# Applications of dynamic nuclear polarization to the study of reactions and reagents in organic and biomolecular chemistry

**Christian Hilty* and Sean Bowen**

***Received 17th March 2010, Accepted 28th May 2010***
***First published as an Advance Article on the web 17th June 2010***
**DOI: 10.1039/c004420m**



Nuclear Magnetic Resonance (NMR) is an important spectroscopic tool for the identification and structural characterization of molecules in chemistry and biochemistry. The most significant limitation of NMR compared to other spectroscopies is its relatively low sensitivity, which thus often requires long measurement times or large amounts of sample. A way of increasing sensitivity of single scan NMR spectra by several orders of magnitude is through hyperpolarization of nuclear spins. Dynamic nuclear polarization allows hyperpolarization of most spins in small molecules encountered in chemistry and biochemistry. NMR spectra of small amounts of samples from natural source, or from chemical synthesis can readily be acquired. Perhaps more interestingly, the availability of the entire hyperpolarized NMR signal in one single scan allows the measurement of transient processes in real time, if applied together with a stopped-flow technique. Through observation of chemical shift, different reactant and product species can be distinguished, and kinetics and mechanisms, for example in enzyme catalyzed reactions, can be elucidated. Real-time hyperpolarization-enhanced NMR is uniquely amenable to correlating atomic positions not only through space, but also over time between reactant and product species. Such correlations carry mechanistic information about a reaction, and can prove reaction pathways. Applications of this technique are emerging in different areas of chemistry concerned with rapid reactions, including not only enzymatic processes, but also chemical catalysis and protein folding.

## Introduction

Molecular structure and dynamics are often probed using a wide array of analytical techniques. X-Ray diffraction and nuclear magnetic resonance (NMR) are tools that provide detailed, yet static "snapshots" of a three-dimensional structure. Alternatively, high time resolution can be achieved by stopped-flow or temperature-jump experiments, typically in combination with optical spectroscopy. The drawback of most optical methods, however, is that they allow only a limited number of molecular sites to be probed at a given time. Based on the distinct chemical shifts of individual atoms, and its ability to work in solution; NMR would be an ideal method for the detection of rapid dynamic processes

*Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, TX 77843, USA. E-mail: chilty@chem.tamu.edu*

**Christian Hilty**

*Christian Hilty obtained a Dr sc. degree in Physics in 2004 from the ETH Zürich, where he studied protein structure by NMR under the direction of Prof. Kurt Wüthrich. He completed postdoctoral research in hyperpolarized NMR with Prof. Alexander Pines at the University of California, Berkeley. Currently, he is an Assistant Professor in the Chemistry Department at Texas A&M University. His research interest lies in the development and application of technology for Nuclear Magnetic Resonance. He was the recipient of the Raymond Andrew Prize (2005), the Camille and Henry Dreyfus Award (2006), and a CAREER Award from the National Science Foundation (2009).*

**Sean Bowen**

*Sean Bowen received his B.S. in Chemistry from Purdue University in 2006. In the fall of 2006, he began his Ph.D. studies at Texas A&M University in the laboratory of Prof. Christian Hilty. Since then, he has earned a fellowship from the Chemistry Biology Interface training program, the Bruno J. Zwolinscki Award in Physical Chemistry and the Dow Chemical Graduate Fellowship in Chemistry. His research interests include development of new hardware and methods to harness the power of hyperpolarized NMR.*

such as chemical transformations. Because of this, stopped-flow applications have been proposed even early on in the development of NMR.[1] In order to apply high-resolution NMR techniques to the real-time study of dynamic processes, several limitations must be overcome. Namely, sensitivity constraints often necessitate the averaging of multiple transients in order to obtain NMR spectra of sufficient quality. Secondly, typical multi-dimensional NMR experiments require multiple scans for coherence selection and for chemical shift evolution in indirect dimensions. These multiple scan approaches severely reduce the time resolution of an experiment, and are not readily amenable to real-time studies of rapid processes.

