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Electronic supplementary information for:

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

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Sample preparation

Typical gel samples were prepared by using 400 µl of the solution to titrate. This volume was chosen so that the gel spans approximately 0.7 cm above the center of the coil as determined by the Bruker sample depth gauge, with the top of the gel approximately aligned with the top of the active volume used for measurements (typically 1.3 cm, see the quantification section).

The agar (BioConcept, LE7-01P02-R) gels were prepared at 0.5, 1 and 2 % (weight/volume). None of them lead to any observable changes in the spectra of CD or any other compound tested. The gels were quite stable and could be kept and manipulated outside of the NMR magnet without any observable disturbances in the concentration gradient development (see also Figure S5 for titration curves obtained after 24, 48 and 72 hours). Titration samples were prepared by using 4 mg of agar suspended in 400 µl of 7 mM cyclodextrin in D_2O and titrated with 200 µl of 70 mM solutions of paracetamol in D_2O.

The concentrations of the interacting compounds should be chosen in the same manner as for a classical NMR titration experiments. The gel approaches limits the range of concentration to those obtained by free diffusion within the gel (see Table 2). But if a single acquisition is not sufficient to cover the desired range of concentration, additional acquisition of the slice-selective experiments can be recorded a few hours before and/or after the "optimal" diffusion delay and combined (see Figure S5 for an example). The sample changer of Bruker allows to easily program multiple acquisitions at the user's wish.

Experimental parameters

All NMR spectra were recorded at 298 K on Bruker DRX 500 equipped with a 5-mm DCH ^{13}C–$^{1}H/D$ cryogenic probe equipped with a *z*-gradient coil. All spectra for quantification were recorded using Bruker *baseopt* digitization mode to ensure optimal baseline and calibrated using the H-C(1) chemical shift at 5.08 ppm as an internal reference. The concentration profiles of the sample were built by conversion of absolute integral values to concentrations. Solution samples with known concentration were used as reference for determination of the calibration curve.

Locking and shimming were not affected by the presence of the gel and were obtained as for normal D_2O samples. Note that spectra recorded using slice-selective experiments show slightly better resolution than normal spectra because the field inhomogeneities along the z axis are not affecting the spectra of narrow slices. If the pH of the titrant is expected to differ from the one of the main solution, the pH-sensitivity of D_2O may impose to record spectra without lock or to introduce a capillary with a distinct reference solvent (such as acetone-d_6) to lock and shim.

Note that it should be technically possible to adapt the automatic shimming to take into account variation of pH, but such a feature is still to be developed.

All slice-selective ^{1}H experiments were performed at 500.13 MHz with a pulse-field gradient (PFG) strength of 17.62 G/cm. The selective excitation was performed using a G4 Gauss-Cascade[1] pulse with duration of 1.0445 ms. Combined with the PFG, its bandwidth of 7'500 Hz produced 1 mm slices. Frequency offsets for these pulses were systematically changed from 45'012 Hz to -45'012 Hz with a step of 7'502 Hz, resulting in 13 adjacent slices covering a total spectral width of 97'526 Hz.

Signal quantification

In order to avoid unnecessary loss of signal intensity (and hence integration precision) near the edges of the NMR coil, only the central part of the active volume was used for quantification and the integrals were rescaled according to the gradient profile of the probehead. The profile obtained using 600 µl solution sample revealed that the central region between -50 and 50 kHz exhibits a uniform response covering *ca.* 1.3 cm.

