Sample Concentration and Separation for Nanoliter-Volume NMR Spectroscopy Using Capillary Isotachophoresis

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Full characterization of trace level analytes in complex mixtures is vital to diverse areas such as cellular analysis, natural product extraction, and combinatorial chemistry. Since nuclear magnetic resonance (NMR) spectroscopy can provide structural and dynamic information unattainable by other means, it has found broad utility for compounds ranging from small organic molecules to biopolymers. Despite major advances in performance, NMR systems still require much greater sample amounts than other molecular characterization methods. We present here a technique that concentrates analytes by capillary isotachophoresis (cITP) to generate the highest mass sensitivity ^{1}H NMR spectra ever reported from low microliter-volume samples.

While increased magnetic field strengths have yielded major NMR sensitivity gains, escalating costs and significant technical obstacles have motivated additional strategies such as polarization transfer techniques, cryogenic probes, and reduced-diameter radio frequency (RF) coils.[1] Since mass sensitivity (S_m, S/N per unit amount) is inversely proportional to coil diameter to a first approximation, commercial NMR probes have been designed for mass-limited samples with volumes in the tens of microliters. Solenoidal microcoil probes, which have observe volumes (V_{obs}) of 5 nL to 1 μL, have shown even further improvements in S_m. However, efficient delivery of nanoliter-volume samples into the microcoil V_{obs} within the magnet remains challenging. Sample handling losses and dilution during transfer are difficult to avoid in this smaller volume regime, so that only a small fraction of the injected sample may reside in the V_{obs}.

We report the first coupling of cITP to NMR. Using cITP, larger (several microliters) sample volumes may be injected and focused to less than 100 nL, within the size regime of a microcoil V_{obs}. This method improves the concentration sensitivity of nanoliter-volume NMR by 2 orders of magnitude. Focusing may also purify analytes from contaminants. Individual components of complex samples may be focused into discrete zones, allowing resolution of their NMR spectra.

Sample-focusing methods are well established in capillary electrophoresis (CE).[2] In cITP, the sample is introduced between leading ions, which have an electrophoretic mobility (μ_e) greater than the analyte, and trailing ions of lower μ_e.[3] When an electric field is applied, sharp and stable boundaries form between discrete zones of ions with different μ_e. Once established, the bands remain in contact and travel at constant velocity. To maintain constant current throughout the capillary, each zone must contract or expand to adjust its analyte ion concentration proportional to the leading electrolyte (LE). Hence, the focused volume of each zone is determined by the quantity of charged analyte ions present. For cITP NMR, this would permit focusing an injected sample plug 10 cm in length into a 1-mm-long microcoil V_{obs}. cITP electrolyte systems are available for either anionic or cationic analytes.[3] In the following experiments, cationic analytes were focused by using 120 mM sodium acetate-d_3 (pH 5) in D_2O as the LE and 10 mM acetic-d_3 acid-d (DAc) as the trailing electrolyte (TE).

The cITP NMR instrumentation was similar to that used previously for microcoil NMR detection of CE.[4] All NMR experiments were performed on a Varian 500 MHz spectrometer with a wide-bore magnet, using a novel microcoil sleeve probe in which the RF coil was wrapped around a thin polyimide sheath.[5] A fused silica capillary was inserted through the sleeve to create a V_{obs} of ∼30 nL. Polyvinyl alcohol (PVA) coated capillaries were used to minimize electroosmotic flow, which degrades the boundaries between analyte bands. Hydrodynamic flow was used for sample injection and for positioning the focused sample band in the coil V_{obs}. Injection and separation conditions to focus a sample into a sharp band in 5−10 min were determined by observing the charged visible dye methyl green (MeG) in a capillary on a benchtop.

Figure 1 compares a spectrum of 5 mM tetraethylammonium bromide (TEAB) acquired without preconcentration (Figure 1A) to a stopped-flow cITP NMR spectrum of 8 μL of TEAB injected at 200 μM initial concentration (Figure 1B).[5] The S/N for the latter was twice as great as in Figure 1A for the same 10 s NMR experimental time and data processing parameters. Based upon integrated peak area, cITP focusing in Figure 1B resulted in ∼100-fold increase in concentration, with an overall increase in sample observation efficiency from 0.5% to ∼50%. Similar concentration factors also were obtained for 7 to 16 μL injections of analytes at initial concentrations of 30 to 200 μM.

