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NMR at the Picomole Level of a DNA Adduct

Roger Kautz^{*,‡}, Poguang Wang[‡], and Roger W. Giese[‡]

[‡]Barnett Institute of Chemical and Biological Analysis, and Department of Pharmaceutical Sciences in the Bouve College of Health Sciences, Northeastern University, Boston, Massachusetts 02115, USA

^{*} Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115, USA

Abstract

We investigate the limit of detection for obtaining NMR data of a DNA adduct using modern microscale NMR instrumentation, once the adduct has been isolated at the pmol level. Eighty nanograms (130 pmol) of a DNA adduct standard, N-(2'-deoxyguanosin-8-yl)-2-acetylaminofluorene 5'-monophosphate (AAF-dGMP), in 1.5 μ L of D₂O with 10% methanol-d₄, in a vial, was completely picked up as a droplet suspended in a fluorocarbon liquid, and loaded efficiently into a microcoil probe. This work demonstrates a practical manual method of droplet microfluidic sample loading, previously demonstrated using automated equipment, which provides a several-fold advantage over conventional flow injection. Eliminating dilution during injection and confining the sample into the observed volume realizes the full theoretical mass sensitivity of a microcoil, comparable to a micro-cryo probe. With 80 ng, an NMR spectrum acquired over 40 hr showed all of the resonances seen in a standard spectrum of AAF-dGMP, with a S/N of at least 10, despite broadening due to previously-noted effects of conformational exchange. Also a 2D TOCSY spectrum (total correlation spectroscopy) was acquired on 1.6 μ g in 18 hr. This work helps to define the utility of NMR in combination with other analytical methods for the structural characterization of a small amount of a DNA adduct.

Keywords

DNA adduct; NMR; microcoil

INTRODUCTION

Nuclear magnetic resonance (NMR) is important in determining the structure of DNA adducts; however, nanomole (microgram) quantities of an adduct have been required for 1D ¹H NMR analysis in a conventional 5 mm NMR tube. While such amounts can be prepared synthetically, only much smaller amounts generally are available from biological sources without enormous scale-up. This limitation has contributed to the problem that the structures of most DNA adducts in biological samples are completely unknown. In principle, less scale-up is required if a micro NMR detector is employed. However, sample handling then becomes more challenging. To make an advance in this area, and to better define how small an amount of a DNA adduct, once isolated, can yield NMR data, we studied the combination of a microcoil probe, as a representative micro NMR detector, and a microdroplet technique, as a way to handle a very small sample volume.

*Corresponding Author Tel: 617-373-8211 Fax: 617-373-2855 r.kautz@neu.edu.

There are two basic categories of micro NMR detectors commercially available: micro-cryoprobes and microcoil probes. In the first category, a 1 mm high-temperature superconducting (HTS) micro-cryoprobe was demonstrated in 2006 to be 24× as sensitive, per microgram of analyte, relative to a traditional 5 mm tube for small samples (5-10 μL).¹ A commercial 1.7 mm “micro-cryo” probe,² thought to use similar or hybrid technology, has been cited as 15× to 20× as sensitive for 30 μL to 20 μL samples, respectively.^{3,4} Micro-cryoprobes combine their exquisitely high sensitivity with the convenience of using familiar NMR tubes. Cryoprobes of any size, however, are a major investment in themselves and further are generally installed permanently in a high-field spectrometer. These facilities require sufficient demand to cover these costs, and micro-cryoprobes are less versatile than 3 or 5 mm probes. Nonetheless two to three dozen micro-cryoprobes have been installed in the US and could be accessible through collaboration, including several in facilities explicitly supporting public research.

Microcoils (small solenoidal coils) are also commercially available for NMR, at a cost comparable to conventional probes.⁵⁻⁸ Changing probes between a microcoil and conventional probe is routine, making microscale NMR readily accessible in local facilities (about 200 are in the field, and they are portable). The commercial microcoils (1-2 μL observed volume/5 μL flowcell volume) theoretically have 18x mass sensitivity within the 1 μL observed volume (6-fold smaller diameter, multiplied by the 3-fold advantage of a solenoid over a saddle coil).^{5,6} However, microcoil probes are built as flow cells, and even careful flow-injection loading of a 1 μL sample through a 8 μL dead volume results in significant dilution, so the effective mass sensitivity is several-fold inferior when loaded using conventional sample handling.