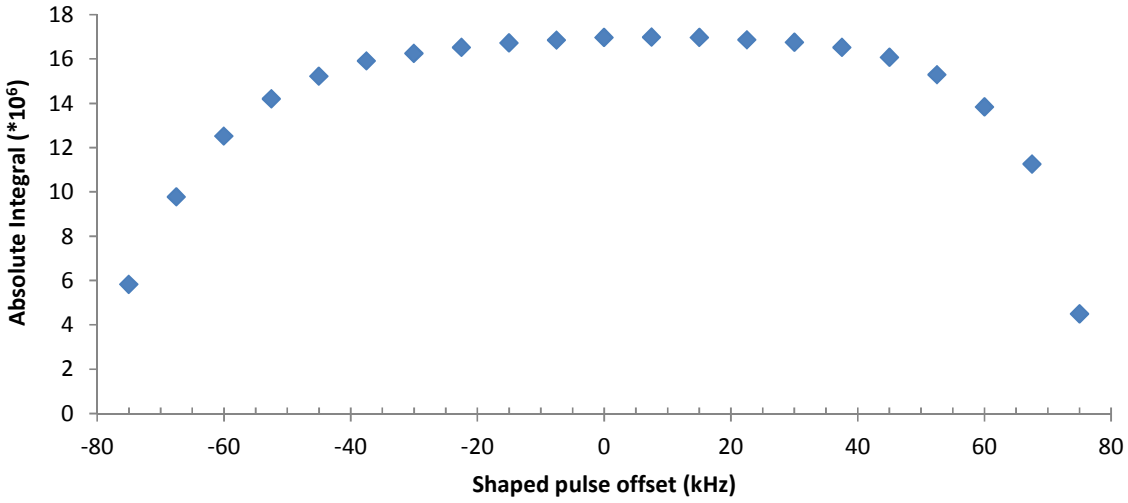

Fig. S1. Intensity profile obtained by slice-selective excitation. The acquisition parameters were identical to the ones described in the experimental section.

Under the typical titration conditions, slice selective experiments with 8 scans on 400 µl 7 mM β-cyclodextrin (CD), titrated with 200 µl 70 mM paracetamol showed a typical signal-to-noise ratio (SINO) of 400:1 for CD and 100-2500:1 for paracetamol, depending on the concentration in the slice and the signal used for integration. In principle, it is possible to analyze the whole active volume (about 2 cm), but more scans (and hence longer experimental times) will be required to achieve reasonable signal-to-noise ratio at the edges of the coil and would

complicate the analysis of the data. The advantage would be to extend the domain of concentration obtained in a single experiment. The concentration profiles of the samples were built after conversion of the absolute integral values in concentrations using homogeneous solution samples with known concentrations as reference (*Fig. S2*)

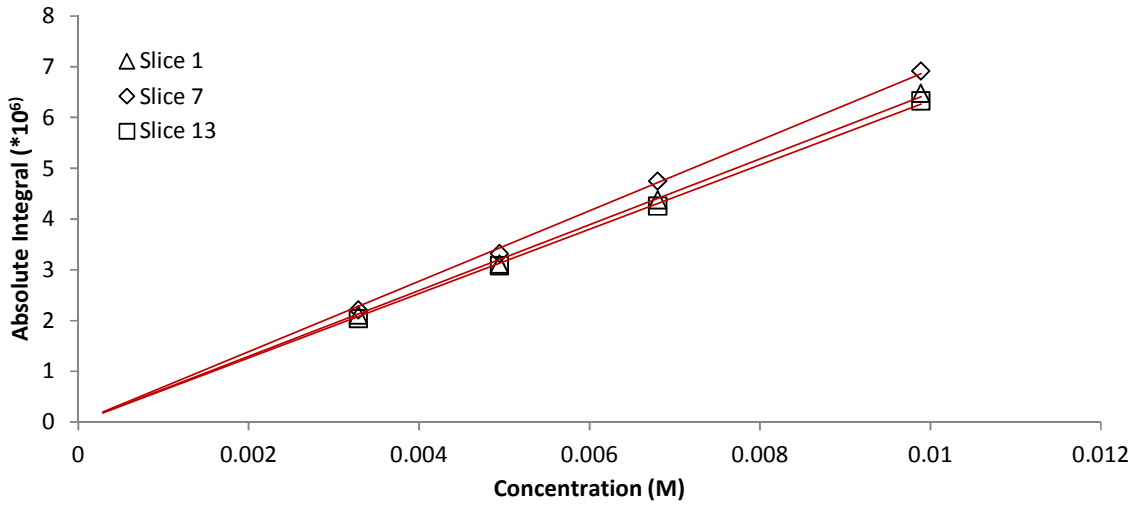

Fig. S2. Typical calibration curves for conversion of absolute integral to concentration. Separate curve was built for each slice and linearly fitted through zero to give the calibration constant as the slope of the line.