One potential concern in using focusing methods with NMR is that local differences in the magnetic susceptibility of the focused zones near the NMR coil may degrade spectral resolution.

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(5) Coated 50-μm-diameter round Cu wire was used to wrap an 18-turn solenoid around a 370 μm i.d./420 μm o.d. polyimide tube. Static analyses yielded line widths between 1 and 2 Hz at 500 MHz. Focusing was performed in an 85-cm-long, 200 μm i.d./360 μm o.d. fused silica capillary internally modified with a covalent PVA coating (Goetzinger, W.; Karger, B. L. US Patent 5840388, 1998). The inlet was placed directly in the TE at the anode 20 cm below the magnet. The outlet capillary, extending 12 cm beyond the NMR coil, was connected to the cathode reservoir of LE by 22 ga Teflon tubing. The capillary was soaked with 0.1% Triton X-100 detergent in D_2O for 30 min after every 3 runs. Before each injection, the capillary was flushed with LE. Sample injection was hydrodynamic: 2−6 μL TE, 7−16 μL sample in 50% TE, 0−7 μL TE. The initial injection of TE aided rapid formation of the LE/sample boundary; the latter injection moved the sample plug closer to the V_{obs}. The applied potential was 20 kV. Since the LE has much greater conductivity than the TE, the relative position of the focused sample can be estimated by the electrophoretic current. Extended NMR acquisitions were obtained by discontinuing the applied electric field and using a stopcock near the cathode vial to stop the flow.

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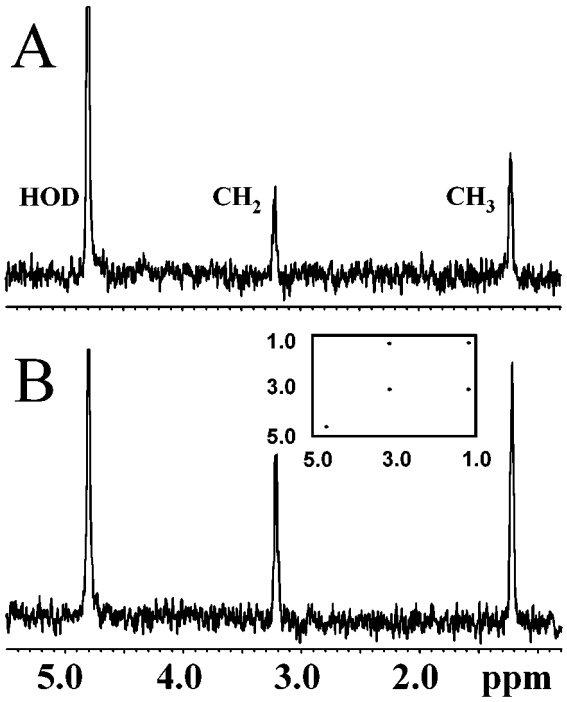

Figure 1. [1]H NMR spectra of TEAB. (A) Capillary filled with 5 mM TEAB without sample stacking (S/N of peak at 1.2 ppm = 13). (B) 8 μL of 200 μM TEAB injected; spectrum after sample stacking by cITP (S/N of peak at 1.2 ppm = 30). The inset shows a COSY spectrum. 1D NMR acquisition parameters: SW = 5000 Hz, NP = 12420, AT = 1.242 s, 55° pulse, delay time = 0 s, NT = 8, total NMR experimental time = 10 s. Data processing conditions: two ZF, linear back prediction (LN) of 4 points based on the first 64 data points, LB = 2 Hz. COSY acquisition parameters: SW = 2271.4 Hz, NP = 1024, AT = 0.225 s, delay time = 1 s, NT = 4, NI = 256, time increment change = 0.44 ms, total NMR experimental time = 22 min. COSY processing conditions: magnitude mode, one ZF along the indirect dimension, sine-bell multiplication in both dimensions.

