Comparative Analysis of a Neurotoxin from *Calliostoma canaliculatum* by On-Line Capillary Isotachophoresis/^{1}H NMR and Diffusion ^{1}H NMR

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NMR spectroscopy has been coupled on-line to capillary isotachophoresis (cITP) to enhance structural analyses of dilute charged species through separation and sample concentration. Microcoils, the most mass-sensitive NMR probes available, provide optimal detection for cITP/NMR. To evaluate the utility of cITP/NMR for natural product analysis, a homogenate of the hypobranchial gland from the marine snail *Calliostoma canaliculatum* containing a cationic neurotoxin (**1**, a disulfide-bonded dimer of 6-bromo-2-mercaptotryptamine) was studied. For comparison, hypobranchial gland homogenate was also examined by diffusion-NMR, an alternative approach for NMR mixture analysis. cITP/NMR concentrated the neurotoxin by almost 20-fold and isolated it from some of the other components present in the matrix. However, a minor component, likely a precursor or degradant, co-migrated with compound **1**. Diffusion-NMR also did not resolve the two, indicating that the compounds possessed similar diffusion coefficients and electrophoretic mobilities. The strengths and limitations of the two approaches for NMR mixture analysis are discussed.

Complete structural elucidation of natural products often requires nuclear magnetic resonance (NMR) spectroscopy. NMR operates best with relatively pure sample solutions. However, the natural product of interest frequently resides in a complex matrix. Moreover, NMR possesses a lower sensitivity than other analytical techniques, thereby limiting its application to small-quantity samples. Unlike synthetic chemicals, natural products cannot always be procured in large amounts.

A number of different strategies to increase NMR sensitivity have been investigated and implemented. For mass-limited samples, reduced-diameter NMR coils offer an attractive approach to enhance NMR sensitivity.[1,2] As predicted theoretically[3,4] and verified experimentally,[3,5] the mass sensitivity (defined as S/N per sample quantity) of an NMR coil is inversely proportional to its diameter to a first approximation. Numerous mass-limited natural products have been successfully interrogated with reduced-diameter NMR coils.[6−14] In the best cases, microcoils, solenoidal NMR coils possessing a diameter of 1 mm or less and detection volumes from 1 μL to a few nL, have demonstrated 40-fold improvements in mass sensitivity compared to conventional 5 mm NMR probes (coil diameter of 6.7 mm and detection volume of 220 μL).[5] With their high mass sensitivity, microcoils have examined osmolytes in single invertebrate neurons.[15]

For NMR mixture analysis, two distinct techniques can be employed. Obviating the need for an actual physical separation, specially designed pulse sequences can discriminate between different components on the basis of their diffusion coefficient[16−18] or electrophoretic mobility (μ_e).[19−26] Alternatively, to present isolated components from a mixture to the detector, NMR can be coupled on-line to different separation techniques such as HPLC,[27−35] capillary electrophoresis (CE),[29,30,36−41] and capillary electrochromatography (CEC).[29,30,37,38,42,43] As compared to off-line detection, on-line observation allows faster analysis with improved separation resolution. Reduced-diameter NMR coils, such as microcoils, have enabled NMR detection of microscale and capillary-scale separations. While the development of appropriate on-line separations for NMR detection can be time-consuming, they afford more versatility in identifying components from complex mixtures than diffusion and μ_e-edited NMR experiments.

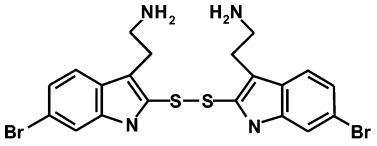

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Figure 1. Structure of the neurotoxin from *Calliostoma canaliculatum*, the disulfide-bonded dimer of 6-bromo-2-mercaptotryptamine (BrMT, **1**).

Recently, capillary isotachophoresis (cITP), a form of CE, has been integrated on-line to microcoil NMR.[44,45] Similar to most other modes of CE, cITP separates charged analytes on the basis of their μ_e. cITP also affords the benefit of sample concentration (by up to 2−3 orders of magnitude), thereby greatly facilitating NMR detection of dilute charged analytes.

