NMR flow cell to maximize the observation efficiency of the RF coil. After cleavage and removal of the stripping solution from the product, the analyte was dissolved in a minimal amount (2.5 μL) of solvent and loaded into the probe. With this particular arrangement, approximately one-third of the total sample resided within the 800 nL V_{obs}, although this sample handling approach has generated observation efficiencies of up to 70% in previous work (39). Such improvements in future work would allow the same quality NMR spectra to be acquired in less than 15 min. While manual sample handling was employed in these experiments, the system is certainly amenable to automation since the use of microliter-volume fluidics is now routine. The coupling of capillary NMR flow probes with automated sample loading would generate significant improvements in the overall throughput of these types of measurements.

Although this custom-built microcoil NMR flow probe demonstrated higher mass sensitivity than commercial probes with larger diameter RF coils, it also produced a significant ^{1}H NMR background signal. While this very broad signal likely arose from epoxy that was used in the probe fabrication process, some of the spectral background can be attributed to residual protons in the magnetic susceptibility matching fluid that

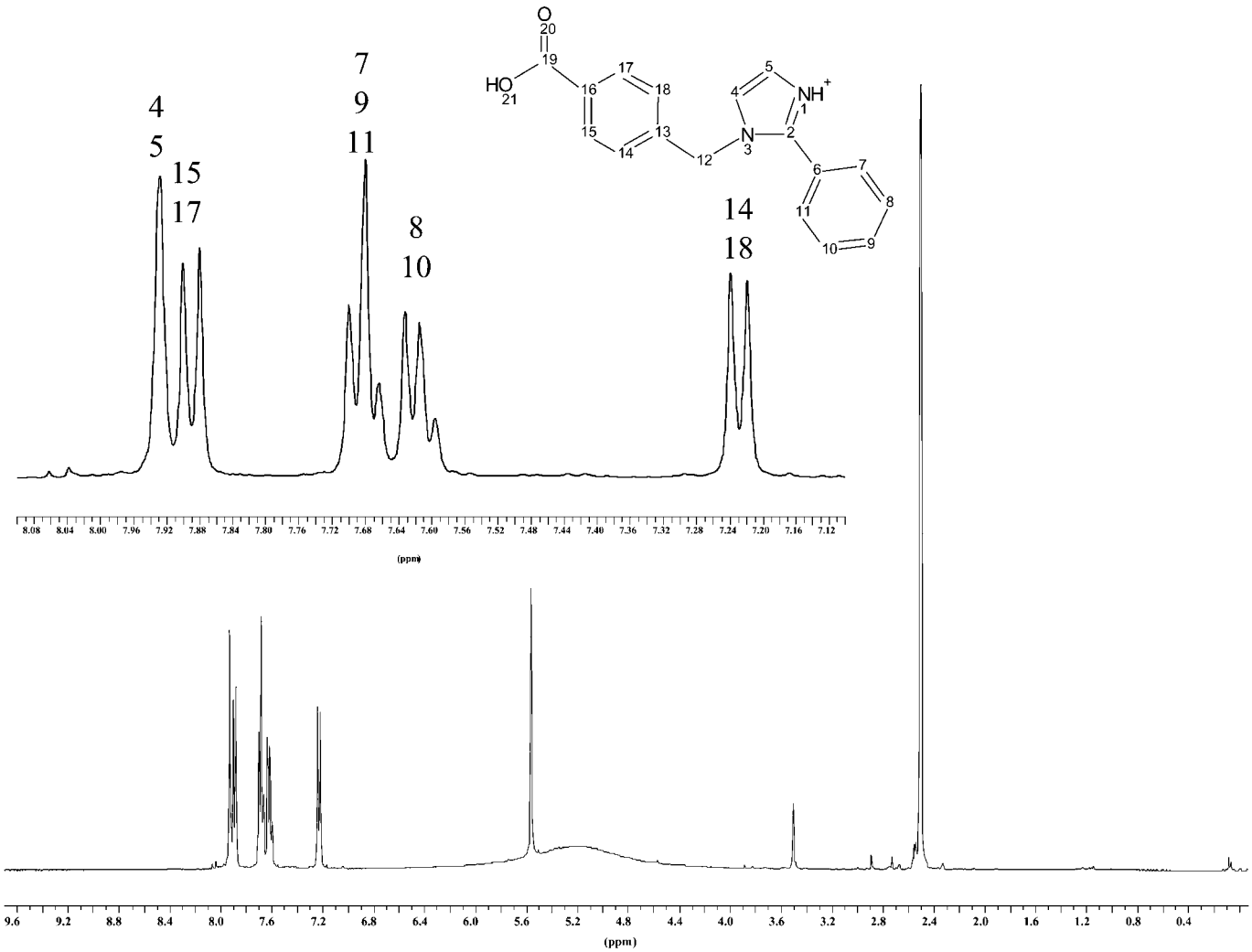


FIG. 2. Reference 400-MHz ^{1}H NMR spectrum of ~580 μg of cleaved product in 600 μL of DMSO-d_6.

surrounded the sample and coil (*31*). As shown in Fig. 3A, the detection of these species resulted in a broad background that was evident even in the presence of resonances with very good S/N. For signals that were closer to the limit of detection, the spectral background proved more of a problem. To alleviate this interference, a CPMG pulse sequence with a very short