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High-Throughput Nuclear Magnetic Resonance Analysis Using a Multiple Coil Flow Probe

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An automated method for high-throughput nuclear magnetic resonance (NMR) spectroscopy has been developed using a four-coil Multiplex NMR probe. The probe is constructed with solenoidal microcoils optimized for detection of small volume, mass-limited samples and a flow-through design. Four samples can be simultaneously injected into the Multiplex probe with a robotics liquid handler and then analyzed in rapid succession using a selective excitation experiment. Due to the simultaneous injection of four samples and the reduced analysis time with rapid selective excitation, the analysis rate achieved thus far is as low as 1 sample/34 s for 1D ^{1}H NMR.

Nuclear magnetic resonance (NMR) spectroscopy is a versatile and powerful analytical tool due to its unique capabilities in structure determination and intermolecular interaction detection as well as its nondestructive and quantitative nature. NMR is routinely used in biomedical and synthetic applications and is increasingly used in clinical analysis, pharmaceutical drug discovery programs, and combinatorial library analysis. With the adoption of combinatorial methods, large numbers of new compounds are being synthesized for areas such as pharmaceutical research, organic synthesis, and catalysis discovery.[1−4] Pharmaceutical companies often have libraries of 500 000 or more compounds with the collection at Pfizer, Inc. exceeding 2 million compounds as reported in 2001.[3] Large libraries of potential drug compounds are screened with NMR techniques in search of interactions with target protein molecules.[5−8] As more libraries are produced with combinatorial reactions, the demand for high-throughput analysis increases. Structural confirmation, yield, and purity estimations are necessary before compounds can be screened for pharmacological activity.[9−11] Pincircoli et al.[10] recently introduced an internal standard to provide purity analysis of combinatorial libraries with 1−2% accuracy with 1D ^{1}H NMR, and Schröder et al.[11] have automated structural verification for combinatorial libraries using 2D ^{1}H, ^{13}C-correlated HSQC (heteronuclear single quantum coherence) NMR data. While NMR is well suited for the analysis of combinatorial libraries, the throughput of NMR is limiting.

Current approaches to high-throughput NMR use automatic sample changers or flow probes with robotic liquid handlers.[12,13] Automatic sample changers are limited by a relatively high failure rate mainly due to the use of glass NMR tubes, which can break and also vary enough that automatic routines such as spinning the sample and finding the ^{2}H lock can fail.[14] The flow probe automation systems commercially available are reported to be more reliable.[12,13] Commercial systems use a flow-through probe design with sample cells aligned parallel to the magnetic field. The flow probes typically incorporate saddle-shaped Helmholtz coils with sample volumes ranging from 100 to 480 μL and active volumes ranging from 40 to 250 μL.[14−17] These systems use liquid handlers that rinse the flow cell, inject the samples from well plates, and, after analysis, retrieve the samples in a serial fashion. The overall recycle time is approximately 2−5 min/sample for these systems, which for 1D NMR analysis is the limiting time factor.[12,18,19] The concentration sensitivity of the flow probes is good and has recently been improved by a factor of 4 using a cryogenic flow probe.[17] Some of the disadvantages of these flow automation systems is carryover caused by sample coating the

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capillary transfer lines, a susceptibility to clogging from low solubility and aggregating samples, and dilution of the sample from push solvent or residual wash solvent.[12]

Another approach to NMR flow probe design is the development of microcoil NMR probes. The microcoil flow probe has been used with several hyphenated techniques, such as microbore HPLC-NMR and capillary electrophoresis (CE)-NMR.[20−22] The advantage of using a microcoil probe is that less sample volume is needed (1 nL−10 μL) and the mass sensitivity (signal-to-noise ratio (S/N) per μmol of analyte) is high.[23] Saddle-shaped coils can also be made for small volume detection as indicated by the recent development of a capillary, 5 μL probe;[24] however, the solenoidal geometry is more sensitive. Microcoil flow probes provide an ideal system for use in cases where small sample volumes are either necessary or desirable, such as natural product libraries or synthetic combinatorial compounds. For example, Eldridge et al. recently used a commercial microcoil flow probe with an active volume of 1.5 μL to determine the structure of a sizable number of biologically active, potential anticancer compounds from a library of 36 000+ natural product molecules.[25] A sophisticated series of high-throughput techniques including HPLC, solid-phase extraction, LC-evaporative light scattering detection-mass spectrometry, and solvent evaporators were used to isolate small molecules from a large, diverse group of plants. Due to the high mass sensitivity of the microcoil probe, only 5−10 μg of pure compound was used for 1D ^{1}H and COSY (correlation spectroscopy) experiments, and sample volumes of only 3 μL were needed. Although most of the preparation steps were automated and conducted in parallel with liquid handlers and 96- and 384-well plate formats, the samples were manually loaded into an injection valve and pushed into the probe with a syringe pump. Even though this microcoil probe flow method is not fully automated, the throughput rate for the microcoil probe is similar to other commercial flow automation methods. In this example, it took 2.5 min to deliver the sample into the probe plus additional time for rinsing and removing the sample.