While cITP/NMR has successfully analyzed samples comprised of relatively simple mixtures, the technique needs to be applied to a real-world sample before its utility and limitations can be properly assessed. Consequently, cITP/NMR has been employed in the interrogation of a recently identified novel neurotoxin from a marine organism. As a defense against predatory starfish, the sea snail *Calliostoma canaliculatum* Lightfoot, 1786 (Calliostomatidae) releases a yellow-colored mucus from its hypobranchial gland[46] that exhibits potassium channel blocking effects.[47,48] Through HPLC isolation and the NMR and MS characterization of the extract from ~15 snails, the active agent has been identified as the disulfide-bonded dimer of 6-bromo-2-mercaptotryptamine (BrMT, **1**).[47] Examining the structure of **1** in Figure 1, the species is positively charged at physiological pH. This study seeks to ascertain if cITP/NMR can isolate and concentrate **1** from the other components present in the raw homogenate from fewer snails. Facilitating the study, a dual serial multiple microcoil ^{1}H NMR probe[44] was employed for obtaining both on-flow and

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stopped-flow cITP/NMR data. As a comparison, the raw homogenate was also examined by ^{1}H NMR diffusion experiments.

Results and Discussion

cITP/NMR. To investigate the ability of cITP/NMR to analyze real-world samples, a partially purified homogenate from the marine species *C. canaliculatum* was analyzed in an effort to concentrate and isolate the charged neurotoxin (dimer BrMT, **1**) from the matrix. The extract was cleaned on a C_{18} cartridge before analysis to remove lipids and inorganic salts, which interfered with cITP. Prior to cITP/NMR, cITP trials of the natural product extract were performed in Teflon tubing to observe the focusing of the yellow-colored neurotoxin. With an applied potential of between 10.0 and 15.0 kV, which was adjusted according to the electrophoretic current, complete focusing of the neurotoxin occurred in approximately 40 min.

Following the injection protocol determined from benchtop trials, cITP/NMR analysis of the natural product extract was performed with a dual serial multiple microcoil probe. Both coils were shimmed individually prior to the run, while the capillary was filled with the leading electrolyte (LE) used for the cITP separations. For this particular dual-coil probe, each coil had a resolution of 4 Hz full-width at half-maximum (fwhm), although such probes have been made with a spectral resolution in the range 1−2 Hz. Initially, the scout coil was tuned to the ^{1}H Larmor frequency while the second coil was detuned. Moreover, the shims were set to optimized values for the scout coil.

After the hydrodynamic injection, a 15.0 kV potential was applied for the duration of the run. For this cITP system and applied voltage, the current was initially 5.2 μA. Electrophoretic current induced a magnetic field gradient that degraded the microcoil NMR spectral resolution.[41] However, at such a low electrophoretic current, microcoil NMR spectral resolution was only slightly broadened, by 0.2−0.4 Hz fwhm.

Surprisingly, for this sample, the current slowly increased over time. We expected the current to decrease as the cITP run progresses as more of the highly resistive trailing electrolyte (TE) replaced the more conductive LE. After 40 min, the current rose to 16.9 μA so that the electrophoretic current-induced broadening became more severe, 2 Hz fwhm. With the greatly reduced EO flow in the coated capillaries, focused cITP analyte zones migrated slowly. To bring the sample into the scout microcoil more quickly, the outlet LE buffer vial was lowered by 0.9 cm compared to the inlet TE buffer vial to create an appropriate hydrodynamic flow.

After 65 min, migrating sample peaks were observed at the scout microcoil. Figure 2 shows a stacked plot of on-flow NMR spectra acquired during passage of the focused cITP analyte zone through the scout coil. As expected, the neurotoxin **1** NMR peaks were observed. However, additional NMR peaks indicated that a minor component(s) co-migrated with the neurotoxin. Thus, under these experimental conditions, cITP did not completely separate **1** from the other components present in the partially purified extract. The focused sample band migrated through the 1 mm long microcoil in 3 min. During this time, the electrophoretic current was 18.0 μA.

Once the focused analyte zone passed through the scout coil, NMR observation was switched from the scout microcoil to the second microcoil. This process involved tuning the second coil to the ^{1}H Larmor frequency while the scout coil was detuned and setting the shims to optimal values for the second coil. Data from each coil were stored in separate files. With the current instrumentation, the entire coil-changing process took about 30 s. By implementing automatic circuit (de)tuning[49−51] rather than manual (de)tuning, this time could be reduced to a few seconds or less.