transverse relaxation time was used. Figure 3B illustrates that this approach eliminated the broad background signals without perturbing the phase of the analyte resonances. Furthermore, since such a short (2 ms) transverse relaxation time was used, the signal intensity of the analyte signals was not measurably affected.

In the course of acquiring the NMR data from single SPS beads, it became apparent that the single-bead spectra did not match the spectrum of the bulk sample. After cleavage and drying of the product from single beads *in vacuo*, the ^{1}H NMR spectra in the microcoil probe consistently resembled that shown in Fig. 4A. While the majority of the resonances could be identified despite slight frequency shifts, the signals from protons 4 and 5 were ambiguous. We hypothesized that the discrepancy between the reference spectrum and the single-bead spectra arose from a difference in pH. The shifts of a model compound which also has an azole moiety (i.e., imidazole) revealed similar spectral shifts as a function of pH. To further examine this hypothesis, a series of single-bead experiments were conducted in which the dried product was dissolved in DMSO-d_6 acidified with DCl. In all cases, the analytes in acidified DMSO-d_6 yielded spectra (see Fig. 4B) that matched the reference spectrum. Based upon these resonances, the structure of the model compound **1** was confirmed according to the spectral assignments listed in Table 1. While the spectra in both Figs. 4A and 4B are consistent with the structure given for compound **1**, the better spectral resolution of the signals in the acidified solution facilitates the resonance assignments.

The flat baseline provided by the CPMG pulse sequence not only eliminated background interference but also aided quantitation (linewidths are very similar). Using the relative intensity