Acquiring NMR spectra in a single scan may, depending on concentration and properties of a sample, necessitate an increase in the signal-to-noise ratio of the experiment. A powerful way of achieving this goal is through hyperpolarization of nuclear spins. Hyperpolarization techniques are applied prior to an NMR measurement, to increase the population difference between the Zeeman levels of the nuclear spins in a magnetic field. The increased population difference translates proportionally into an increase in NMR signal. While some hyperpolarization techniques are substance specific, such as optical pumping for polarization of xenon gas;[2] dynamic nuclear polarization (DNP) is capable of hyperpolarizing the nuclear spins of almost any small molecule.[3–5]

DNP operates by transferring polarization from unpaired electron spins to nuclear spins. Compared to nuclear spins, electron spins have large magnetic moments and therefore achieve comparatively high levels of polarization in a magnetic field. Unpaired electron spins are added to the sample in the form of free radicals, which need not be covalently attached to the sample molecule. The coupled electron/nuclear spin system is then pumped using microwave irradiation. DNP can be applied in a variety of modalities. However, the highest one-time polarization enhancements for liquid state NMR have been achieved by polarizing a frozen sample at cryogenic temperature (~ 1 K), followed by dissolution into a solvent and NMR measurement at ambient temperature.[6] Compared to conventional NMR, a sample polarized using this "dissolution DNP" technique in a single scan can yield a signal-to-noise ratio that is enhanced by a factor of $10^2$ to $10^4$ when compared to a thermally polarized sample at room temperature. This extraordinarily large signal enhancement has the potential to open new application areas for NMR. Specifically, the dissolution DNP technology is actively being developed to provide imaging agents for *in vivo* metabolic imaging.[7,8] In this application, the signal enhancement is harnessed to obtain highly sensitive images of the distribution of small amounts of metabolites in organs and tissue. The focus of this article, however, is to highlight a few of the new applications in chemistry and biochemistry that arise through the ability to obtain high-resolution NMR spectra of samples polarized using DNP.

## Spectroscopy of mass limited samples

Since the time required for signal averaging has a quadratic dependence on the desired signal-to-noise ratio, the signal enhancement available from hyperpolarized NMR enables the measurement of samples that would otherwise not be amenable to NMR spectroscopy. The spectrum of the methyl ester of the natural product fusaric acid shown in Fig. 1 was measured in 3.6 s after a DNP polarization time of 3 h. Obtaining a conventional NMR spectrum of equivalent signal to noise with the same amount of sample and the same NMR instrument would require more than 5 years of signal averaging, even when disregarding additional limitations due to quantization noise that may arise in the measurement of thermally polarized samples with low signal.

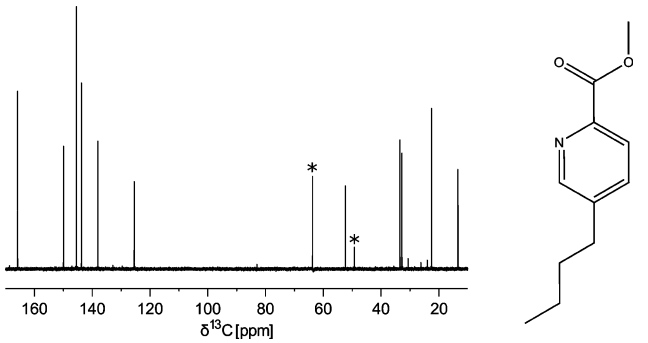


**Fig. 1** DNP-NMR spectrum of methyl fusarate. * denotes resonances from the polarization and dissolution solvents, (ethylene glycol and methanol respectively).

In this experiment a solution of 0.2 μL methyl fusarate with 0.8 μL ethylene glycol and 15 mM Finland radical was spin polarized through the DNP mechanism. After dissolution into methanol, the sample was transferred to an NMR spectrometer using an automated sample injector specifically designed for this purpose.[9,10] From Fig. 1, it can be seen that high-quality NMR spectra are readily obtained using this procedure. In this spectrum, a line width < 0.5 Hz was obtained, rivalling the quality of conventional NMR spectra. The spectrum in Fig. 1 also shows that resonances from a variety of chemical groups are readily observable. The relative intensity of a resonance line in such a spectrum is a function of polarization efficiency, and since the sample needs to be dissolved and injected into an NMR instrument prior to NMR measurement, it is also a function of the amount of signal lost due to spin–lattice relaxation during the time between DNP polarization and NMR measurement.

