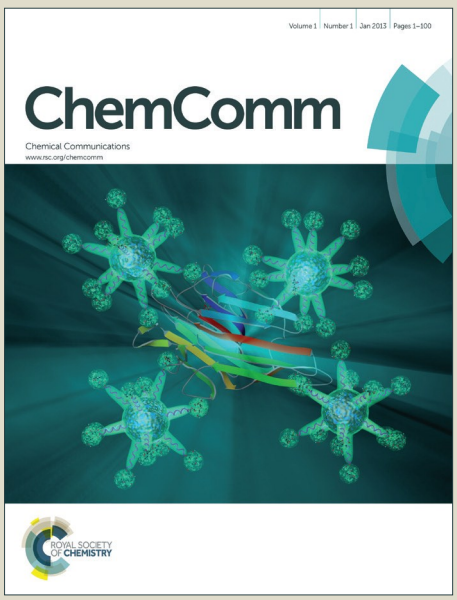
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

NMR analysis of weak molecular interactions using slice-selective experiments via study of concentration gradients in agar gels

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Y. Mitrev,[a,b] S. Simova,[b] and D. Jeannerat[a]

Weak molecular interactions can be localized and quantified from a single NMR experiment analysing concentration gradients generated in agar gels. The spectra from various cross-section along the gradient were obtained using a slice-selective pulse sequence realisable with standard NMR equipement.

NMR titrations are among the most widely applied methods for quantitative investigations of non-covalent interactions in solution. Association parameters (association constants and stoichiometry) can be calculated from the variations of NMR parameters induced by complexation. This requires series of spectra with varying concentration ratio between the interacting species.[1] Titrations are usually performed either as a time consuming "single-tube" titration, incrementing the concentration of the titrant manually, or in a product consuming "multiple-tube" manner, where series of samples with different compositions are prepared. Recently, Niklas *et al*.[2] introduced a single-shot NMR titration by recording slice-selective ^{1}H and ^{7}Li experiments to study concentration gradients created by the dissolution of frozen 12-crown-4 ether in $LiClO_4$/acetonitrile-d_3. Such an approach circumvents simultaneously both disadvantages of the classical titration, allowing for efficient use of chemicals to obtain titration data in a matter of minutes, once the concentration gradient is developed. Despite the promising results, the freezing/mixing scheme is limited to systems where one of the components is solid or could be easily frozen. A more practical medium would be to include one of the components in a porous matrix and to allow the other to diffuse in, thus giving the system more resistance to mechanical stress. Such system is used by the authors[2] to monitor the chemical reaction of *N,N,N′,N″,N″*-pentamethyldiethylenetriamine with *n*BuLi/toluene-d_8 in a swollen polystyrene gel. Combining both approaches, we extend the range of application of single-shot NMR titrations to aqueous samples and present a very robust and practical system based on agar gels.

Agar, and its main component, agarose, are known to form physical gels with nanometre pore size at concentrations lower than 1 %,[3] allowing efficient molecular diffusion even for large molecules.[4] They are commonly used in separation techniques such as electrophoresis.[5] Concerning NMR spectroscopy, these gels have the remarkable property of causing no observable changes in the chemical shifts and lineshapes compared to normal aqueous solutions (Figure 1).[6] Moreover, the small quantity and the favourable relaxation properties of these gels result in the "NMR invisibility" of the matrix. This is not the case of other water compatible gels such as polyacrylamide. The thermoreversible nature of agar and agarose gels allows for quick and easy preparation of uniform samples by a simple heating/cooling cycle. This contrasts with the chemically cross-linked polymer gels such as polystyrene or PBLG requiring several days to obtain a homogeneous swelling.[7] Finally, agar gels are compatible with a wide range of pH (2 to 9 at RT) and provide the attractive opportunity to record spectra of aqueous solutions down to -10 °C because they reduce the freezing temperature of water.[6a]

Slice-selective experiments are gaining popularity as a method to study diverse systems where the composition varies along the NMR tube.[7,8] The NMR spectrum of a single

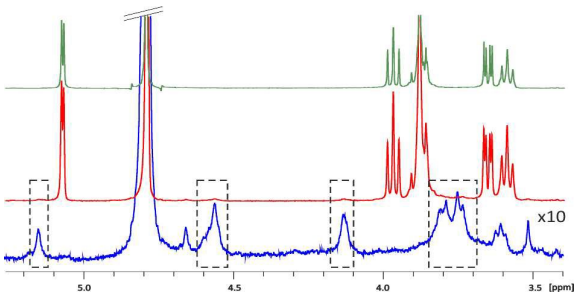


Fig. 1 1D ^{1}H spectra of 7 mM β-cyclodextrin in the absence (top) and presence (middle) of 1% (w/v) agar gel. Spectrum of the gel alone (bottom) with 10 times increase of the vertical scale.