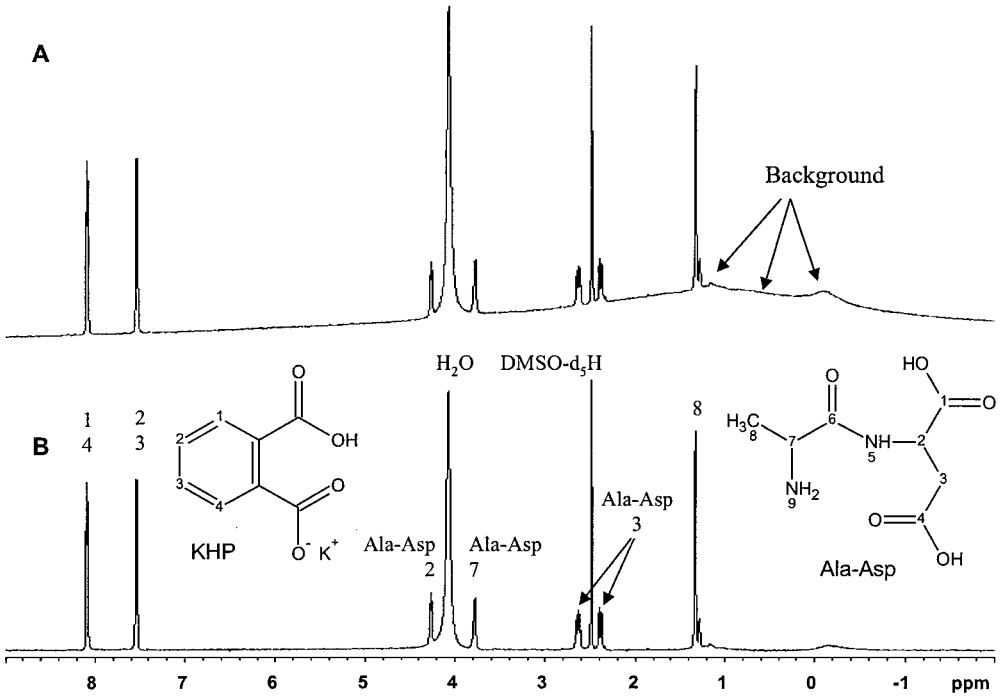


FIG. 3. (A) Standard single-pulse NMR spectrum of a solution of 23.4 mM KHP and 18.5 mM Ala-Asp in 60% DMSO-d_6/40% D_2O. (B) The CPMG spectrum measured for this solution shows a significant reduction in the probe background signal during the 2-ms transverse relaxation time.

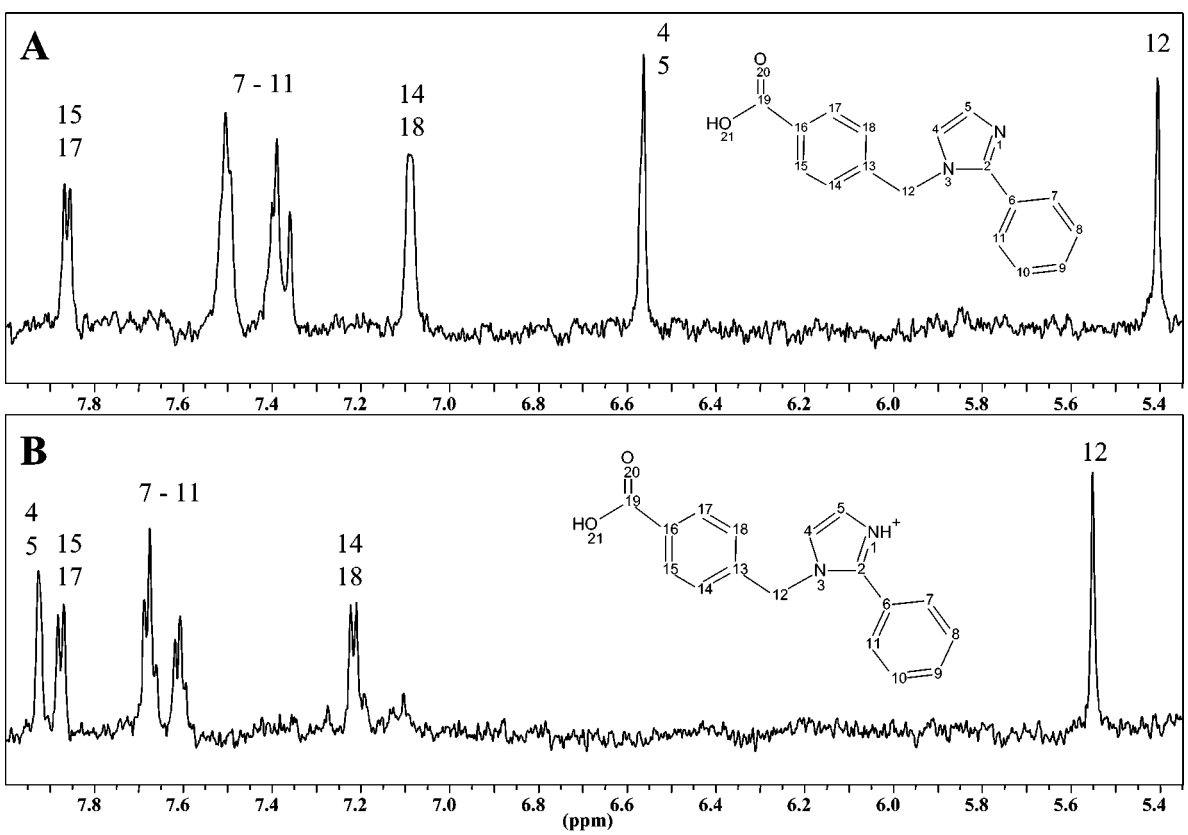
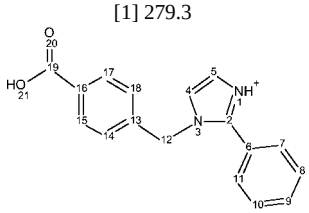