Microcoils provide another avenue to increase NMR throughput via parallel NMR detection.[26−28] Because solenoidal microcoils are aligned perpendicularly to the magnetic field and are smaller than most saddle-shaped Helmholtz coils, multiple microcoils can be stacked along the magnetic field axis in a single NMR probe. Parallel analysis is common for other analytical techniques but has only recently been explored for NMR.[26−34] Various approaches have been attempted, including isolated circuits,[26,28] rapid selective sample excitation,[32] and imaging methods.[32,34] We previously introduced the multiple-coil Multiplex NMR probe for parallel NMR analysis, which is capable of analyzing four samples using chemical shift imaging or, in rapid succession, using a selective excitation approach.[32] In this article we present an automated injection system incorporating an enhanced version of our Multiplex NMR probe and a Gilson multiprobe 215 liquid handler. Since four samples are injected simultaneously, the limiting time factor for flow automation systems—the time it takes to retrieve and deliver a sample—is immediately reduced by a factor of 4 with the Multiplex probe automation system. For this reason and the time advantages of sequential detection and small sample size, the overall recycle time for one sample can be greatly reduced compared to current NMR flow automation methods.

EXPERIMENTAL SECTION

The complete automated system is shown in Figure 1 and includes a multiple probe 215 liquid handler (Gilson, Inc.), two 12-port valves (Valco Instruments Co. Inc.), the Multiplex NMR probe, and the NMR spectrometer with a 300 MHz magnet.

Multiplex NMR Probe. Included in Figure 1 is a photograph of the Multiplex NMR probehead with the sample detection region consisting of four solenoidal microcoils fabricated by wrapping polyurethane coated high purity (99.99%) 42-gauge (63.5 μm diameter) copper wire (California Fine Wire Co.) around glass capillaries (1.4 mm i.d., 1.7 mm o.d.). The microcoils are attached to the capillary tubes using a cyanoacrylate adhesive (Krazy Glue, Borden, Inc.). Each coil consists of eight turns and has a length of 0.65 mm, resulting in an active volume of 1 μL. The sample capillaries are mounted on a rectangular support made of white Delrin or PVC, which holds the capillary tubes at an intercoil spacing (center to center) of 3.3 mm. The resonant circuit consists of the coils connected in parallel with two variable capacitors (Voltronics Corp.) for tuning and matching the probe. The quality factor value (Q) of the Multiplex probe is 70. Since the four microcoils are connected in parallel, the Multiplex probe is compatible with standard NMR hardware and does not require any additional equipment for operation. The probe is also equipped with a pair of actively shielded pulsed field gradient (PFG) coils, which can produce a linear, axial field gradient.[32] The sample coils and pulsed field gradient coils are housed in a removable PVC container, which is filled with Fluorinert FC-43 (3M Company), a magnetic susceptibility matching fluid shown to improve spectral line width.[35]

Liquid Handler. The Multiplex NMR probe is interfaced to a multiple probe liquid handler (Gilson, Inc.). The liquid handler consists of four syringe pumps controlled in parallel, four beveled

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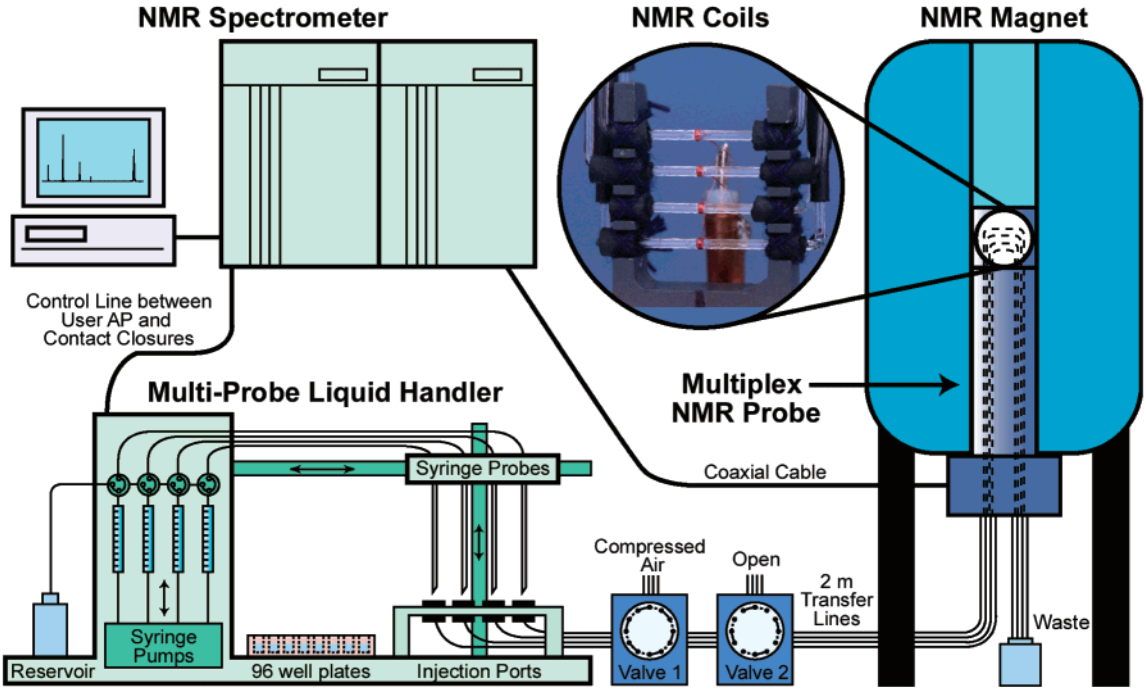

Figure 1. The automation system includes a multiprobe liquid handler, two 12-port valves, the Multiplex NMR probe, and the NMR spectrometer. The liquid handler and spectrometer are electrically connected and communicate through the contact ports on the liquid handler and the user analogue port on the spectrometer. The 12-port valves are controlled by the liquid handler and are used to control and remove the samples during the automation routine. The liquid handler can inject four samples at a time into the Multiplex probe through four transfer lines. The Multiplex flow probe, which is placed inside an NMR magnet, has four sample capillaries each with an NMR excitation/detection coil as shown in the photograph.