For typical one-dimensional cITP NMR spectra for the sleeve probe, the line widths ($w_{1/2}$, full width at half-maximum) degraded from 2 to 4 Hz. Microcoils wrapped directly on fused silica capillaries can achieve $w_{1/2} < 1$ Hz;[1e] however, the sleeve probe described here facilitates capillary exchange. Some broadening is acceptable when focusing makes possible two-dimensional experiments, which use lower digital resolution. The inset in Figure 1B shows a stopped-flow COSY spectrum acquired in 22 min. The increased concentration produced by cITP enabled this spectrum to be collected 10 000 times faster than without sample focusing with this probe. Compared with the smallest volume ($\sim$30 μL) commercially available probes and/or NMR accessories, this approach represents more than an order of magnitude improvement in S_m and a corresponding 100-fold time savings.

CE techniques have found widespread application through their ability to separate minute quantities from complex mixtures with high efficiency.[2] In addition to sample stacking, cITP is a well-established means to separate mixtures of analytes with different μ_e.[3] Figure 2 shows a stacked plot of NMR spectra from a cITP separation. The 7 μL sample consisted of 200 μM each TEAB, MeG (85% purity), and the dipeptide alanine-lysine (Ala-Lys).

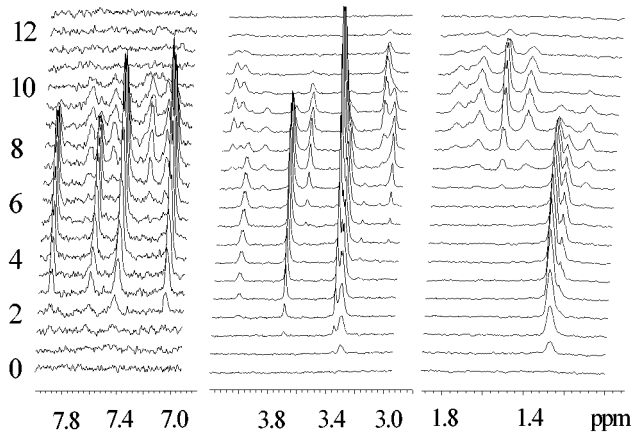

Figure 2. Continuous-flow [1]H NMR spectra of a cITP separation of TEAB, MeG, and Ala-Lys (200 μM each, 7 μL injected). Spectra were acquired in 10-s intervals; for displayed data, 4 slices were co-added with the separation time (min) labeled on the left. TEAB appears from 40 s to 9 min 40 s, MeG from 1 min 20 s to 9 min 20 s, MeG impurity from 5 min 20 s to 10 min, and Ala-Lys from 7 min 20 s to 12 min. The region from 6.80 to 8.00 ppm is scaled 4 times higher than the other regions. The top spectrum is offset by 10% along the frequency axis. Spectral parameters: SW = 5000 Hz, NP = 12420, AT = 1.242 s, 55° pulse, delay time = 0 s, NT = 8. Data processing conditions: LN = 2 points based on first 64 data points, LB = 4 Hz.

The NMR data shown are a series of 40-s-long acquisitions collected sequentially, without interruption of the applied voltage or flow as the focused bands passed through the NMR microcoil via slow hydrodynamic flow. Because of the finite size of the microcoil, adjacent zones overlap in the detector even though the cITP zone boundaries may be sharp. However, the NMR resonances are resolved, and the individual spectra of the four compounds can be seen from their four distinct sets of rise and decay times. Of interest, cITP separated an impurity in the MeG, which migrated as the third component.

cITP NMR has demonstrated significantly improved sample concentration sensitivity over any prior capillary-scale NMR experiment. This simple yet powerful system takes advantage of electrophoretic methods to effectively focus microliter injection volumes into nanoliter-volume zones of electrophoretically purified analytes. It thus enables nanoliter-volume microcoils, the highest mass-sensitivity NMR probes, to be used for structural elucidation of charged trace components. The strong resonances of TEAB may be used as a marker to trap even lower concentration analytes not detectable in on-flow acquisition. Such a system has immediate applicability to areas of drug discovery and biochemical research where mass-limited analyses of ionizable species are crucial.

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