The problem of sample dilution during loading of a small volume into an NMR microcoil has been overcome by a segmented-flow loading method (now called “microdroplet NMR”, since the establishment of droplet microfluidics as an independent field).⁹⁻¹⁴ In this technique, the sample is carried through the loading tube as a droplet in an immiscible liquid, namely Fluorinert FC43, a perfluorinated fluid. FC43 is immiscible with most organic as well as polar solvents, and wets Teflon surfaces aggressively, so the moving droplet is separated from the tubing inner surface by a layer of FC43 (“zero-dispersion” conditions).^{15,16} This avoids the losses or carryover that can occur if the sample contacts a solid surface. The technique can deliver a small (1 μL) volume sample without dilution or dispersion, and then confine it into the NMR observed volume.^{9,17} FC43 has magnetic susceptibility similar to aqueous solvents and functions as a Shigemi tube for the flow system, allowing sample volumes to be nearly as small as the observed volume without causing shimming problems.^{9,18} Here we extend the microdroplet technique by conducting it in a simple, manual way on a small sample of a DNA adduct: a 1.5 μL total sample volume (initially in a vial) is fully picked up and handled efficiently, leading to an NMR spectrum at the pmol (ng) level.

MATERIALS AND METHODS

Chemicals

N-(2'-Deoxyguanosin-8-yl)-2-acetylaminofluorene 5'-monophosphate (AAF-dGMP) was synthesized as previously described.¹⁹ Fluorinert (FC43) was from 3M Corp (St. Paul, MN). Deuterium oxide and deuterated methanol were from Cambridge Isotope Laboratories (Andover, MA). Other chemicals were from Fisher Scientific (Pittsburgh, PA).

Instrumentation

NMR spectra were acquired using a Varian INOVA 500 MHz NMR instrument (currently Agilent, Santa Clara, CA). The microcoil NMR probes were made by Protasis/MRM (Marlboro, MA): The 1D spectra were acquired using a model H/500/1.5, with a 1.0 μL observed volume and 6 μL dead volume (including flowpath from probe inlet). 2D spectra were acquired using an inverse carbon gradients (ICG) probe with a 2 μL observed volume, an 8 μL dead volume, and with temperature control. The silica flowpath was internally coated with tridecafluoro-1,1,2,2-tetrahydrooctyl-triethoxysilane (fluorooctylsilane, FOS, United Chemical Technologies, Lewistown, PA) as previously described.⁹ The Baby-Bee syringe pump was from BASi (West Lafayette, IN).

Injection Device

To pick up the sample and inject it into the NMR probe, a homemade injection device was used as shown in Fig 1A: The needle of a 10 μL syringe (Hamilton, Reno, NV) was fitted into one end of a 30 cm length of PTFE tubing (1/16" o.d., 200 μm i.d.; Cole-Palmer, Vernon Hills, IL). The other end of this tubing was connected, via a zero-dead-volume union (P720, Upchurch, Oak Harbor, WA), to a 5 cm segment of polyimide-clad silica capillary (200 μm i.d. / 360 μm o.d; Polymicro Technologies, Phoenix, AZ), that had been coated on the inside with FOS.⁹

Probe Connections

The NMR probe inlet was fitted on the inlet side with 5 cm of FOS-silica capillary (as above), then a filter (Upchurch M520) and finally 3 cm of the FOS-silica capillary. The probe outlet was fitted to a waste line of 30 ga Teflon tubing, into which plugs of dye could be injected to measure flow through the microcoil probe with a ruler. The NMR probe was flushed with 0.5 mL of methanol- d_4 , blown dry, and then prefilled with FC43 prior to use.