Developments of the DNP technique are likely to further improve both of these factors. Polarization efficiency depends for example on the properties of the free radical used for DNP polarization. In dissolution DNP, where the sample is transferred from the solid to the liquid state, the sample transfer time will govern the loss of signal before NMR measurement. For the spectrum shown in Fig. 1, the sample was transferred between two superconducting magnets in 2 s, a procedure that allows high-quality DNP-NMR spectra to be obtained from spins with a spin–lattice relaxation time larger than ~700 ms, which includes the commonly used NMR active nuclei such as $^1$H, $^{13}$C or $^{15}$N in most small organic molecules,[11] as well as in polypeptides and under certain conditions even in proteins. A final consideration specific to dissolution DNP is the dilution factor encountered during dissolution, which was *ca.* 600 in the present experiment, requiring the use of a small, but highly concentrated sample for dissolution.

Due to the substantial gain in sensitivity for spectroscopy with small molecules, DNP-NMR also appears well suited for ligand binding studies, as demonstrated recently by Lerche *et al.*[12] Even without further development of the technique, dissolution DNP

 

enables highly sensitive NMR spectroscopy, for the elucidation of the structure of minute amounts of substance from natural or synthetic sources.

## Real-time measurements

Beyond the measurement of highly sensitive NMR spectra, a potentially even more powerful application of the DNP technique arises from the fact that the entire signal from hyperpolarization is available at one time. This observation points towards a way of determining kinetics and mechanisms of non-equilibrium processes through the measurement of NMR spectra in real-time. If applied together with stopped-flow mixing, DNP-NMR holds promise for studying complex chemical and biochemical reactions. Proof-of-concept for a real-time measurement of a biochemical process is shown in Fig. 2, on the example of the trypsin catalyzed hydrolysis of $N_\alpha$-benzoyl-L-arginine-ethyl ester (BAEE).[13] In this experiment, substrate polarized through DNP was mixed rapidly with a solution of the trypsin enzyme. Following mixing, the conversion of the substrate into the product $N_\alpha$-benzoyl-L-arginine (BA) was monitored over a period of 3 s by a sequence of single NMR scans using small flip angles for excitation. From the intensities of the substrate resonance that is being depleted with time (peak #1 in Fig. 2), or from the product resonance that builds up (peak #2 in Fig. 2), the time-course of the reaction can be followed. The reaction rate can be extracted by normalizing the obtained signal intensities against a reference dataset of unreacted compound, which serves to remove the dependence on the relaxation rate of the observed spins. The dataset shown in Fig. 2 was acquired using a 3.3 mM solution of substrate that contained the NMR-active $^{13}C$ isotope only at the level of the natural abundance of 1.1%. Therefore, the NMR spectra were measured at an effective $^{13}C$ isotopomer concentration of 36 μM. A comparison with a conventional NMR spectrum of a sample of BAEE indicates that measurement of a spectrum equivalent to the first scan in the hyperpolarized dataset would take more than 100 days. Since the hydrolysis of BAEE is a chromogenic reaction, the rate obtained for this reaction by DNP-NMR may be compared to a measurement by optical spectroscopy for validation. However, the NMR technique enjoys broad applicability towards a variety of enzyme catalyzed reactions, even if the naturally occurring substrates or products involved do not contain chromophores.

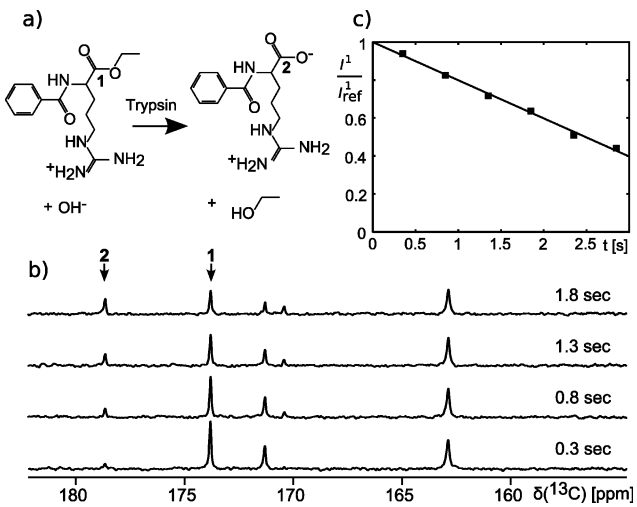


**Fig. 2** Illustration of time-resolved, DNP enhanced NMR. a) Trypsin catalyzed conversion of BAEE into BA (see text). b) time-course of the reaction, observed by $^{13}C$ NMR at natural abundance, using 3.3 mM DNP enhanced BAEE and 54 μM Trypsin. c) Linear fit of normalized intensities from b, yielding the rate constant $k_{cat} = 12.1\ s^{-1}$. (reproduced in part from ref. 13, with permission).