Quantification with interleaved acquisition

The presence of only one selective pulse in the sequence offers the possibility to record the slice-selective experiments in an interleaved manner, effectively circumventing the usual requirement to use long relaxation delays to achieve reliable quantification. This was obtained using a Bruker "AU" program executing the series of experiment so that the time between two consecutive scans for a given slice is used to record the spectrum from the other parts of the sample. Besides the obvious advantage of significant reduction of the experimental time, the interleaving also ensures that any change in the system during the NMR experiment is properly averaged. This allows one to consider all slices in the sample as being recorded at the same time.

The claim of fast and accurate integration using interleaved acquisition was verified (see Figure S3 and Table S1) by comparing the data obtained using the normal mode of acquisition with recovery delays of 20 s (> 5 T_1 determined by inversion-recovery experiment) with data obtained using interleaved experiments with 2 s acquisition time and 0.1 s recovery delays.

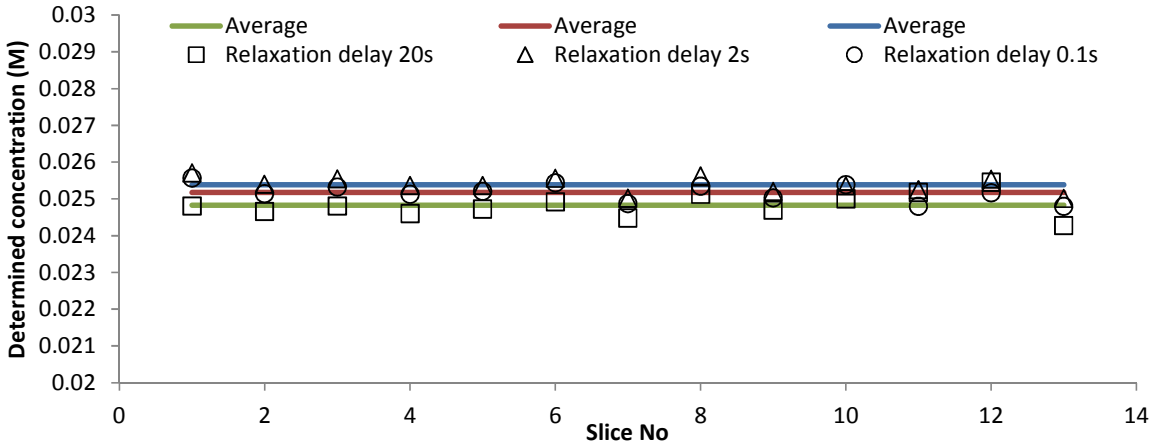

Fig. S3. Comparison of the quantification obtained using normal and interleaved modes of acquisition for a sample of 25 mM paracetamol in D_2O recorded with 8 scans per slice.

Table S1. Relaxation delays, overall experimental time and determined concentration for normal and interleaved slice selective quantification.

Relaxation delay (s)	Total experimental time (min)	Determined concentration (mM)
20	40	24.83 ± 0.31
2	9	25.38 ± 0.22
0.1	6	25.17 ± 0.24

The total experimental time could be further reduced by decreasing the acquisition time (duration of the recorded FID), which was 3.2 s in all case and was the main contribution to the overall experimental time in the case of interleaved acquisition.