stainless steel syringe probes (1.5 mm o.d., 1.1 mm i.d.), a platform that holds up to 10 96-well microtiter plates, four injection ports, a solvent reservoir, and electrical contact ports (see Figure 1). The syringe pumps have a 250 μL capacity and can be used to aspirate or deliver liquid or air in the stainless steel syringe probes or to deliver solvent from the reservoir via three-way valves located in series with the syringe pumps. The syringe probes can be moved in three-dimensions to retrieve samples from multiple well plates and deliver them to the injection ports. The four injection ports are connected to two 12-port valves connected in series. Most of the connections between the components of the Multiplex NMR automation system are made with 320 μm i.d. glass capillary (Supelco) and standard 1/16 in. fittings and capillary sleeves from Upchurch Scientific. Compressed air is connected to valve 1 with 20 gauge Teflon tubing (Small Parts Inc.), and valve 2 is connected to the base of the Multiplex probe with four, 2 m glass capillary transfer lines (see Figure 1). Upchurch union fittings are used at the base of the probe for easy detachment of the transfer lines. Within the probe, the glass capillaries continue to the sample region at the top of the probe. Due to space restriction around the sample region, the sample borosilicate glass capillaries (1.4 mm i.d., 1.7 mm o.d.) are connected to the transfer glass capillary lines with Teflon tubing (300 μm i.d., 1.58 mm o.d., Supelco). Standard fittings and heat shrink Teflon tubing were used to make the connections, and 20 gauge Teflon tubing was used as the exiting waste line.

Spectrometer/Liquid Handler Interface. Control and communication between the NMR spectrometer and liquid handler were realized to provide complete automation. A separate computer (not shown in Figure 1) loaded with the Gilson 735 Instruments Control Software was used to program and control the liquid handler. Electrical communication between the NMR spectrometer and the liquid handler was achieved by using input/output contact closures on the liquid handler and User AP (Analogue Port) connectors on our Varian INOVA NMR spectrometer. The liquid handler software includes "wait" macros that are triggered via the contact closures and are placed in the program to prevent the liquid handler from performing any operations before the spectrometer has completed the analysis. Likewise, software was written for the spectrometer to input the number of samples to be analyzed, wait for and receive signals from the liquid handler, execute the NMR experiments in queue, save the data with a unique filename, and trigger the liquid handler to continue with its program. This communication system provides flexibility in the liquid handler programming and the design of the NMR experiments.

Reagents. D_2O (99.9% D) was purchased from Cambridge Isotope Laboratories. Sixteen water-soluble amino acids (L-alanine, L-arginine, L-asparagine, L-cysteine, L-glutamine, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-threonine, L-valine), D-(+)-glucose, maltose, mannose (all from Sigma-Aldrich), and sucrose (Mallinckrodt Laboratory Chemicals) were used to make a library of samples. Methanol was purchased from Mallinckrodt Laboratory Chemicals. A magnetic susceptibility matching fluid, Fluorinert FC-43, was obtained from 3M Company. All chemicals were used as received without further purification.

Procedure for Samples. Samples (100 mM) of the amino acids and sugars listed in the Reagents section were made in D_2O. One 96-well plate was filled with 30 μL of each sample, and the

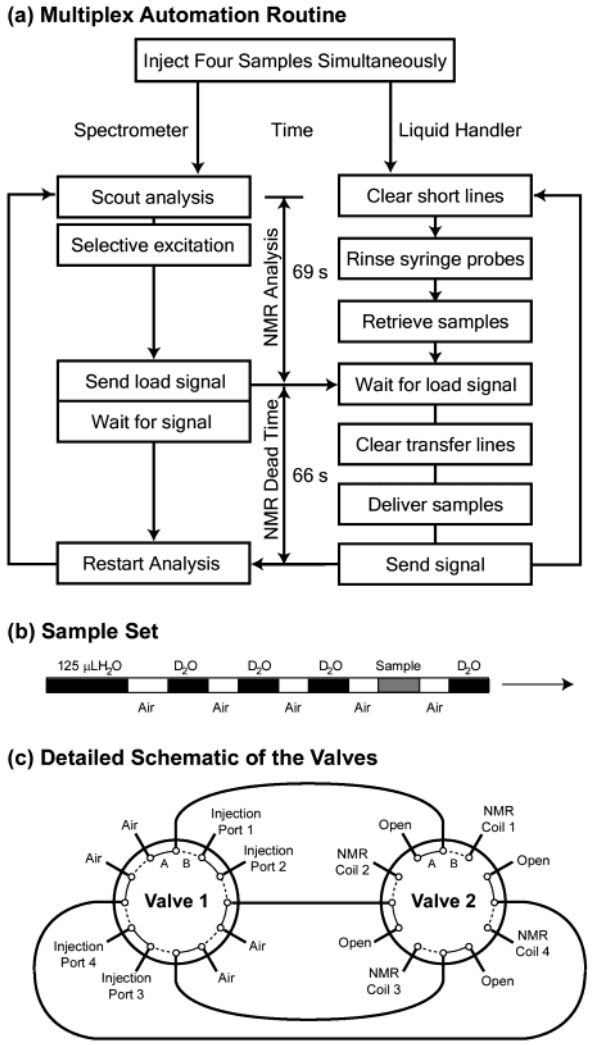

Figure 2. (a) A block diagram of the Multiplex automation scheme is presented with the spectrometer operations listed on the left and the liquid handler operations listed on the right. The NMR analysis time and NMR dead time are listed in the center. See text for a detailed description. (b) Each sample set consists of a 25 μL sample surrounded with four, 35 μL D_2O plugs separated by 15 μL air bubbles and pushed into the Multiplex probe with 125 μL of H_2O. (c) A detailed drawing of the valves shows that valve 1 is a regular 12-port two-position valve (A and B), while valve 2 is a special two-position valve (A and B) that acts as four, three-way valves. The lines that are connected to the 12-port valves include the injection ports, compressed air, open lines, and transfer lines that lead to the sample capillaries in the Multiplex NMR probe.