Sample Injection and NMR Spectrum

The injection device was prefilled with FC43 and then 3.5 μL was ejected to waste. The AAF-dGMP sample (1.5 μL of 0.1 mM AAF-dGMP in D_2O : methanol- d_4 , 90:10, v/v) was prepared in a polypropylene microcentrifuge tube (freshly rinsed twice with 200 μL of methanol- d_4 , followed by shaking out and air-drying). The 1.5 μL came from a larger stock solution prepared by weighing AAF-dGMP. The entire sample was drawn into the injection device (with FC43 ahead of it), as illustrated in Figure 1B, using the syringe manually and viewing with a stereo microscope, according to the following sequence of steps: (1) place the tip of the pickup tube, filled with FC43, to the bottom of the sample droplet; (2) draw the entire sample droplet into the pickup tube (ejecting any air drawn after the sample); (3) move the pickup tube to a second vial of FC43 and draw 2 μL . The injection device was then connected to the probe inlet through a union so that the two ends of fused silica capillary butted against each other (2 cm length of 30 ga Teflon tubing, designated as "Single-Use Union" in Figure 1). The probe and union were prefilled with FC43 prior to making the connection, to avoid an air bubble, as well as fill any gap in the resulting butt joint. The syringe was then mounted onto the syringe pump, which provided smooth, constant movement of the aqueous droplet of sample into the NMR coil, as illustrated in Figure 1A.

To position the sample in the NMR observed volume, progress of the sample was roughly monitored by movement of a dye droplet in the outlet tubing (taped to a ruler) as well as by time. Single-scan NMR spectra at 2 second intervals showed solvent NMR signals as the head of the plug arrived at the coil, and sharpened as it filled it. The most precise indicator of position was the lock level rising to a similar level seen in practice injections of solvent

without analyte. The pump could be stopped a few seconds early, and the sample nudged forward by turning on the pump for a few seconds. If the droplet was injected too far, it could be returned to an optimal position by withdrawing the injection syringe, then hastening the backflow of solvent by applying gentle pressure to the waste end of the outlet line with a second syringe filled with FC43. One mm movements of the dye mark in the 30 ga outlet line reported the position of the sample within 0.1 μL ; touching up the X and Y shims could compensate 0.2 μL variations in positioning a 1.5 μL sample. After positioning and shimming, acquisition of NMR spectra then was begun. A “blank” spectrum of solvent handled similarly to the AAF-dGMP sample was also acquired.

NMR Spectra

1D spectra were acquired with a 45-degree pulse, 1.02 sec acquisition time and 0.5 sec relaxation delay. Solvent suppression, where noted, was effected by setting the transmitter on the solvent resonance and pre-saturating at low power during the relaxation delay. 2D sequences were as provided in the vnmr6.1C library. Because full phase cycles were acquired, the indicated sequences were chosen that do not use gradients within the preparation or evolution time, only homospoil prior to the relaxation delay. The TOCSY at 1 mM acquired 512 points, relaxation delay 1 sec, 64 scans per increment $\times$ 200 complex increments. The 2D spectra presented were processed with MestreNova 8.0.2, applying a gaussian of 8.5 based on a detected T_2^* of 0.05 sec.

RESULTS AND DISCUSSION

Any microscale NMR technique requires preparing a concentrated solution of the analyte in a small volume. Towards this goal, and also seeking to have the DNA adduct in a highly polar form in a highly polar solvent, we selected to work with a model DNA adduct in a nucleotide form, and to employ D_2O containing 10% methanol- d_4 as the sample solvent. FC43 was selected as the immiscible carrier fluid due to its prior success in this area. A potential concern is that a nonpolar, bulky DNA adduct still might have limited solubility even in this solvent. Further, a bulky adduct might tend to localize at the aqueous/fluorous interface or bind to the tubing wall, leading to its loss from the droplet during transfer to the microcoil NMR observed region. Thus, we selected the bulky nonpolar DNA adduct, N-(2'-deoxyguanosin-8-yl)-2-acetylaminofluorene 5'-monophosphate (AAF-dGMP), as a good test case. This choice was also motivated by the fact that this adduct has been characterized previously by NMR, for comparison.¹⁹⁻²¹