A major advantage of using hyperpolarized NMR to follow kinetic processes is that it enables the detection of less sensitive nuclei such as $^{13}C$ or $^{15}N$. These nuclei exhibit larger chemical shift dispersion than protons, and thus improve the ability to distinguish peaks from different molecular sites. As conventional stopped-flow NMR is limited to high sensitivity nuclei such as $^{1}H$, the dispersion provided by these nuclei is an invaluable tool for the elucidation of kinetics and mechanisms of reactions. The minimum time resolution achievable with this technique is given by the time that is needed to record a meaningful spectrum, which is approximately 10 ms, where a compromise between spectral and spatial resolution is made. On the other hand, the maximum time over which a reaction can be followed is on the order of the spin–lattice relaxation time of the observed nucleus, ranging from seconds to tens of seconds. For slower processes, this method seamlessly integrates with conventional NMR using signal averaging. Altogether, hyperpolarized $^{13}C$ NMR presents an attractive alternative to optical stopped-flow techniques for the study of dynamic processes, because of the additional benefit of NMR being specific to molecular structure.

## Correlations in space and time

Much of the power of NMR spectroscopy derives from the ability to record spectra that contain information about correlations between atoms. Chemical shift correlations, which permit the identification of the relative positions of atoms in a molecule, are typically obtained from multi-dimensional NMR spectroscopy.[14] Measurement of these spectra relies on acquiring a number of transients with changing chemical shift evolution time. However, once the available polarization from a hyperpolarized sample has been converted into observable coherences, the spin system does not return to the hyperpolarized state. Because of this, additional transients cannot be measured, and it is necessary to explore new NMR techniques for obtaining structural information. Various techniques for enabling the recording of chemical shift correlations from hyperpolarized samples have been proposed. Spectral information can be encoded in a spatial dimension along an axis in the sample using pulsed field gradients, allowing recording of a correlation spectrum in a single scan.[15,16] An alternative scheme that is particularly easy to implement, uses small flip angle excitation to record a two-dimensional spectrum from a single hyperpolarized sample with sequential sampling of the indirect dimension.[17] Finally, chemical shift can indirectly be obtained from as little as four sequentially acquired transients recorded from a single hyperpolarized sample, using differential scaling of observed coupling constants by off-resonance decoupling.[18] These and other similar sequences may become invaluable in the structure determination of mass limited samples, as well as of reaction intermediates, by enabling the assignment of chemical shifts and the identification of functional groups.