corresponding wells of another plate were each filled with 150 μL of D_2O. The liquid handler has four syringe probes spaced to retrieve every other sample from a row in a 96-well plate (8 wells in a row by 12 wells in a column), so that each row contained two sets of samples that were injected consecutively before the next row was injected. Glycine samples (100 mM) were injected in all four sample capillaries at the beginning and end of each set of samples as a quality control to monitor the spectral line width for each coil. The line width for each of the four glycine samples was required to be < 2 Hz to be considered acceptable.

Sample Delivery Method. A block diagram of the liquid handler and spectrometer operations for delivering samples and acquiring data is shown in Figure 2a. The liquid handler was programmed to retrieve four samples simultaneously from 96-well plates. Plugs of D_2O separated by air bubbles surrounded the samples. The D_2O was used to wash the transfer lines and syringe probes of residual H_2O, which would otherwise dominate the spectrum. The liquid handler was programmed to simultaneously retrieve four sample sets consisting of three 35 μL D_2O plugs, 25 μL of sample, and another 35 μL of D_2O each separated by 15 μL of air as shown in Figure 2b. Before injecting the samples into the injection ports, the liquid handler was programmed to wait for an electrical signal from the spectrometer, which sent a signal to the liquid handler when it was ready for the next set of samples, i.e., when the analysis of the previous samples was complete. The valves were switched (valve 1 = A, valve 2 = B, see Figure 2c) to open the transfer lines to filtered, compressed air, which forced the previous samples from the lines and sample capillaries to the waste container. After the lines were free of liquid, valve 1 was switched (valve 1 = B) to open the injection ports to the transfer lines. The four sample sets (sample, D_2O plugs, and air) were then injected into the transfer lines. Without moving the syringe probes from the injection ports, 125 μL of H_2O was injected from the reservoir into each of the transfer lines to center the samples in the sample capillaries of the Multiplex NMR probe.[36,37] The 125 μL of H_2O also served to wash the lines and sample capillaries as it was forced from the lines after analysis with compressed air. Immediately after injection, the liquid handler was programmed to send a signal to the spectrometer, which initiated the analysis, and valve 2 was switched (valve 2 = A) to prevent a siphon from draining the samples from the Multiplex probe. In this position valve 2 also served to open the short lines between the valves and the injection port so that the syringe probes could pull the remaining H_2O from the short lines between the valves in preparation for the next samples. After the short lines were cleared, the syringe probes were moved to the rinse station of the liquid handler to eject the extra H_2O and rinse the inside and outside of the syringe probes with 100 μL of H_2O each. The liquid handler then immediately retrieved the next four samples and waited for the NMR spectrometer to finish taking data before the new samples were loaded.

NMR Analysis. Spectra were collected using a Varian INOVA spectrometer operating at 300 MHz for ^{1}H. Individual spectra of the samples were collected using the selective excitation pulse

(36) Before any experiments could be run, all four lines had to be calibrated with respect to one another. All lines were calibrated by pushing 500 μL of water through the empty lines at a flow rate of 0.48 mL/min while acquiring spectra every 0.125 s corresponding to a spectrum every 1 μL. The spectrum with the first evidence of the water peak for each line was recorded and compared for all lines. The experiment was run 7 times for each line, and the lines were cut until the spectral appearances which occurred were within one unit (1 μL) of each other. The standard deviation of 7 runs was 2 μL units.

(37) The volume of push water that was needed to center the sample in the sample capillary around the excitation/detection coils was determined for each line by arraying the push volume by 1 μL increments over a 20 μL range. Glycine (100 mM) in D_2O was used as the sample because it has one, singlet peak from which the line width at half-height is easily measured. The line width of the glycine peak was used to gauge whether the sample was centered around the coil. If the sample was not centered and the air bubble on either end of the sample was within 0.5 cm of the coil, then the line width would broaden from its normal value of 1−2 Hz until the peak disappeared into the baseline. With a 25 μL sample, it was found that the push volume could fall within an 8 μL window, and the glycine line width would still be < 2 Hz for each coil. The windows for each line overlapped in the range of 121−128 μL. Thus, 125 μL of push solvent was used to center the samples.