Based on our prior experience with our microcoil NMR system, including acquisition of a preliminary spectrum of 1 mM AAF-dGMP, we expected that a 1D ^1H NMR spectrum could be obtained of this compound by overnight acquisition on a sample concentration as low as 0.1 mM. We then set up the injection device illustrated in Figure 1A. Starting with 1.5 μL of 0.1 mM AAF-dGMP in D_2O , 10% methanol- d_4 , containing 80 ng (130 pmol), in a microcentrifuge tube, we used our segmented flow sample loading method to obtain the microcoil NMR spectrum shown in Figure 2B. The lack of any difference in the signal at 7.4 ppm between the first 4 and last 4 of the 40 hours of acquisition (inset) demonstrates that there is no diffusion of analyte out of the observed volume during the measurement of the spectrum. By comparison of spectra of a droplet of 1 mM AAFdG to overfilling the microcoil probe with a stock solution of the DNA adduct standard (injecting 8 μL into a dry probe so the observed volume is unambiguously filled with full stock concentration), we calculate that the recovery of the adduct from the vial into the probe is essentially quantitative. (Additional evaluation of recovery is described later.)

Shown in Figure 2C is a reference spectrum, of a much larger amount of AAF-dGMP acquired in a conventional 5 mm NMR tube (39 μg in 0.55 mL, 512 scans, 10 min

acquisition). As can be seen, the signal-to-noise ratio (S/N) of the microcoil spectrum in Figure 2B is satisfactory for unambiguous comparison with the reference spectrum (Figure 2C), or for searching in a spectral database, and the two spectra are equivalent aside from the solvent and background peaks detected in the solvent blank (Figure 2A). The weakest peak of AAF-dGMP, at 6.4 ppm, has a S/N of 9.6 in the microcoil spectrum and 25 in the reference spectrum; the microcoil spectrum is of 1/488 the amount of material but was acquired for 195 times longer, so the effective mass sensitivity of the microcoil probe with a 1.5 μ L sample is 13 times higher than that of the 5 mm tube, consistent with an expectation of 18 times higher within its 1 μ L observed volume. The solvent peaks near 4.9 ppm (the OH moieties of water and methanol) vary between the three spectra due to different amounts of H₂O in the samples, and because solvent suppression (presaturation) was applied to B and C. The peaks for the DNA adduct are broad (relative to sharp contaminant peaks, which indicate the shims were acceptable) because this adduct exists as a mixture of conformers that interconvert on the intermediate NMR time scale at room temperature, as described before and discussed further below.^{20,21} This explanation is confirmed in the reference spectra by resolving the conformers in slow exchange at -15°C , and merging them in fast exchange at 55°C (inset of Figure 2C). Although the microcoil used in this 1D work does not have temperature control, newer microcoil probes in the field do, and such control can be important in optimizing resolution, as seen here with a conventional 5 mm probe.

It would be appropriate to demonstrate the ability to acquire 2D NMR is commensurate with the 1D NMR sensitivity. For example, a common rule of thumb is that obtaining a S/N of 4 in a 1D spectrum indicates the minimal sample concentration and number of scans per increment to obtain a COSY or TOCSY, under normal conditions. While AAF-dGMP was chosen to test the worst-case scenario of a bulky hydrophobic adduct in dissolution and sample transfer, this compound presents even greater challenges for 2D NMR because of the chemical exchange noted above. Chemical exchange can dissipate 2D NMR signals if the lifetime of a species is less than the evolution time of the 2D pulse sequence. As discussed in detail by Evans, AAF-dGMP has slow rotation about two bonds, the guanyl-nitrogen bond and the amide bond, resulting in four resolved conformations when exchange is slowed by cooling to -50°C .²⁰ A single set of resonances is observed on heating to $+50^{\circ}\text{C}$, although the broadness of the lines indicates it is still in “intermediate exchange”, where the exchange rate perturbs NMR spectra. Chemical exchange in NMR is a deep topic, but the results here can be clarified with only one of its conclusions.²⁵ The “fast exchange” regime is when multiple conformations present as a single sharp resonance — like most pH equilibria or bond rotations — and the average structure has a long NMR relaxation time T_2^* . The transition between intermediate exchange and fast exchange is different for each proton, and depends on how many Hz apart are the resonances of the resolved conformations at -50°C , called $\Delta\delta$. In the 1D spectra at $+50^{\circ}\text{C}$ of Figure 3A, the sharper lines (6 Hz line widths) have a $\Delta\delta$ on the order of 20 Hz; the broader F1 and F3 lines (60 and 80 Hz line widths) have $\Delta\delta$ over 80 Hz.