[a] *Department of Organic Chemistry, University of Geneva, 1211, Geneva 4, Switzerland. e-mail: Damien.Jeannerat@unige.ch*
[b] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.*

† Electronic Supplementary Information (ESI) available: Detailed procedures, experimental parameters, pulse sequence, AU programs and additional investigations. See DOI: 10.1039/x0xx00000x

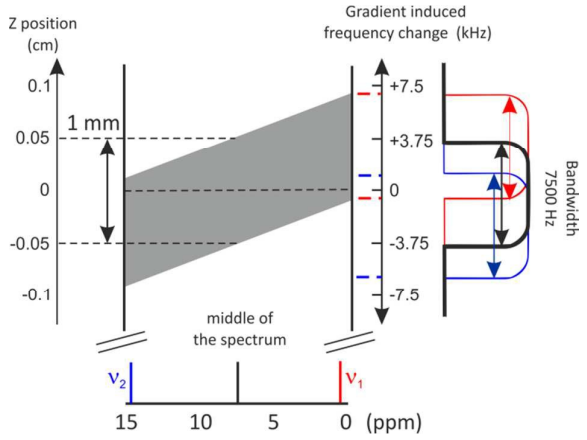


Fig. 2 Schematic representation of a section of the NMR tube in a slice-selective experiment. During a pulse-field gradient (PFG) of 17.62 G/cm, a linear field gradient along the direction of the NMR tube causes a spatial dependence of the Larmor frequency relative to the mid-tube frequency of 500 MHz (see the vertical scale in kHz). The application of a selective G4 gaussian cascade9 in the middle of the spectrum selects a bandwidth of 7.5 kHz (see the excitation profile on the right) which selects only a 1 mm slice of the NMR tube (filled in grey in the center). Note the slightly different location of the signals depending on the chemical shift (tilt of the selected region). This effect could be neglected in our case (< 0.07 mm per ppm), but may require a correction when using weaker PFG.

slice across the NMR tube is obtained by combining a controlled inhomogeneity of the B_0 field (called pulsed-field gradient or PFG) with a frequency selective pulse. Both are standard features in any modern NMR spectrometer (Figure 2). The same principle is used in homodecoupling techniques,[10] with one major difference when studying inhomogeneous samples is the requirement of less selective pulses (min. bandwidth equal to the spectral width) in order to obtain information from a discrete region of the sample. Recording series of spectra by systematically varying the carrier frequency of the selective pulse scans the NMR tube along its axis over *ca.* 1.3 cm (13 slices, approx. 1 mm each). This sample height was chosen to ensure a uniform excitation, thus avoiding loss of signal intensity at the boundaries of the active volume of the detection coil. (see ESI†) The sensitivity of the experiment is proportional to the selected volume of the individual slice, *i.e.* ≈ 5 % of a normal ^{1}H spectrum. But this reduction of the signal-to-noise ratio can be compensated by the opportunity to record the full series of 5-20 spectra without relaxation recovery period, thus significantly decreasing the overall data acquisition time (see ESI† for details on interleaved acquisition). This approach was successfully used to study kinetics of fast reactions in solution,[11] but we present the first application to the analysis of inhomogeneous samples.

As a model system to demonstrate that agar gels can be used for quantitative analysis we choose the determination of the affinity of β-cyclodextrin (CD) for paracetamol. It exhibits a simple 1:1 complexation behaviour with a relatively low association constant of about 150 M^{-1}.[12,13] The preparation of 1% gels is straightforward: 4 mg of agar are directly suspended in a standard 5 mm NMR tube with 400 μL of the D_2O solution of cyclodextrin. A 5 min stirring of the suspension in the NMR tube immersed in hot water bath (*ca.* 90 °C) with a Pasteur pipet insures a perfectly homogeneous solution. The tube is then taken out of the bath and fixed in a vertical position. The gel forms during a 15 min cooling period towards room temperature and is stabilized during 1 h at 4 °C.[14] The concentration gradient was obtained after adding 0.2 ml of the titrant solution on top of the gel. To evaluate the temporal stability and overall performance of the system, gel containing 7 mM cyclodextrin was titrated with a 70 mM paracetamol solution and slice selective spectra were recorded during a 3 days period. Using interleaved acquisitions, each set of 13 quantitative spectra, covering 13 mm of sample was acquired in c.a. 6 minutes (Figure 3). After 24 hours, simple integration of paracetamol aromatic proton signals and H-C(1) from the cyclodextrin showed paracetamol/cyclodextrin ratio from 0.7 to 7.6. The differences of the chemical shifts of H-C(5) and of H-C(3) provides the necessary variation to follow the complex