sequence[32] with presaturation of the water signals. Analysis was initiated after the samples were injected into the Multiplex probe by a signal from the liquid handler that triggered the NMR spectrometer to start the queued experiments. Before the selective excitation experiment could be performed, the frequency of the water peaks in each coil had to be determined for optimum suppression of the water signal. This was accomplished with a quick scout scan of each of the samples.[38] One scan of each sample was collected with no presaturation pulse and a shortened acquisition time (0.5 s). The spectrometer then automatically executed a macro that was written to locate the water peak in each spectrum and set the presaturation pulse frequency to an array of the four values determined. Through a set of macros, the spectrometer was programmed to start the selective excitation experiment with the new presaturation pulse frequency array. Figure 3a illustrates the pulse sequence that was used to analyze the samples with a presaturation pulse of 1.5 s. Since the presaturation pulse was not a selective pulse, all four coils were excited with the same excitation bandwidth, which could occur at slightly different chemical shifts for each sample if the magnetic field at each coil was different. To maintain good resolution and to avoid potentially saturating the analyte signals in other coils, the coils were shimmed individually with the stipulation that the frequency scales of all the coils must overlap within 5 Hz (0.02 ppm) for each shim set and the line width at half-maximum of each coil must be <2 Hz. These parameters were determined using four glycine samples. Therefore, the shims could be arrayed with the presaturation pulse frequency to maximize the S/N of each coil and to prevent saturation of resonances far from the solvent frequency in each coil. Only fast responding shims were arrayed to avoid artifacts from equilibrating shim currents.

After presaturation a large field gradient (6.6 G/cm) was applied to shift the samples into separate spectral regions (compare parts b and c of Figure 3).[39] During the gradient, a selective 90° Gaussian-shaped pulse (1.7 ms) was applied with a transmitter offset that was specific to one of the four spectral regions (see Figure 3c). After excitation, a smaller, reverse gradient (3.5 G/cm) was applied to refocus any partially dephased magnetization, and the free induction decay (FID) signal was acquired (2 s). The simulated result shown in Figure 3d is a spectrum with signal from only one coil. Each coil is excited sequentially by arraying the transmitter offset of the Gaussian pulse with the presaturation pulse frequencies and the shims. Signal averaging was achieved by repeating the array multiple times and storing the data in four, separate free induction decay (FID) data files. The selective excitation approach provides a time advantage compared to a single coil probe because it utilizes the time needed for the samples to relax back to equilibrium. While one sample is relaxing, scans for the other three samples can be acquired. The samples that were analyzed have a relaxation rate that was fast enough that the 1.5 s presaturation pulse provided enough time between scans that no relaxation delay was needed.

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(39) The Gaussian pulse width, transmitter power, and gradient strengths were determined. The pulse width and transmitter power were chosen to give a 90 degree pulse with a 1350 Hz excitation width at half-height and 8200 Hz at 1% height. The gradient was chosen to separate the centers of the spectral windows of neighboring coils by 9000 Hz. The reverse gradient strength was chosen to give the best S/N for each coil.

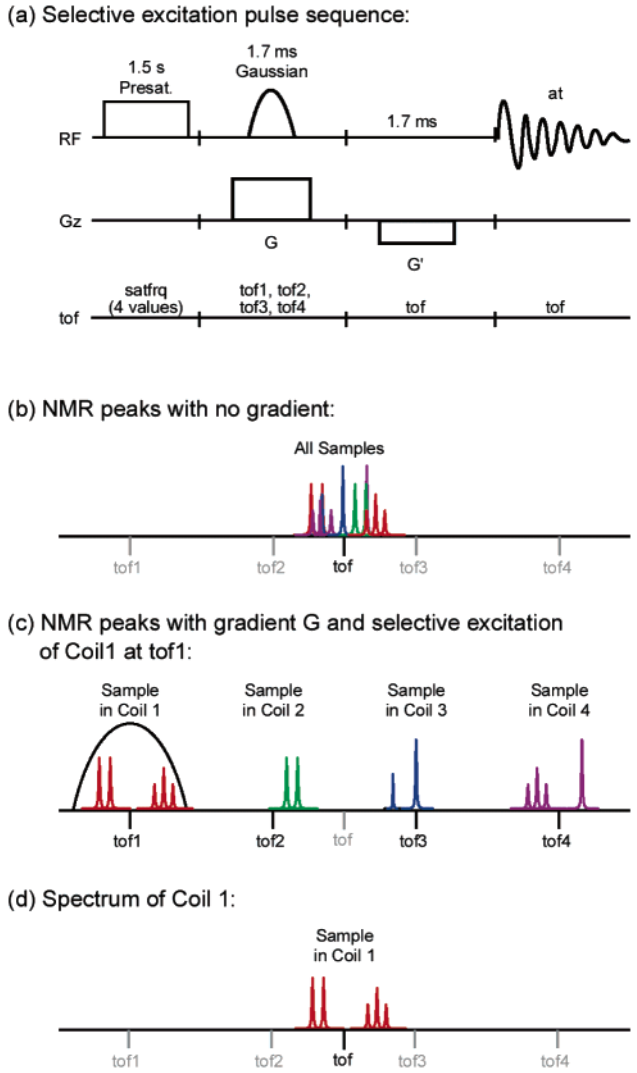

Figure 3. (a) The selective excitation experiment can be described by three components acting simultaneously: the radio frequency channel (RF) where radio frequency pulses are activated to excite protons in the samples, the gradient channel (Gz) which creates a linear, axial magnetic field gradient via the pulsed field gradient coils, and the transmitter offset channel (tof) which controls the center frequency of the radio frequency pulses. The sequence consists of a presaturation pulse centered at four different frequencies (satfrq), a selective Gaussian-shaped pulse applied during a gradient pulse with four different transmitter offset frequencies (tof1, tof2, tof3, tof4), a refocusing gradient, and acquisition (at) of the NMR signal. (b) If no gradient is applied during the pulse sequence, then the spectrum acquired will contain signal from all four coils. (c) When a large axial field gradient is applied, the spectral windows of the four coils separate in frequency. A selective Gaussian radio frequency pulse is applied to excite the sample from a single coil by centering the pulse within the spectral window of that coil using tof1. (d) After a refocusing gradient, the excited signal is acquired, resulting in a spectrum of the single coil that was excited (coil 1). Each coil is excited and detected sequentially by repeating the pulse sequence and arraying the Gaussian pulse transmitter offset values. After analysis, the spectrum from each coil is saved in a separate free induction decay (FID) data file.