To determine which 2D sequences would work with AAF-dGMP under its exchange conditions, a preliminary survey of common 2D NMR spectra was made at a relatively high concentration (35 μg in 3 μL , 22 mM) so sample datasets could be acquired quickly. An overnight acquisition afforded a COSY (1 hr), TOCSY (2.5 hr), DQCOSY (1 hr), NOESY (4 hr) and ROESY (5 hr). At best partial spectra were observed: what cross peaks were seen were only from resonances with $\Delta\delta$ below 40 Hz — at the ends of the ribose and AAF moieties, furthest from the slow-rotating bonds. The TOCSY was able to elucidate the full spin systems of the AAF moiety (Figure 3A). Although a cross peak was not observed directly between the broadened F1 and F3 protons, the meta coupling between them was observable through the sharper F4 proton.

To acquire a TOCSY spectrum near the limit of detection, we calculated that 1.6 μg in 3 μL (1 mM) would provide a S/N of 4 for the 6-Hz-wide aromatic protons in 64 scans. This spectrum was acquired (18 hr) and is shown in Figure 3B. Despite the F1 and F3 protons broadening further, relative to 20 mM, and moving under the F8 and F6 resonances, this spectrum was also able to resolve all cross peaks observable at higher concentration (Figure 3A). The changes in F1 and F3 between the 1D spectra at different concentrations indicate intermolecular associations are an additional challenge in studying this adduct, likely due to aromatic stacking of the AAF and/or dG moieties in aqueous solvent altering equilibria between the multiple conformations. (Note also the midpoint of the aromatic cluster has shifted by 0.25 ppm). The amount of material used to acquire the TOCSY spectrum was confirmed by UV absorption of the recovered sample. The sample was recovered by back flushing it into a clean tube, followed by a clean solvent plug (that had been injected prior to the sample for this purpose of rinsing probe surfaces during recovery). Comparing UV measurements of the recovered sample to the stock solution revealed a recovery of 98%.

The sensitivity when acquiring the TOCSY spectrum was consistent with that observed in the 1D at the limit of detection. With 1.6 μg , the S/N of the aromatic region was 5 in 64 scans. The 80 ng spectrum above gave a S/N of 10 in 100K scans, which should give 35 times better S/N than 64 scans; 1.6 μg is 20 times more material than 80 ng. A limit of detection of 1.6 μg for a TOCSY spectrum is actually about 5-fold higher than would be expected for a microcoil using droplet loading, or a micro-cryo probe. However, here the lines were over 6 Hz wide, with relaxation times of 50 msec and less. The limit of detection would be at least 5-fold lower concentration for a more typical sample with 1 Hz line width.

The above work shows how a small amount of a DNA adduct can be transferred into a microcoil probe with negligible losses, and 1D or 2D NMR spectra acquired. Other concerns about identifying an *in vivo* DNA adduct (the most difficult case as a reference point) are discussed below. These include the amount of tissue that would have to be extracted; the integration of NMR with other analytical techniques during purification; further discussion of the scale-up required to obtain NMR beyond 1D; and the aspect of balancing the effort and cost of scale-up with that of obtaining the highest NMR sensitivity. For an adduct formed *in vitro* (where the exposure typically can be increased to form more adduct), the task in general would be much easier than what is presented below.