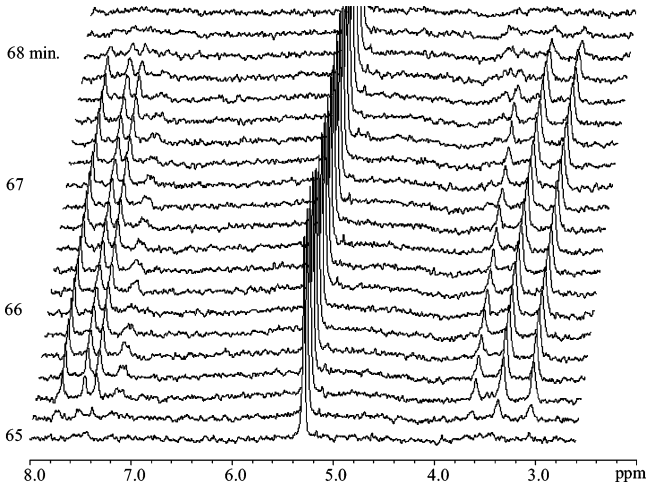

Figure 2. Array of on-flow cITP/NMR spectra displaying the focused analyte zone containing the neurotoxin (the dimer of BrMT, **1**) and a minor component migrating through the scout microcoil.

By observing its peak shape as a function of relative migration time at the scout coil, the migrating analyte zone was trapped at peak maximum in the second coil for optimal stopped-flow detection. While applying hydrodynamic pressure forced the analyte zone back into the detector after it had migrated past, reversing the direction of flow in cITP substantially degraded focusing. As both coils possessed the same detection volume and sensitivity, the peak maximum gave the same S/N at the two detectors. Once the peak reached the second coil, flow was discontinued by closing the valve above the LE buffer reservoir and turning off the applied voltage. Figure 3A illustrates a stopped-flow NMR spectrum of the focused cITP analyte zone captured at the peak maximum.

As shown in Figure 3A, NMR peaks were identified to be from the neurotoxin **1** and a minor component(s). Simple pulse-and-acquire 1-D ^{1}H NMR experiments probably could not differentiate between the monomer and the dimer of BrMT. However, the dimer possessed a bright yellow color, while the monomer was colorless. Thus, a large portion, if not all, of BrMT was in the dimer form. cITP had previously separated components based on differing μ_e.[45] Consequently, the neurotoxin and the minor component(s) had a similar μ_e.

To quantitate the concentration of the neurotoxin in the focused cITP analyte zone, a 100 mM indole CD_3OD solution was loaded into the cITP separation capillary. Certain indole and neurotoxin (dimer BrMT, **1**) NMR peaks possessed similar longitudinal relaxation times (determined in separate 5 mm NMR tests). Comparing spectra from the focused cITP analyte zone and the indole standard acquired and processed with the same spectral parameters, the neurotoxin **1** was stacked to a concentration of approximately 70 mM (assuming BrMT is exclusively in the dimer form).

An NMR spectrum of the natural product extract was obtained to evaluate the performance of cITP/NMR. A 15 μL aliquot of the natural product extract was dissolved in 650 μL of TE and transferred to a 5 mm NMR tube for analysis on a 500 MHz NMR spectrometer with a 5 mm probe. With an observed volume of 220 μL, the 5 mm NMR