In addition, the delay allowed any accidental presaturation of signals near the water frequency of the other three samples to relax at least partially back to equilibrium. Experiments were performed where 1, 2, and 4 scans were acquired for each sample,

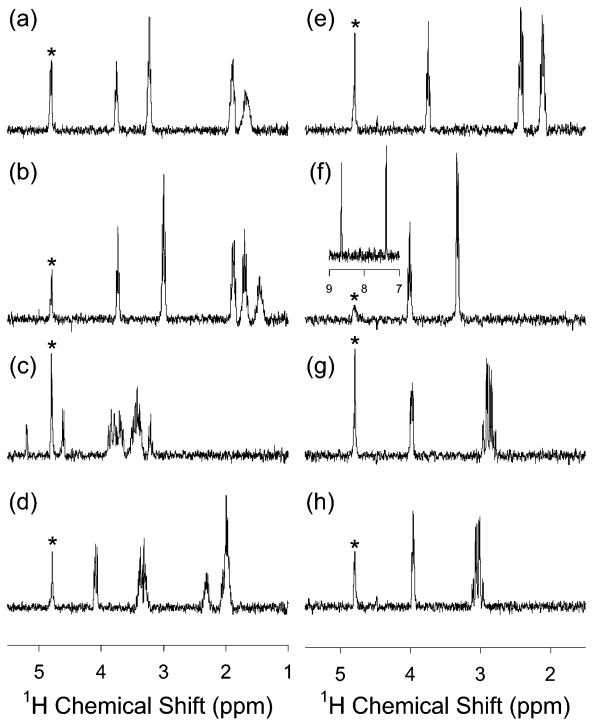

Figure 4. Spectra of eight samples automatically injected into the Multiplex NMR probe were acquired with the automated flow injection routine: (a) L-arginine (b) L-lysine, (c) D-(+)-glucose, (d) L-proline, (e) L-glutamine, (f) L-histidine, (g) L-asparagine, and (h) L-cysteine, all at 100 mM. The asterisks indicate the residual HDO peaks after suppression with a presaturation pulse.

and the spectra were processed with 1 Hz line-broadening, zero-filled twice, and baseline corrected.

RESULTS AND DISSCUSSION

Twenty samples were automatically injected and analyzed four at a time using the automated Multiplex system. Figure 4 shows eight of these spectra taken during the analysis with each column containing one spectrum from each coil in the Multiplex probe. The line width in each spectrum is between 1 and 2 Hz, and presaturation was effective in suppressing the water peaks by a factor of approximately 40. While the spectrometer is analyzing samples with the scout scan and the selective excitation experiment (Figure 2, left column), the liquid handler requires 69 s to clear the short lines after the samples are delivered, rinse the syringe probes, and retrieve the next samples and D_2O plugs from the well plates (see Figure 2, right column). Using the current methodology this is the minimum time available for NMR data acquisition. After the liquid handler retrieves the next set of samples, it waits until the spectrometer is finished acquiring data. Depending on the number of scans needed for a particular sample, any time beyond 69 s will add time to the overall automation routine. In the interest of speed, only one scan was acquired for most of our experiments so that the liquid handler would be immediately triggered.[40] The liquid handler requires 66 s to clear the four transfer lines with compressed air, deliver the samples, and center the samples in the Multiplex probe sample capillaries with push solvent (see Figure 2, right column). The automation procedure is repeated for each set of four samples and requires only 34 s/sample.

As with the current flow automation routines, one goal is to minimize spectrometer dead time, i.e., the time it takes to remove the analyzed samples, rinse, and deliver the next samples. The samples are delivered at a rate of 0.5 mL/min, which was found to be the fastest rate allowable without significant mixing or breaking up of the samples and D_2O plugs. If a more viscous solvent such as dimethyl sulfoxide (DMSO) were used, a slower flow rate would be necessary to avoid mixing effects and increased backpressure. The volume of the transfer lines from the injection ports to the sample capillaries was measured to be 302 μL, largely due to the use of 320 μm i.d. capillaries. This volume could be reduced if a shielded magnet were available because the liquid handler could be moved closer to the Multiplex probe and shorter transfer lines could be used. Additionally, if a high-pressure system were used, the capillary inner diameter could be reduced. Both approaches would significantly decrease the total volume between the injection ports and the NMR sample capillary and the time needed to deliver the samples perhaps by a factor of 4. Even though further improvements could be made, the current dead time of 16 s/sample is a significant improvement over the approximately 2 min/sample typically available with current methods.