Based on ^{32}P -postlabeling, many adducts are at the attomole to femtomole level in a few micrograms of DNA.²² Obviously, it would be essential to isolate an adduct at this level from much more DNA. Issues with chemical noise and recovery would be critical. To obtain 100 pmol of a DNA adduct present in DNA at a level of 1 adduct in 10^7 nucleotides, for example, one would need about 1.5 g of DNA, assuming a 20% yield of the adduct. In turn, this would require starting with about 1 kg of tissue. Clearly this would be a daunting project, perhaps comparable in magnitude and effort to the experiments conducted many years ago to identify hormones. No doubt successive rounds of HPLC on different columns would be central to the purification, using the technique that discovered the adduct in the first place to follow the adduct of interest. Ideally the latter technique would be mass spectrometry (MS), for example an MS technique has been reported that can discover unknown adducts and partially characterize them at the fmol level, furnishing accurate mass values for the molecular ion and fragment ions, based on an isotopologue mass-tagging method.^{23,24} Because NMR cannot selectively analyze a low-level analyte in a mixture as well as MS, the capability to obtain microscale NMR locally would be important in guiding the later stages of the purification strategy in response to the NMR interferences observed, until an interpretably clean spectrum could be acquired.

In determining the structure of a novel adduct, MS, aside from a potential discovery role, would be the first technique to employ, contributing structural information especially via fragmentation data. 1D NMR would then provide complementary information, for example the sites of addition to an aromatic ring. This would likely lead to hypothetical structures or partial structures for the adduct, and define specific questions for targeted structural analysis. Determining chiral centers by a nuclear overhauser effect could require 10-30× more material than a 1D; long-range proton-carbon correlations, by means of heteronuclear single quantum coherence/heteronuclear multiple bond correlation, another 10-30× more. If additional scale-up were needed, having 1D NMR and MS data can show that an adduct, discovered at a trace level *in vivo*, is identical to an adduct present at a higher level in an alternate source, such as an *in vitro* sample subjected to an exposure that gives the adduct.

The effort and cost of scale-up of an adduct would in practice be balanced against that of obtaining higher NMR sensitivity. In principle, NMR sensitivity might be increased as much as 7x beyond what is reported here, reducing the amount of tissue required from 1 kg (see above) to about 150 g. Increasing the field strength from 500 MHz (used here) to a 900 MHz magnet would provide a 2.8-fold boost in sensitivity, if outfitted with a similar microcoil detector. A micro-cryo probe would give comparable mass sensitivity, as well as facilitate loading and recovery of a precious sample by using a 1.7 or 1 mm tube. The highest mass sensitivity would come from a small microcoil: a 360 μm microcoil should give 2.5 times better mass sensitivity than a 1 mm microcoil, although it would require concentrating the sample to 50 nL at about 1 mM (in contrast with 1.5 μL at 0.1 mM tested here).^{5,6,8} Whatever balance is chosen between the efforts of scale up and NMR sensitivity, a prerequisite to accessing or developing more sensitive NMR would be demonstrating that the purity and quantity would be adequate for the proposed NMR studies.

Conclusion

Modern microscale NMR can play an important role in determining the structures of DNA adducts isolated from biological sources. The efficient sample handling technique described here for microcoil NMR in which a small aqueous volume (1.5 μL), containing a DNA adduct in a nucleotide form at the pmol level (for 1D ¹H NMR), or nmol level (2D NMR), is completely picked up and transferred from a vial as a microdroplet in a fluorocarbon carrier liquid, can be performed with readily available equipment. The 3-fold improvement in sample efficiency is comparable to the advantage of a cryoprobe over a conventional probe, and makes a microcoil probe as sensitive as a micro-cryo probe, enabling a 1D ¹H NMR spectrum to be obtained on less than 100 ng in a local facility without such specialized instrumentation.