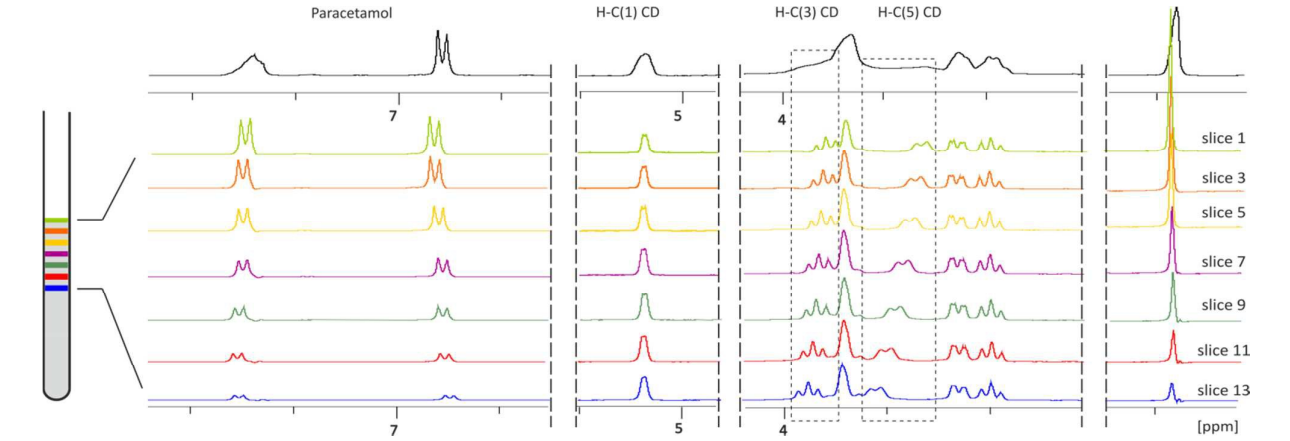


Fig. 3 ^{1}H spectrum (black) and slice-selective spectra of gel, containing CD (only odd slices are shown). The spectra are recorded 24 hours after the addition of paracetamol on the top of the gel. Note the slight reduction of the CD signals intensity in slice 1 (30 % decrease in the absolute integral value), indicating diffusion of CD in the titrant volume.

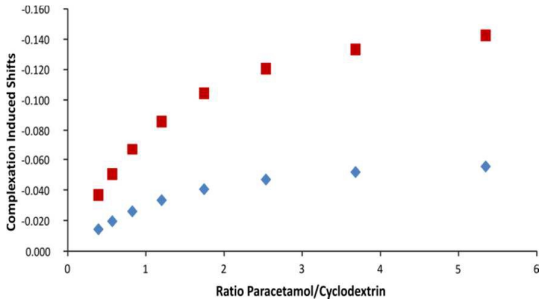


Fig. 4 Chemical shift variation of cyclodextrin H-C(5) (red) and H-C(3) (blue) protons after 24 h diffusion time of paracetamol in the gel containing CD. For convenience the chemical shift variations are presented as $\Delta\delta = \delta_{obs.} - \delta_{free}$.

formation (from 30 % to 84 %) over the analysed 1.3 cm of concentration gradient (Fig. 4). Beside a sensitivity reduced by a factor 20, the only significant difference with spectra obtained using a classical titration is a modest broadening of the drifting signals caused by the concentration gradient after spectral averaging within the slice thickness (see ESI†).

The association constant and the complexation induced shifts (CIS, $\Delta\delta = \delta_{complex} - \delta_{free}$) were calculated according to a 1:1 model by simultaneous least square fitting of the experimental data for H-C(3) and H-C(5). Table 1 compares the constants determined by the proposed gel titration method with the values obtained from conventional solution titration and literature data. In all cases excellent agreement is observed.

Because of its lower sensitivity, our approach is most appropriate for systems with concentration in the mM range. Optimal titration conditions and necessary diffusion times are presented in Table 2, to demonstrate its preferential use for systems with association constants between 10 and 10^3 (see Table 2 and ESI† for details on diffusion dynamics).

A single-shot NMR gel titration method was introduced to study weak molecular interactions in aqueous solutions. It utilizes the practical and quickly produced agar gels as medium for high-quality liquid state NMR measurements. Concentration gradients can be generated by the simple addition of the titrant solution on top of the gels. After an appropriate diffusion time, the series of spectra can be recorded using a slice-selective 1D ^{1}H experiment to scan the concentration gradients. This approach cannot replace standard NMR titration when sensitivity or signal enlargement are limiting. But the fact that a full set of spectra can be recorded in a few minutes from a single NMR tube should make it very useful for qualitative screening of small libraries

Table 1. Association constants, complexation induced shifts and free energies, obtained by diverse methods at 298 K.