Another consideration for automatic injection of samples is contamination and dilution from the solvent used to push the sample and rinse the system. Although it is useful to use D_2O as a solvent to reduce the ^{1}H solvent signal, for cost considerations, it was appropriate to use H_2O to wash the lines and syringe probes and to use as a push solvent. To avoid ^{1}H contamination of the sample, D_2O plugs were used to separate the sample from the push solvent in the syringe probes. It was found that the majority of the ^{1}H contamination came from aspirating the samples into the stainless steel syringe probes of the liquid handler. To solve this problem, three 35 μL plugs of D_2O were aspirated into the syringe probes before the sample. Another source of ^{1}H contamination was in the transfer lines themselves. Since 125 μL of H_2O was used to push the samples to the center of the Multiplex probe sample capillaries, small droplets of H_2O were left behind at various points such as the connectors and the walls of the capillaries. Thus, a 35 μL D_2O plug was aspirated after the sample and was the first liquid to travel through the transfer lines (see Figure 2b). With this method, the ^{1}H concentration in the form of HDO was only increased by 175% compared to a sample that was manually injected into the probe. If the cost of D_2O could be tolerated, then D_2O could be used in the solvent reservoir and the extra D_2O plugs would not be necessary; thus, reducing the time needed to retrieve the samples, lessening the ^{1}H contamination, and possibly reducing the need for presaturation.

During delivery of the sample, dilution can occur from the rinse solvent and D_2O plugs at connections where dead volumes and inner diameter mismatches retain small droplets of liquid. Using Upchurch fittings minimized the connection dead volumes, and the inner diameters were matched as closely as possible. The samples were diluted to only 92% compared to a sample that was

(40) Currently, when the shim values are arrayed, an internal delay between scans results with the Varian INOVA spectrometer. The delay is 4−6 s/scan. When this problem is circumvented, the number of scans that can be performed for each sample in the 69 s window of analysis time can be increased from 1 to 5 without adding time to the automation method.

manually injected (100%). Other automation procedures use nitrogen or air to dry the lines between samples, which is effective but very time-consuming.[13] Eliminating the Teflon shrink-wrap connections and 300 μm i.d. Teflon tubing at the sample capillaries and replacing them with standard fittings should reduce the dilution effect. Also, smaller inner diameter sample capillaries (with etched sample chambers)[41] that more closely match the transfer line inner diameter would improve the sample integrity.

Since microcoils are best suited for mass-limited samples, it is important for the automation method to use as little sample as possible. In this way samples that can be easily concentrated, through extraction or evaporation techniques, would not be limited by the volume requirements of the Multiplex automation method. Cone-shaped well plates and beveled syringe probes were used so that all but 5 μL of sample could be retrieved from the well plate. Therefore, only 30 μL of sample was needed in the well plate to retrieve and inject a 25 μL sample plug. Using smaller inner diameter syringe probes and carefully calibrating the syringe probe depth in the well plate could further reduce this residual sample volume.

The sample size for the Multiplex automation method is limited by the proximity of the air bubbles that surround the sample to the NMR coil. The air bubbles locally distort the magnetic field and can result in poor line shapes and reduced sensitivity if they are not several millimeters from the coils. The line width at half-maximum is a good indicator of the proximity of the air bubbles and was used in several experiments to calibrate the push solvent volume, determine the minimum sample requirement, and determine the reproducibility of sample delivery. To center the 25 μL samples in the sample capillaries, the push solvent volume was calibrated by varying the volume by 1 μL increments for each sample capillary.[37] Through these experiments it was determined that the push solvent volume produced acceptable results from 121 to 128 μL, indicating that 25 μL of sample was sufficient to easily center in the sample capillary and prevent distortions from the air bubbles. In fact, the large margin in push volume implies that the sample volume could be decreased to a minimum of 17 μL. The reproducibility of sample injection was tested by analyzing 308 samples (25 μL) over 2 days using 125 μL of push solvent to center the samples. Only two samples were not centered in the sample capillary giving a reproducibility rate of over 99%.

Several methods were tested to eliminate the need for air bubbles around the sample so that smaller sample volumes could be used. One approach was to use FC-43 to surround the sample since FC-43 is not miscible with water and does not distort the magnetic field at the sample/FC-43 interface.[42] However, it was found that the FC-43, D_2O, and sample mixed within the glass capillary lines creating bubbles of FC-43 in the sample. Teflon lines with a 300 μm i.d. were also tested and caused less mixing but only at relatively slow flow rates. Since air bubbles cannot be eliminated with the current system, a better approach would be to reduce the inner diameter of the transfer lines and use a smaller diameter sample capillary with an etched sample chamber.[41] With these approaches and a high-pressure system, the sample volumes for the Multiplex probe could become as small as a few microliters without the need for air bubbles as has been demonstrated.[25]

A significant issue in NMR analysis is the sensitivity. Microcoils provide several advantages to flow-through probes because of their good mass sensitivity and small volume requirements.[23] Good mass sensitivity is achieved at the expense of concentration sensitivity (S/N per unit concentration). A useful comparison can be made with the limits of detection (LOD). The concentration LOD of the Multiplex probe is approximately 3 mM at 300 MHz for a single scan and is about a factor of 15 worse than the LOD of a typical 5 mm probe. Nevertheless, when only small sample amounts are available, it is a better strategy to concentrate those samples and use a smaller detection coil, as the mass sensitivity of the Multiplex NMR probe is approximately 100 times greater than that of a typical 5 mm probe.