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Abbreviations

FC43	Fluorinert
AAF-dGMP	N-(2'-Deoxyguanosin-8-yl)-2-acetylaminofluorene 5'-monophosphate
perfluorooctylsilane, PFOS	Tridecafluoro-1,1,2,3-tetrahydrooctyl-triethoxysilane

COSY	Correlation spectroscopy
TOCSY	Total correlation spectroscopy
DQCOSY	Double quantum filtered COSY
NOESY	Nuclear Overhauser enhancement spectroscopy
ROESY	Rotating-frame nuclear Overhauser effect

REFERENCES

1. Brey W, Edison AS, Nast RE, Rocca J, Saha S, Withers R. Design, construction, and validation of a 1-mm triple-resonance high-temperature-superconducting probe for NMR. *J. Mag. Reson.* 2006; 179:290–3.
2. Hilton BD, Martin GE. Investigation of the experimental limits of small-sample heteronuclear 2D NMR. *J. Nat. Prod.* 2010; 73:1465–1469. [PubMed: 20812673]
3. Gonella, NC. Chromatographic Science Series. Vol. 105. CRC Press.; 2013. LC-NMR: expanding the limits of structure elucidation.
4. Molinsky TF. Nanomole-scale natural products discovery. *Curr. Opin. Drug Disc. Dev.* 2009; 12:197–206.
5. Olson DL, Peck TL, Webb AG, Magin RL, Sweedler JV. High-resolution microcoil ¹H-NMR for mass-limited, nanoliter-volume samples. *Science.* 1995; 270:1967–1970.
6. Wolters AM, Jayawickrama DA, Sweedler JV. Microscale NMR. *Curr. Opin. Chem. Biol.* 2002; 6:711–716. [PubMed: 12413558]
7. Olson DL, Norcross JA, O'Neil-Johnson M, Molitor PF, Detlefsen DJ, Wilson AG, Peck TL. Microflow NMR: concepts and capabilities. *Anal. Chem.* 2004; 76:2966–74. [PubMed: 15144211]
8. Jones CJ, Larive CK. Could smaller really be better? Current and future trends in high-resolution microcoil NMR spectroscopy. *Anal. Bioanal. Chem.* 2012; 402:61–8. [PubMed: 21879299]
9. Kautz RA, Goetzinger W, Karger BL. High-throughput microcoil NMR of compound libraries using zero-dispersion segmented flow analysis. *J. Comb. Chem.* 2005; 7:14–20. [PubMed: 15638474]
10. Lin Y, Schiavo S, Orjala J, Vouros P, Kautz R. Microscale LC-MS-NMR platform applied to the identification of active cyanobacterial metabolites. *Anal. Chem.* 2008; 80:8045–8054. [PubMed: 18834150]
11. Gathungu RM, Oldham JT, Bird SS, Lee-Parsons CW, Vouros P, Kautz R. Application of an integrated LC-UV-MS-NMR platform to the identification of secondary metabolites from cell cultures: benzophenanthridine alkaloids from elicited *Eschscholzia California* (California poppy) cell cultures. *Anal. Methods.* 2012; 4:1315–1325. [PubMed: 22707983]
12. Limtiaco JF, Beni S, Jones CJ, Langeslay DJ, Larive CK. The efficient structure elucidation of minor components in heparin digests using microcoil NMR. *Carbohydr. Res.* 2012; 346:2244–54. [PubMed: 21824609]
13. Teh SY, Lin R, Hung LH, Lee AP. Droplet microfluidics. *Lab Chip.* 2008; 8:198–220. [PubMed: 18231657]
14. Niu X, deMello AJ. Building droplet-based microfluidic systems for biological analysis. *Biochem. Soc. Trans.* 2012; 40:615–23. [PubMed: 22817704]
15. Nord L, Karlberg B. Extraction based on the flow-injection principle. Part 6. Film formation and dispersion in liquid-liquid segmented flow extraction systems. *Anal. Chim. Acta.* 1984; 164:233–249.
16. Patton, CJ.; Wade, AP. *Analytical Instrumentation Handbook*. 2nd ed. rev.. Ewing, GW., editor. Marcel Dekker; New York: 1997. p. 153-155.
17. Lacey ME, Sweedler JV, Larive CK, Pipe AJ, Farrant RD. ¹H NMR characterization of the product from single solid-phase resin beads using capillary NMR flow probes. *J. Magn. Reson.* 2001; 153:215–222. [PubMed: 11740897]