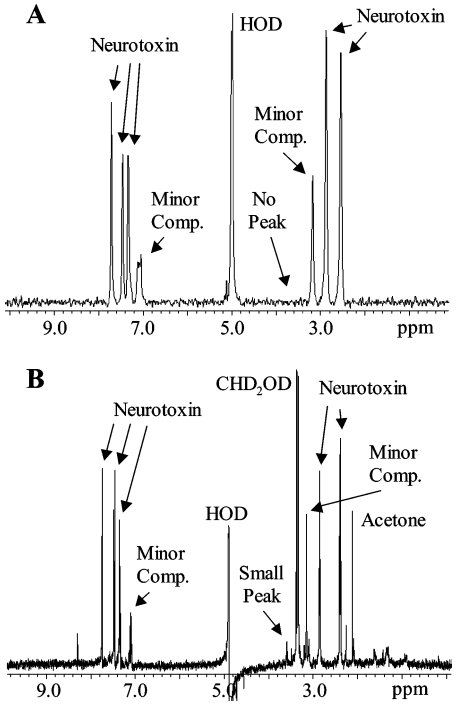

Figure 3. (A) Stopped-flow cITP/NMR spectrum of the focused analyte band containing the neurotoxin (the dimer of BrMT, **1**) and a minor component, trapped in the second microcoil. (B) Conventional 5 mm tube NMR spectrum of the natural product extract. Both spectra were acquired with similar spectral parameters and processed with filtering matched to the line width.

probe contains approximately the same volume of the original natural product extract (~5 μL) in its coil that was injected for the cITP/NMR run. Figure 3B shows a 5 mm NMR spectrum of the natural product extract acquired with solvent suppression, a presaturation pulse (duration of 1.5 s) on the intense HOD resonance prior to each transient. Aside from the application of the presaturation pulse, both spectra in Figure 3 possessed identical data acquisition parameters. Integrating the resonances present in the spectrum, the minor component aromatic peaks were small enough that they could be assigned as a separate species from the neurotoxin. However, the minor component aliphatic peak could be construed as belonging to the neurotoxin on the basis of its relative peak integration. By comparing the 5 mm NMR spectra of the natural product extract and the 100 mM indole standard (obtained with the same data acquisition parameters), the neurotoxin was at a concentration of approximately 3.7 mM (assuming BrMT was exclusively in the dimer form) in the homogenate.

Examining the two spectra in Figure 3, cITP separated the neurotoxin **1** from some of the other species present in the natural product extract (e.g., the extra peak highlighted in Figure 3B not observed in Figure 3A). Moreover, cITP concentrated the neurotoxin by ~19-fold before NMR detection. Thus, assuming constant concentration in the focused cITP analyte zone, the neurotoxin was focused to an approximately 8 mm long band from the initial 15 cm long sample plug. During stopped-flow analysis, only 1/8 of the peak was within the microcoil detection volume so that an 8-fold improvement could be obtained by better matching the size of the microcoil to the volume/length of the focused analyte band.

Diffusion NMR. As an attractive alternative to the on-line hyphenation of separation methods to NMR, diffusion NMR could analyze mixtures without the need to perform an actual physical separation. By implementing specially designed pulse sequences, NMR spectra with diffusion weighting could be obtained to identify the individual components of mixtures. Consequently, the natural product extract was examined by diffusion ^{1}H NMR to determine if the NMR resonances belonging to the neurotoxin (dimer BrMT, **1**) and the minor component(s) could be properly assigned.

Table 1. Measured Diffusion Coefficients of Sample Peaks in a Natural Product Extract from BPP-LED STE NMR Experiments

chemical shift (ppm)	diffusion coefficient (cm^2/s)	neurotoxin (**1**)	minor component
2.37	$(3.36 \pm 0.08) \times 10^{-6}$	×	
2.81	$(3.33 \pm 0.08) \times 10^{-6}$	×	
3.10	$(3.20 \pm 0.08) \times 10^{-6}$		×
7.04	$(3.13 \pm 0.08) \times 10^{-6}$		×
7.06	$(3.07 \pm 0.08) \times 10^{-6}$		×
7.08	$(3.01 \pm 0.08) \times 10^{-6}$		×
7.21	$(3.32 \pm 0.08) \times 10^{-6}$	×	
7.43	$(3.37 \pm 0.08) \times 10^{-6}$	×	
7.69	$(3.40 \pm 0.08) \times 10^{-6}$	×	

For the NMR diffusion experiments, the natural product extract was loaded into a specially designed 5 mm NMR tube for analysis on a 500 MHz spectrometer equipped with a 5 mm probe that produced magnetic field gradients along the *z*-axis (axial to magnet bore). Varying the applied magnetic field gradient strength, a series of NMR spectra (18 in total) were obtained with the BPP-LED STE pulse sequence to measure the diffusion coefficients of the different NMR resonances. To determine the values of the diffusion coefficients, the peak heights from each spectrum were measured and the data fitted to eq 1 (Experimental Section). (Peak area measurements did not provide accurate results.)