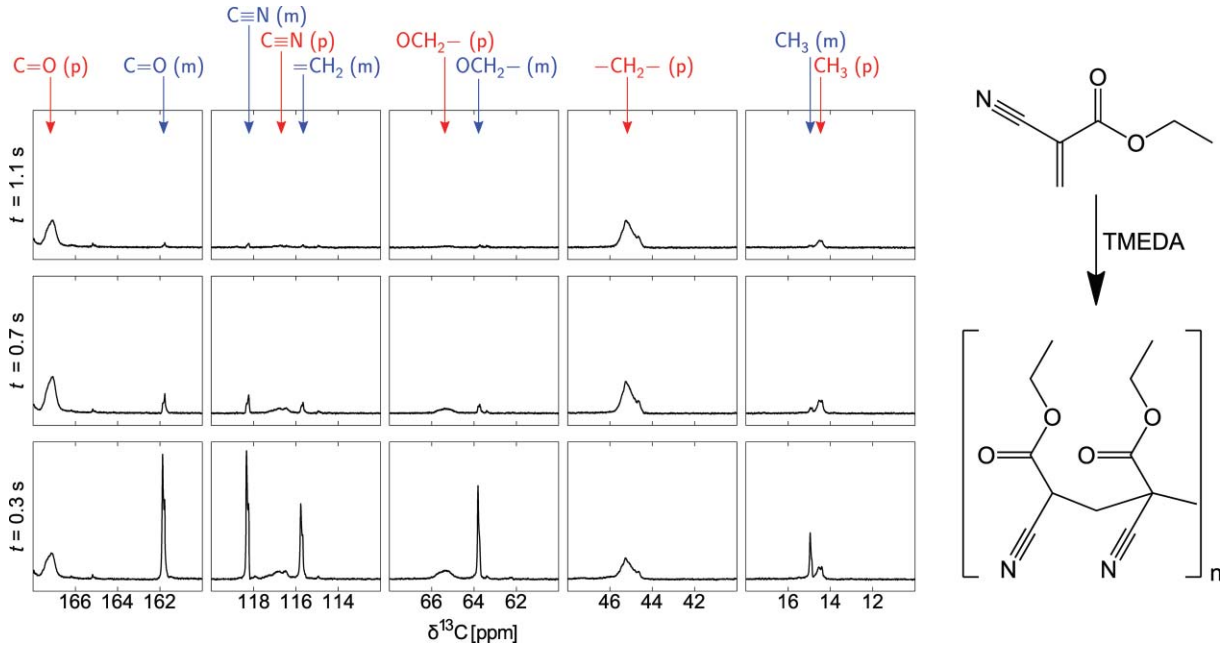


**Fig. 3** DNP-NMR spectroscopy of a polymerization reaction. Hyperpolarized ethyl-2-cyanoacrylate monomer in THF was injected into a solution of TMEDA initiator to yield a final concentration of *ca.* 60 mM ethyl-2-cyanoacrylate and 5 mM TMEDA. 32 variable flip angle scans were acquired over 11 s.

One type of correlation that is unique to stopped-flow hyperpolarized NMR is a correlation between the chemical shift of a nuclear spin in different environments at multiple points in time; as encountered in an atom during the course of a reaction. This experiment is based on the fact that unlike in conventional NMR, in hyperpolarized spectroscopy all of the polarization that can be converted into an observable coherence is present at the beginning of the experiment. Therefore, it is possible to "encode" information into a spin state, for example by selective inversion of one molecular site, at the beginning of the reaction. This spin-state is preserved even if the atom of interest participates in a chemical reaction and forms a new bond.[19] Through observation of this spin-state, the rearrangement of atoms from the reactant to the product, or if present to a reaction intermediate, can directly and unambiguously be followed. In addition to allowing the unambiguous assignment of the chemical shifts of reaction intermediates, these correlations carry important mechanistic information about the reaction. Even in reactions with multiple steps or multiple pathways, corresponding atomic positions can be identified directly, and without the need for isotope labeling.

## Scope of application

By providing a sensitivity enhancement of several orders of magnitude, dissolution DNP enables NMR measurements of previously unimaginable samples. DNP-NMR can be used for the rapid characterization of mass limited small molecule samples; such as those purified in limited quantities from a natural source (Fig. 1), as well as products from combinatorial organic synthesis. Through one- and two-dimensional spectroscopy, such compounds can be identified and structurally characterized. Furthermore, synthetic pathways may be elucidated through NMR based quantification of selective isotope incorporation.

Time-resolved spectroscopy of hyperpolarized samples allows for the investigation of kinetics and mechanisms of rapid reactions. Reactions that exhibit kinetic lifetimes in the observable sub-second to second time scale include a large number of enzyme catalyzed reactions (Fig. 2), as well as chemically catalyzed reactions. An example DNP-NMR dataset of such a chemical reaction, an anionic polymerization reaction forming poly(ethyl-2-cyanoacrylate), is shown in Fig. 3. The DNP sample consisted of 40 μL of a 50 : 50 (v/v) mixture of ethyl-2-cyanoacrylate and methyl methacrylate containing 15 mM $\alpha,\gamma$-bisdiphenylene-β-phenylallyl (BDPA) radical; the sample was polarized for 3 h, dissolved in tetrahydrofuran (THF) and mixed with 25 μL of 100 mM tetramethylethylenediamine (TMEDA) in THF in the NMR tube during injection. In the subsequently acquired time-resolved set of NMR spectra, the formation of polymer is observed, and all of the resonances from monomer and polymer are identified (Fig. 3). Under the given reaction conditions, long polymer chains were rapidly formed, as evidenced by the broad product peaks. However, under reaction conditions where slower kinetics are favored, real-time NMR is particularly well suited to study the kinetics of the first steps after polymerization initiation. In this approach, addition of initial monomer units may be followed without the need for selectively isotope enriched substrates, due to distinct $^{13}C$ chemical shifts. For catalytic polymerization processes, the DNP technique has the potential to elucidate catalyst specificity through the observation of transient catalyst complexes.