With the Multiplex probe, as the number of coils in the probe increases, the S/N decreases by a factor of $n^{1/2}$, where n is the number of parallel coils, assuming the sample of interest is in only one coil. This reduced S/N results from only one sample coil contributing to the signal while all contribute to the noise. A probe with one coil was tested for S/N using a sample of 0.5 M CH_3OH in D_2O with a sample volume of approximately 60 nL and was found to have a S/N of 140. When additional coils were added in parallel, the S/N from one coil dropped to 120 for a two-coil probe and to 70 for a four-coil probe. While a decreased S/N is undesirable, this fact does not affect the efficiency of the automation routine significantly even when multiple scans are necessary. By using the selective excitation approach, four samples can be analyzed in the same amount of time as one sample, assuming that the relaxation delay required by the samples is equal to or greater than 3 times the acquisition time. Therefore, even though 4 times more scans are required for each sample to maintain the same S/N, the scans are taken 4 times faster because the normally unused time for relaxation is being utilized with the selective excitation method. With 69 s available for acquisition, at least five scans can be acquired without slowing down the current automation procedure (assuming faster shim resetting is possible[40]). By taking advantage of this available scan time one can more than make up for the reduced S/N. The net result is that the four-coil probe is about a factor of 4 faster than a single coil probe of the same dimensions, assuming a similar automation procedure is used. At higher numbers of scans the advantage diminishes somewhat, but unless the scan time is much longer than the load time, the advantage is significant. Of course, when there are many samples to be analyzed, it is beneficial to work at the highest analyte concentration possible to reduce the overall analysis time by keeping the number of scans relatively low.

Nevertheless, improvement of the concentration LOD is desirable and can be improved by reducing the line width, increasing the filling factor (sample volume/coil volume), or using higher magnetic fields. However, the least expensive and perhaps easiest method to increase the concentration LOD is to increase the coil volume. Coils were prepared with 5, 8, 10, and 14 turns around identical sample capillaries, and the S/N was recorded for a single scan. As summarized in Table 1, as the coil volume increases with the number of turns, the concentration sensitivity increases and the mass sensitivity stays essentially constant. Since the inductance of four identical coils in parallel is a factor of 4

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Table 1. Four Sets of Coils Made for the Four-Coil Multiplex NMR Probe, Each Set with a Different Number of Turns[a]

number of coil turns	S/N for the four-coil probe	S_m (S/N per μmol ^{1}H)	S_c (S/N per M ^{1}H)
5	84	1093	56
8	155	1267	103
10	198	1291	132
14	262	1223	175

[a] The coils were wrapped around 0.4 mm i.d., 0.8 mm o.d. glass capillaries with 63.5 μm copper wire and connected in parallel to the Multiplex probe circuit. One of the sample capillaries was filled with 0.5 M CH_3OH in D_2O, and the others were filled with D_2O. The S/N of the methanol peak was determined for a single scan Bloch decay experiment without line-broadening and was used to determine the mass and concentration sensitivities.

less than a single coil, the number of turns of the coils can be increased and still be tuned as a single coil. Therefore, it is possible to recuperate some concentration sensitivity for a multiple coil probe by increasing the coil volume.

The automation of a multiple coil flow probe provides some unique possibilities over a single coil probe. For instance, there is an opportunity to optimize each coil for a different solvent. Changing the solvent in a single-coil configuration requires flushing the tubing of the liquid handler and the probe, recalibrating the push solvent volume for solvents with different viscosities, and reshimming the probe for a particular solvent. With the Multiplex probe each sample capillary could be connected to a different solvent reservoir with calibrated line volumes and optimized shim sets for each solvent. A multiple solvent system would provide flexibility for instruments with multiple users and when issues of sample solubility are important. Another application for the Multiplex probe is to better match the coil volume to the sample volume. A multiple coil probe can be used to customize the coil volume to the sample volume. Since all four coils are connected in parallel, they can be excited/detected simultaneously as one, larger coil, which would result in an improvement in sensitivity by a factor of 2 and a decrease in the experiment time by a factor of 4 compared to a single, microcoil probe. Likewise, two or three sample capillaries could be filled with the same sample and analyzed to provide customized coil volumes. This would be particularly advantageous for peak-trapping LC NMR experiments, where eluent peaks can have different volumes and probe changes are time-consuming. With four sample coils that can be treated either independently with the selective excitation experiment or simultaneously, the Multiplex NMR probe provides flexibility and convenience in the NMR experiments that cannot be obtained with a single coil probe.

CONCLUSIONS

An automated system for high-throughput, parallel NMR analysis has been developed. Four samples are injected and analyzed in parallel using the unique design of the multiple coil Multiplex NMR probe. The main time advantage of the Multiplex probe is a reduction in the spectrometer dead time because four samples are delivered simultaneously and capillary rinsing is integrated with the delivery and expulsion of the samples. In addition, the four samples are analyzed in rapid succession leading to a reduced relaxation delay and faster analysis times. The routine is currently capable of analyzing the samples at an overall rate of 1 sample/34 s to produce 1D ^{1}H NMR spectra with the presaturation-selective excitation pulse sequence. More importantly, the time that the spectrometer is being used is maximized since only 16 s/sample is required to introduce new samples into the Multiplex probe. The method is completely automated with samples retrieved from well plates, electrical communication between the liquid handler and spectrometer, and spectrometer programs to scout water frequencies and save spectra automatically. Improvements to the probe and liquid handler configuration are being investigated to increase the speed of analysis and the sensitivity of the probe, to decrease the volume of sample that is needed, and to provide a method for sample recovery. As discussed above, with further development, it is not unreasonable to expect additional reduction of sample volumes by 4, increases in sensitivity by a factor of 2−3, and increases in throughput of 2 or more in the near future. With the union of automated sample handling and the microcoil flow Multiplex NMR probe, mass limited samples can be analyzed with a method that significantly lowers the time necessary for NMR analysis, paving the way for truly high-throughput NMR analysis of large numbers of samples.

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