Table 1 lists the measured diffusion coefficients for the sample peaks from the natural product extract. A reasonable estimate of the error for diffusion measurements from the BPP-LED STE pulse sequence was 5%.[52] All the neurotoxin peaks possessed similar diffusion coefficients within this error. Examining the peaks from the minor component(s), the three aromatic resonances had different diffusion coefficients than the neurotoxin, thereby indicating that they belonged to another component. However, the minor component aliphatic resonance could not be excluded from being from the neurotoxin on the basis of its diffusion coefficient. The diffusion data were inconclusive as to whether the minor component(s) peaks belong to a single species or not.

Conclusion and Future Directions

The cITP/NMR and diffusion-NMR analyses of the natural product containing the neurotoxin **1** (Figure 1) illustrate well the strengths and limitations of the two techniques. The 6-bromoindole-related compounds investigated here, as in the case of other natural products, are often found in complex mixtures of related compounds even when extracted from a single species,[53] and the ability to distinguish related compounds is important. cITP/NMR concentrates charged compounds and separates them based on their μ_e. Thus, a difference in μ_e must exist between the components in order to resolve them. To establish a difference in μ_e, the pH (pD) could be adjusted and/or other agents, such as cyclodextrin,[54–56] could be added to the electrolyte solutions. Diffusion-NMR offers a straightforward means for mixture analysis. However, the diffusion coefficient usually cannot be as readily manipulated as the

electrophoretic mobilities. To increase the resolving power of diffusion-NMR, micelles could be introduced into the solution to observe relative partitioning.[16]

Further improvements in cITP/NMR instrumentation will greatly facilitate its application. The addition of other on-line detectors such as ultraviolet/visible absorption,[57] diode arrays,[58] conductivity detectors,[59] potential gradient detectors,[60,61] and MS[62] to cITP/NMR will afford complementary data. For complete structural assignment, cITP/NMR probes capable of conducting $^{1}H-^{13}C$ heteronuclear multidimensional experiments can be constructed.[63,64] As a further enhancement for natural product analysis, the addition of a sample collector at the outlet end of the cITP/NMR separation channel will enable off-line bioassays in conjunction with structural identification.

Experimental Section

Chemicals. All chemicals were used as purchased from the manufacturer without further purification. Acetone, acetonitrile, CD_3OD, glacial HCl, and trifluoroacetic acid were acquired from Aldrich Chemical Co. (Milwaukee, WI). Methyl green was purchased from Sigma Chemical Company (St. Louis, MO). Indole was acquired from Fisher Scientific (Fair Lawn, NJ). D_2O (D, 99.9%) was obtained from Cambridge Isotope Laboratories (Andover, MA).

Natural Product Collection. Specimens of *Calliostoma canaliculatum* Lightfoot, 1786 (Calliostomatidae) were both supplied by Sea Life Supply (Sand City, CA) and also collected from kelp forests at and adjacent to Hopkins Marine Life Refuge (Pacific Grove, CA) during the summers of 1998−2002. Reference specimens are retained at Hopkins Marine Station. For the experiments reported here, eight animals were used.

Natural Product Extraction. Surgical dissection and isolation of the hypobranchial glands, which contain the neurotoxin, were performed at Hopkins Marine Station. After removal, the glands were frozen in liquid nitrogen for preservation. The samples were then shipped freeze-dried to the University of Illinois for analytical analysis.

Established protocols were used to extract chemical components from the hypobranchial glands.[47] Briefly, eight glands were situated in a glass micro-homogenizer. An acidified acetone solution (40 mL of acetone, 6 mL of distilled H_2O, and 1 mL of glacial HCl) was used as the extraction solvent. After pulverizing the glands, the extraction solvent was transferred to a centrifugation vial for particulate removal. The efficacy of the extraction procedure easily could be ascertained, as the neurotoxin possesses a bright yellow color. The extraction vials were centrifuged at a rate of 9700*g* for 5 min. The resulting supernatant was then collected in a test tube. The extraction protocol was repeated until the yellow hue disappeared from the glands and the extraction solvent.