The information obtained from NMR will be of particular value for distinguishing relative rates of competing reaction pathways, as well as identifying transient species based on their differing chemical shifts. Here, an interesting application would be the elucidation of protein folding pathways, starting from hyperpolarized polypeptide chains. Due to the complexity of

 

polypeptide molecules, protein folding remains one of the most formidable problems in biochemistry, and its investigation could benefit most significantly from real-time DNP-NMR due to the structural specificity of the method.

## Acknowledgements

Startup funds from Texas A&M University are gratefully acknowledged. CH thanks the Camille and Henry Dreyfus foundation for a new faculty award. SB thanks a Chemistry Biology Interface training grant administered by Texas A&M University, and a Diversity Fellowship from Texas A&M University. Financial support was obtained from the Welch Foundation (Grant A-1658), and from the National Science Foundation (Grant CHE-0840464). We thank Dr Howard Williams of Texas A&M University and Dr Robert D Stipanovic of the United States Department of Agriculture for the sample of methyl fusarate.

## References

1 R. O. Kuhne, T. Schaffhauser, A. Wokaun and R. R. Ernst, *J. Magn. Reson.*, 1979, **35**, 39–67.
2 T. G. Walker and W. Happer, *Rev. Mod. Phys.*, 1997, **69**, 629–642.
3 R. A. Wind, M. J. Duijvestijn, C. Vanderlugt, A. Manenschijn and J. Vriend, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1985, **17**, 33–67.
4 A. Abragam, *The Principles of Nuclear Magnetism*, Clarendon Press, Oxford, 1961.
5 D. A. Hall, D. C. Maus, G. J. Gerfen, S. J. Inati, L. R. Becerra, F. W. Dahlquist and R. G. Griffin, *Science*, 1997, **276**, 930–932.
6 J. H. Ardenkjaer-Larsen, B. Fridlund, A. Gram, G. Hansson, L. Hansson, M. H. Lerche, R. Servin, M. Thaning and K. Golman, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 10158–10163.
7 K. Golman, R. in't Zandt and M. Thaning, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 11270–11275.
8 A. Viale and S. Aime, *Curr. Opin. Chem. Biol.*, 2010, **14**, 90–96.
9 C. Hilty, S. Bowen Rapid Nuclear Magnetic Resonance Analysis System. US Patent application 12/539,486: 2009.
10 S. Bowen and C. Hilty, *Phys. Chem. Chem. Phys.*, 2010 DOI:10.1039/C002316G.
11 G. C. Levy and D. J. Kerwood, in *Encyclopedia of Nuclear Magnetic Resonance*, ed. D. M. Grant and R. Harris, Wiley, Weinheim, 1996, vol. 2, pp. 1147-1156.
12 M. H. Lerche, S. Meier, P. R. Jensen, H. Baumann, B. O. Petersen, M. Karlsson, J. O. Duus and J. H. Ardenkjaer-Larsen, *J. Magn. Reson.*, 2010, **203**, 52–56.
13 S. Bowen and C. Hilty, *Angew. Chem., Int. Ed.*, 2008, **47**, 5235–5237.
14 R. R. Ernst, G. Bodenhausen and A. Wokaun, *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, Oxford University Press, Oxford, 1986.
15 L. Frydman and D. Blazina, *Nat. Phys.*, 2007, **3**, 415–419.
16 L. Frydman, T. Scherf and A. Lupulescu, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 15858–15862.
17 H. Zeng, S. Bowen and C. Hilty, *J. Magn. Reson.*, 2009, **199**, 159–165.
18 S. Bowen, H. Zeng and C. Hilty, *Anal. Chem.*, 2008, **80**, 5794–5798.
19 S. Bowen and C. Hilty, *Anal. Chem.*, 2009, **81**, 4543–4547.