	Ka(M^{-1})	$\Delta\delta^a$ H-C(5) (ppm)	$\Delta\delta^a$ H-C(3) (ppm)	ΔG (kJ/mol)
Gel titration	170	-0.061	-0.167	-12.7
Solution titration	170	-0.062	-0.159	-12.7
Fluorescence [b]	148	n.a.	n.a.	-12.4

[a] $\Delta\delta = \delta_{complex} - \delta_{free}$; [b] reference [13].

Table 2. Calculated optimal conditions for gel titration according to Fick's second law.

Ka	Analyte (M)	Titrant (M)[a] Max.	Min.	Diffusion time [c] (hours)
10	0.001	0.4008	0.0252	10[d]
	0.01	0.408	0.027	10
	0.1 [b]	**0.48**	**0.045**	**12**
100	0.001	0.0408	0.0027	10
	0.01 [b]	**0.048**	**0.0045**	**12**
	0.1	0.12	0.0225	20
1000	**0.001** [b]	**0.0048**	**0.00045**	**12**
	0.01	0.012	0.00225	18
	0.1	0.084	0.02025	24

[a] Minimum and maximum concentrations of titrant required to observe complexation between 20% and 80%; [b] Optimal titration conditions[15] are presented in bold; [c] Calculated diffusion time of titrant to achieve the desired concentrations at the top and the bottom of the gel respectively for a titrant with D = $6.2*10^{-4}$ mm^2/s (e.g. paracetamol at 25 °C);

of compounds and opens the possibility of high-throughput analysis.

Financial support from the State of Geneva and the Swiss National Foundation 200021_147069 and 206021_128746 are acknowledged. The authors thank Marta Brucka and Marion Pupier for comments on the manuscript.

Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

1 (a) Y. Cohen, L. Avram and L. Frish, *Angew. Chem. Int. Ed. Engl.*, 2005, **44**, 520; (b) A. Pastor and E. Martínez-Viviente, *Coord. Chem. Rev.*, 2008, **252**, 2314; (c) P. Thordarson, *Chem. Soc. Rev.*, 2011, **40**, 1305.
2 T. Niklas, D. Stalke and M. John, *Chem. Commun. (Camb)*, 2015, **51**, 1275, 469.
3 M. Maaloum, N. Pernodet and B. Tinland, *Electrophoresis*, 1998, **19**, 1606.
4 A. Pluen, P. A. Netti, Rakesh K. Jain and D. A. Berk, *Biophys. J.*, 1999, **77**, 542.
5 P. Serwer, *Electrophoresis*, 1983, **4**, 375.
6 (a) A. M. Spring and M. W. Germann, *Anal. Biochem.*, 2012, **427**, 79; (b) A. Pastore, S. Salvadori and P. A. Temussi, *J. Pept. Sci.*, 2007, **13**, 342.
7 A. C. Poppler, S. Frischkorn, D. Stalke and M. John, *ChemPhysChem*, 2013, **14**, 3103.
8 (a) J. Allen and K. Damodaran, *Magn. Reson Chem*, 2015, **53**, 200; (b) P. Trigo-Mourino, C. Merle, M. R. Koos, B. Luy and R. R. Gil, *Chem. Eur. J.*, 2013, **19**, 7013.
9 L. Emsley and G. Bodenhausen, *Chem. Phys. Lett.*, 1990, **165**
10 L. Castañar and T. Parella, *Magn. Reson. Chem.*, 2015, **53**, 399.
11 (a) G. E. Wagner, P. Sakhaii, W. Bermel and K. Zangger, *Chem. Commun. (Camb)*, 2013, **49**, 3155; (b) J. Kind and C. M. Thiele, *J. Magn. Reson.*, 2015, **260**, 109.
12 N. S. Moyon, T. S. Singh and S. Mitra, *Biophys. Chem.*, 2008, **138**, 55.

13 M. El-Kemary, S. Sobhy, S. El-Daly and A. Abdel-Shafi, *Spectrochim. Acta. Mol. Biomol. Spectrosc.*, 2011, **79**, 1904.
14 N. Kusukawa, M. V. Ostrovsky and M. M. Garner, *Electrophoresis*, 1999, **20**, 1455.
15. K. Hirose, Quantitative Analysis of Binding Properties, in *Analytical Methods in Supramolecular Chemistry*, ed. C. A. Schalley, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, Second Edition, 2012, 27-66.