To remove inorganic salts and lipids, the sample extract was cleaned by a reversed-phase C_{18} cartridge (1 g of absorbent, Alltech Associates Inc., Deerfield, IL). The sample extract was blown dry under nitrogen gas and was then dissolved in distilled H_2O (+ 0.1% trifluoroacetic acid). Prior to usage, the C_{18} cartridge was cleaned with acetonitrile (+ 0.1% trifluoroacetic acid) and conditioned with distilled H_2O (+ 0.1% trifluoroacetic acid). After loading the sample extract onto the C_{18} cartridge, inorganic salts were removed by thorough rinsing with a 95% H_2O/5% acetonitrile (+ 0.1% trifluoroacetic acid) solution. The cleaned yellow-colored sample extract was then collected with an 80% acetonitrile/20% water (+ 0.1% trifluoroacetic acid) solution. The sample was then blown dry under nitrogen gas and redissolved in a 90% D_2O/10% CD_3OD solution for analysis.

cITP. The cationic cITP electrolyte system employed in this study was similar to those used in previous cITP/NMR experiments.[44,45] In contrast to the cITP electrolyte systems from the aforementioned work, an organic modifier, CD_3OD, was added to both the leading electrolyte (LE) and trailing electrolyte (TE) to improve the solubility of the hydrophobic neurotoxin. The LE consisted of 160 mM sodium deuterated acetate (d_3) buffered to pD = 5.5 with deuterated acetic acid (d_4) in a 90% D_2O/10% CD_3OD solution. (The sample pD is equal to the pH meter reading + 0.40 for D_2O solutions.[65]) Similarly, the TE was 10 mM deuterated acetic acid (d_4) in a 90% D_2O/10% CD_3OD solution. In this cationic cITP electrolyte system, Na^+ served as the high-μ_e leading cation, while D^+, which complexes with the counterion acetate, functioned as the low-μ_e trailing cation.[66−68] For cITP analysis of the extract, the equivalent of one gland of extract was dissolved in 90% D_2O/10% CD_3OD with 5 mM deuterated acetic acid (d_4). The preparation of the sample in 5 mM acetic acid was necessary to ionize the sample components, provide proper counterion continuity, and increase the conductivity to the appropriate level.

For the initial cITP separations of the natural product extract, a 140 cm long piece of 304 μm inner diameter (i.d.)/762 μm outer diameter (o.d.) Teflon tubing served as the separation channel. The yellow-colored neurotoxin could not be observed through the darkly tinted fused-silica capillary with polyimide coating. The transparent Teflon tubing enabled observation of cITP focusing of the yellow-colored neurotoxin. Prior to use, the Teflon tubing was filled with 0.1% Triton X-100 detergent (Fisher Scientific; Fair Lawn, NJ) for 30 min to suppress electroosmotic (EO) flow during cITP, which tended to degrade focusing, and was then flushed with several column volumes of LE.[69]

To conduct cITP, a high-voltage power supply (Series 230; Bertan Associates; Hicksville, NY) applied an electric field across the separation channel. The outlet end of the separation channel was placed in a LE buffer reservoir, while the inlet end was situated in a TE buffer reservoir. As the cITP system was designed for cation analysis, a positive potential was applied at the inlet TE buffer vial, while the outlet LE buffer vial was kept at ground. Platinum electrodes were used at the buffer reservoirs to avoid unwanted electrochemical reactions. A digital multimeter monitored the current on the ground side.

The cITP injection protocol consisted of an initial TE plug, followed by sample, and then another TE plug into the separation channel, which was initially filled with LE. By injecting TE between the LE and sample, charged sample components were focused away from neutral species present in the injected sample band and closer to the boundary between the LE and first TE plug. The second TE plug served to move the sample band toward the NMR coil. Injections were performed using hydrodynamic injections by changing the relative heights of the two buffer reservoirs. The focused sample band could be moved more rapidly by applying slight hydrodynamic pressure.

Microcoil NMR. As mentioned previously, this cITP/NMR experiment used a dual serial multiple microcoil ^{1}H NMR probe.[44] With its two coils arranged in series on the separation channel, the migrating cITP analyte zone of interest could be observed on-flow at the first (scout) coil and then judiciously trapped at the second coil for extended stopped-flow NMR analysis. (This dual serial multiple coil probe could be employed for the on-line coupling of NMR to any separation mode, including HPLC, CE, and CEC.) The probe consisted of two 1 mm long microcoils wrapped around a segment of 370 μm i.d./430 μm o.d. polyimide tubing. The two copper wire microcoils were separated by 1 cm. The polyimide sleeve facilitated easy separation capillary exchange.