FIG. 4. The ^{1}H CPMG spectra obtained from the cleaved product of a single bead: (A) dissolved in neat DMSO-d_6 and (B) dissolved in acidified DMSO-d_6.

TABLE 1
NMR Spectral Assignments for the Product Cleaved from the Bead

Structure and m/z ion observed	Assignment and nucleus in bold		Chemical shift (δ ppm)	Multiplicity and coupling constants (J Hz $\pm$ 0.2)	
[1] 279.3	4	CH	7.915	d	2.0
	5	CH	7.924	d	2.0
	7, 9, 11	CH	7.68	m	
	8, 10	CH	7.61	t	7.3
	12	CH_2	5.32	s	
	14, 18	CH	7.21	d	8.2
	15, 17	CH	7.88	d	8.2
	21	COOH	12.2	b	

Note. b, broad; d, doublet; m, multiplet; s, singlet; t, triplet.

of the DMSO-d_5H versus the analyte signals in a fully relaxed CPMG spectrum obtained from one of the single-bead samples, the amount of product cleaved from the bead was determined to be 540 $\pm$ 170 pmol (with approximately 180 pmol actually present within the V_{obs}). This result is comparable to the value of 500–600 pmol estimated from the typical loadings provided by the manufacturer of the Tentagel resin. The error in the NMR measurement was calculated from the standard deviation of the result obtained for each resolved resonance of the analyte. Although this error is considerably greater than can be achieved by NMR in other situations (*40*), it is reasonable considering the S/N of the spectrum and the purity of the sample. Finally, as discerned by comparison of the resonance integrals in the spectra of a number of beads, the amount of sample recovered from the neat DMSO-d_6 significantly exceeded the sample recovery from the acidified DMSO-d_6. Since glass surfaces were used in the cleavage and dissolution, and in the microcoil flow probe, the lower yield in the acidified solution most likely resulted from adhesion of the positively charged analyte to the walls of these containers. Despite the lower amounts of material in the acidified solutions, the S/N ratios in these spectra were approximately equivalent to the neat DMSO-d_6 because the linewidths were substantially narrower. The use of plastic vials in the sample preparation steps may significantly improve the quality of the NMR data through higher sample recovery.

To assess the ability to obtain more than NMR data from a single-bead sample, the analyte solutions were collected in vials following the NMR experiments and examined by ESI-MS. The accurate molecular ion was present for these samples. Although MS was performed offline in this example, the microcoil NMR flow probe can easily be connected either in series or in parallel to an MS to generate online multidimensional data. Such systems have already demonstrated success on a conventional size scale (*41*, *42*). Additionally, other characterization techniques such as FT-IR, UV–Vis, microelectrodes, and Raman can be added so that a single bead would provide enough material for a rapid structural elucidation and screening. Although NMR has the lowest sensitivity of these techniques, this study shows that single-bead structural characterization is feasible for standard Tentagel combinatorial beads. As such, the primary bottleneck in the molecular characterization of single SPS beads by multiple analytical techniques has been substantially widened by these capillary NMR flow probes. With the development of microfluidics suitable for automated cleavage and sample delivery to the NMR probe, this approach has broad implications for single-bead structural characterization. While this approach generated high-quality ^{1}H NMR spectra from single combinatorial beads, sample throughput could be increased further by improvements in sample loading, reduced analyte adsorption to the vials, and automated cleavage.

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