18. Behnia B, Webb AG. Limited-sample NMR using solenoidal microcoils, perfluorocarbon plugs, and capillary spinning. *Anal Chem.* 1998; 70:5326–31. [PubMed: 9868921]
19. Kriek E, Miller JA, Juhl U, Miller EC. 8-(N-2-fluorenyl-acetamido)-guanosine, an arylamidation reaction product of guanosine and the carcinogen N-acetoxy-N-2-fluorenylacetamide in neutral solution. *Biochemistry.* 1967; 6:177–182. [PubMed: 6030315]
20. Evans FE, Miller DW, Levin RA. Conformation and dynamics of the 8-substituted deoxyguanosine 5'-monophosphate adduct of the carcinogen 2-(acetylamino)fluorene. *J. Am. Chem. Soc.* 1984; 106:396–401.
21. Isgro T, Mathew N, Topper RQ. Structural characterization of N-acetyl-2-aminofluorene (AAF) adducts to guanine and deoxyguanosine via a molecular mechanics, semi-empirical, and density functional theory cascade. *J. Molecular Struct.* 2004; 710:31–43.
22. Phillips DH, Arlt VM. The ^{32}P -postlabeling assay for DNA adducts. *Nature Protocols.* 2007; 2:2772–2781.
23. Wang P, Fisher D, Rao A, Giese RW. Nontargeted nucleotide analysis based on benzoylhistamine labeling-MALDI-TOF/TOF-MS: Discovery of putative 6-oxothymine in DNA. *Anal. Chem.* 2012; 84:3811–3819. [PubMed: 22409256]
24. Wang P, Gao J, Li G, Shimelis O, Giese RW. Nontargeted analysis of DNA adducts by mass-tag MS: reaction of p-benzoquinone with DNA. *Chem. Res. Toxicol.* 2012; 25:2737–2743. [PubMed: 23113579]
25. Bain AD. Chemical Exchange in NMR. *Progress in Nuclear Magnetic Resonance Spectroscopy.* 2003; 43:63–103.

Figure 1.

Microdroplet loading method. (A) Schematic drawing of the injection device, with connection to microcoil NMR probe; the inset illustrates the elongated droplet in the PTFE tubing, surrounded by FC43. (B) Photograph of a conical glass vial (for visualization purposes; a plastic vial was used for AAF-dGMP) with a model sample (aqueous dye solution) being fully drawn into the 380 µm o.d. silica capillary of the injection device. (C) Structure of AAF-dGMP (numbered according to Evans et al.).²⁰ Highlighted bonds are color coded to TOCSY spectrum annotations of Figure 3.

Figure 2.

NMR spectra. (A) solvent blank, 40K scans, acquired in the microcoil. Solvent peaks are greyed. (B) AAF-dGMP, 80 ng, 100K scans acquired in the microcoil over 40 hours. Inset: 7.4 ppm peak from first 4 hours (start) and from last 4 hours (end). (C) Reference spectrum of 39 μ g AAF-dGMP acquired using a conventional 5 mm NMR tube. The inset shows the aromatic region from spectra acquired at higher and lower temperatures. All of the signals have been assigned previously, for example the signals above 7 ppm are: 6.3 ppm (CH at ribose C-1), 4.2 ppm (CHs at ribose C-3 and C-4), 3.9 ppm (bridge CH₂ of fluorene and CH₂ at ribose C-5), and 2.2 ppm (acetyl, and CH₂ at ribose C-2).²⁰

A) TOCSY 35 μg (20 mM), 50 C B) TOCSY 1.6 μg (1 mM), 50 C **Figure 3.**

A) aromatic region of a TOCSY spectrum of 35 μg AAFdG in 3 μL (20 mM) at 50 °C. The 1D spectrum aligned above it is 16 scans. B) aromatic region of TOCSY spectrum of 1.6 μg AAF-dGMP in 3 μL (1 mM) at 50 °C. The 1D spectrum aligned above it is 8k scans. Fluorene resonances are numbered as in Figure 1C.