Each microcoil possessed its own impedance matching circuit tuned for ^{1}H observation at 500 MHz. As only one coil was in operation at a time, the inactive coil could be selectively detuned, by closing a high-capacitance bridge, to significantly improve NMR signal isolation between the two coils. This detuning operation can be performed while the probe was situated in the NMR magnet.

To attain high-resolution NMR spectra from microcoils constructed from copper wire, the surrounding environment must possess a magnetic susceptibility similar to that of copper.[5] Consequently, the dual microcoil probe was encased by a plastic bottle that was subsequently filled with MF-1 (Magnetic Resonance Microsensors Corporation; Savoy, IL), a

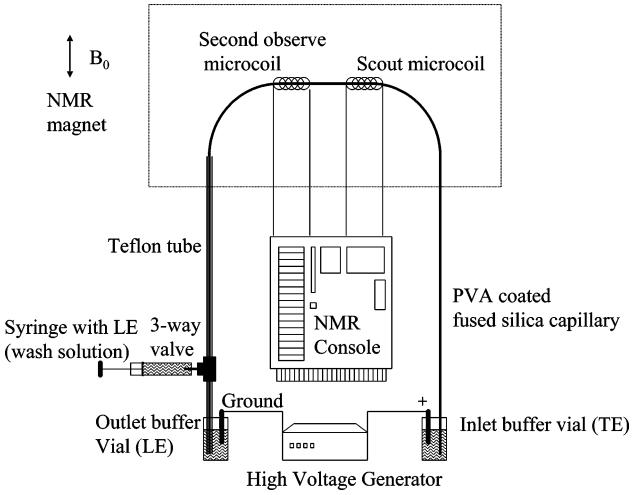

Figure 4. Schematic of the cITP/NMR instrument highlighting the relationship of the separation capillary and fluidic system to the NMR detection coils.

perfluorinated organic liquid possessing a volume magnetic susceptibility within 3% that copper. Further details concerning the construction and performance of this probe were previously reported.[44]

cITP/NMR. All cITP/NMR experiments were conducted on a Varian 500 MHz spectrometer with a wide-bore (89 mm diameter) magnet. The instrumental arrangement for cITP/NMR, depicted in Figure 4, was previously described.[44,45] With this configuration, all cITP operations were conducted with the probe already situated in the NMR magnet bore.

To integrate microcoil NMR detection for cITP, the separation channel was simply threaded through the probe's polyimide sleeve. For this experiment, the cITP separation channel consisted of a 100 cm long piece of 200 μm i.d./360 μm o.d. fused-silica capillary internally modified with a covalently bonded poly(vinyl alcohol) coating that minimized EO flow.[70] With this size capillary inserted into the polyimide sleeve, each microcoil possessed a detection volume of ~30 nL. The distance from the capillary inlet to the first coil was 90 cm. For connection to the LE buffer reservoir, the separation capillary was attached to the following segments of Teflon tubing: 5 cm of 305 μm i.d./762 μm o.d. and 85 cm of 813 μm i.d./1422 μm o.d. The tubing connections were sealed with Teflon heat shrink. The relatively large cross-sectional area of the Teflon tubing ensured that the majority of the electric field was dropped across the separation capillary.

The Teflon tubing was connected to a three-way valve positioned directly above the LE buffer vial. During sample injection and on-flow cITP, the valve was open to the LE buffer reservoir. The flow could be discontinued for stopped-flow NMR analysis by closing the valve to the separation channel. The other outlet of the three-way valve was connected to a syringe filled with LE, which was used for quick flushing of the Teflon tubing and separation capillary. Again, prior to cITP/NMR, the cITP channel was filled with 0.1% Triton X-100 detergent for 30 min to suppress EO flow and was then flushed with several column volumes of LE.[69]

Prior to the actual cITP/NMR experiments on the natural product extract, trials were performed with the visible dye methyl green (initial concentration of 200 μM) to determine an appropriate hydrodynamic injection protocol. (Methyl green and the yellow-colored neurotoxin exhibited similar focusing in initial cITP trials using Teflon tubing.) With this capillary system, an injection of 5 μL of TE (plug length of ~15 cm), followed by 5 μL of sample (plug length of ~15 cm), and 18 μL of TE (plug length of ~54 cm) focused the dye on the inlet side near the first NMR coil.

For the cITP/NMR run, successive spectra were acquired every 10 s during on-flow detection. Standard ^{1}H NMR data acquisition and processing parameters were used: spectral width (SW) = 5000 Hz, number of data points (NP) = 12 800, acquisition time (AT) = 1.28 s, recycle delay (D) = 0 s, pulse width (PW) = 55°, number of acquisitions (NA) = 8, and line broadening (LB) = 6 (matched filtering). For the stopped-flow NMR analysis, the data acquisition parameters and processing parameters were the same except for NA = 128.

Diffusion-NMR. The ^{1}H NMR diffusion measurements were conducted on the same 500 MHz wide-bore NMR spectrometer as the cITP/NMR experiments. Capable of producing magnetic field gradients along the *z*-axis (axial to magnet bore), a Nalorac Z-Spec probe designed for 5 mm NMR tubes was used. The natural product extract, dissolved in 90% D_2O/10% CD_3OD with 5 mM deuterated acetic acid (d_4), was added to a 5 mm Shigemi microtube magnetic susceptibility matched to D_2O. By confining the sample to a length of 0.9 cm (volume of 124 μL) in the microtube, the sample could be positioned in the linear region of the *z*-gradient from the probe, thereby resulting in more accurate diffusion measurements.

NMR diffusion experiments were conducted with the bipolar pulse pair longitudinal encode-decode stimulated echo (BPP-LED STE) pulse sequence.[71] For this particular pulse sequence, the diffusion coefficient, *D*, was related to the observed signal peak height (or peak area) by the following equation:

$$S(q) = S(0)\exp[-D{\cdot}q^2(\Delta - \delta/3 - \tau/2)] \quad (1)$$

where $q = \gamma{\cdot}g{\cdot}d$, γ is the gyromagnetic ratio of the nuclei of interest [26,751.2 rad/(Gauss·s) for ^{1}H], g was the applied gradient strength, δ was the duration of the bipolar gradient pulse, Δ was the time between the two bipolar gradient pulses, and τ was the delay time between the gradient pulse and the radio frequency pulse.[71] To determine *D*, a series of spectra were acquired in which the gradient strength (g) was varied and the resulting data were fitted using a least-squares fitting routine. The probe's gradient strength was calibrated by acquiring diffusion measurements of 1% H_2O/99% D_2O, which has an approximate diffusion coefficient of 2.0×10^{-5} cm^2/s. The measured diffusion coefficients of the components in the natural product extract were approximate values, acceptable for differentiating between different species in the homogenate. Standard acquisition and data processing parameters were employed: SW = 8000 Hz, NP = 30272, AT = 1.892 s, D = 12 s, NA = 16, δ = 0.001000 s, Δ = 0.102044 s, τ = 0.000500 s, and g (18 spectra array) = 2.00, 6.94, 13.89, 18.17, 25.63, 31.35, 36.19, 40.45, 44.30, 47.84, 51.14, 54.24, 57.16, 59.95, 62.62, 65.17, 67.63, and 70.00 G/cm.

Five Millimeter NMR of Natural Product Extract. To evaluate the cITP/NMR results, a ^{1}H NMR spectrum of the natural product extract was acquired on a 500 MHz Varian Unity-Inova spectrometer equipped with a standard 5 mm NMR probe (220 μL detection volume) in a narrow-bore magnet. A 15 μL aliquot of the natural product extract was dissolved in 650 μL of TE and transferred to a 5 mm NMR tube. Aside from the application of a presaturation pulse (duration of 1.5 s) on the HOD resonance for solvent suppression prior to each transient, the ^{1}H NMR data acquisition parameters employed were identical to those used for the stopped-flow cITP/NMR spectrum. During the acquisition, the sample tube was spun and the spectrometer locked on the solvent deuterium signal. For data processing, LB = 0.7 was used for matched filtering.

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