Gel titration performance

The gel system was found to exhibit excellent mechanical stability over time, allowing the NMR tubes to be kept outside the magnet during the concentration gradient development. The presence of the gel matrix does not introduce any changes in chemical shift or in line width (Figure S4), again confirming that any interactions between the agar and the solutes are negligible. The signals from the gel were very broad and could not significantly influence signal integration of CD. Would this be a concern, quantification of signals based on lineshape deconvolution would eliminate broad signals and other baseline offset.

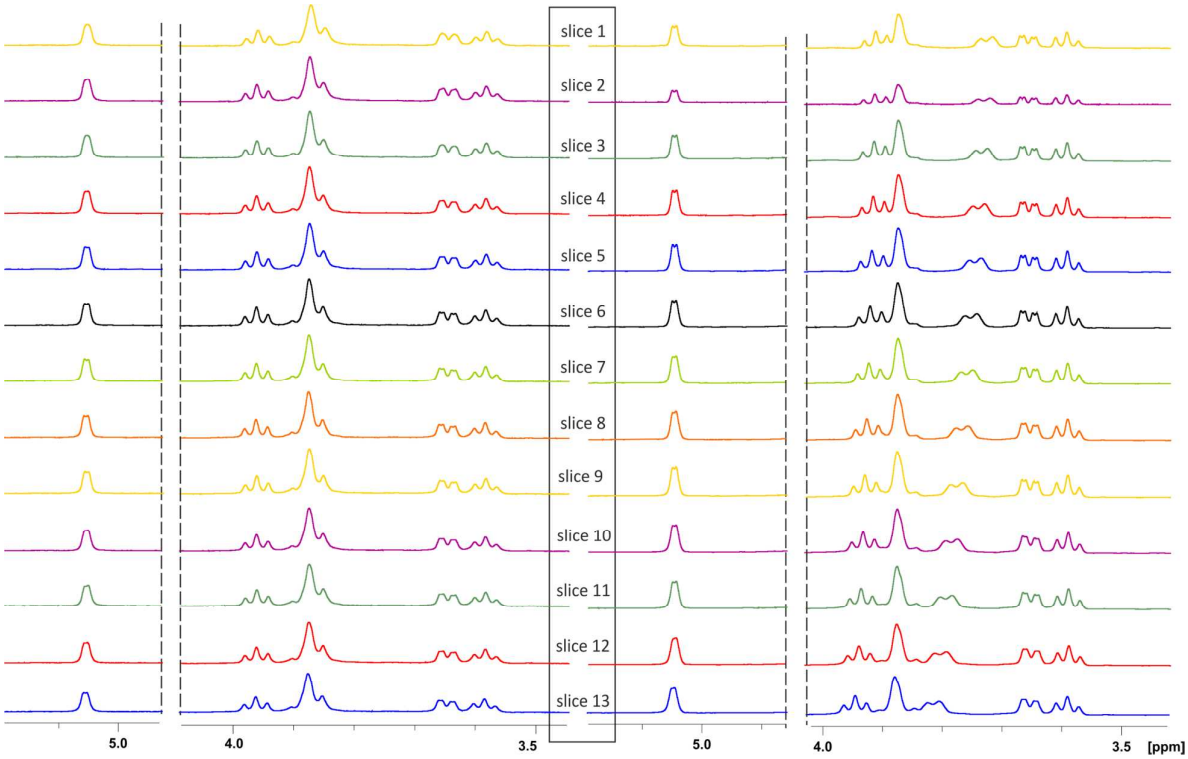

Fig. S4. Cyclodextrin region of slice-selective titration spectra obtained before addition of the titrant (left) and after 24 hours (right). The spectra were recorded in interleaved mode of acquisition for total experimental time of 6 min.

For calculation of the association constant the chemical shifts of H-C(3) and H-C(5), located inside the cyclodextrin cavity, were plotted against the paracetamol/cyclodextrin ratio, as determined by integration (Figure S5). The consistency of the titration data, obtained at different times demonstrates the excellent mechanical stability of the gel and allows also to combine data from different measurements and to use them, if needed, in the association constant determination.

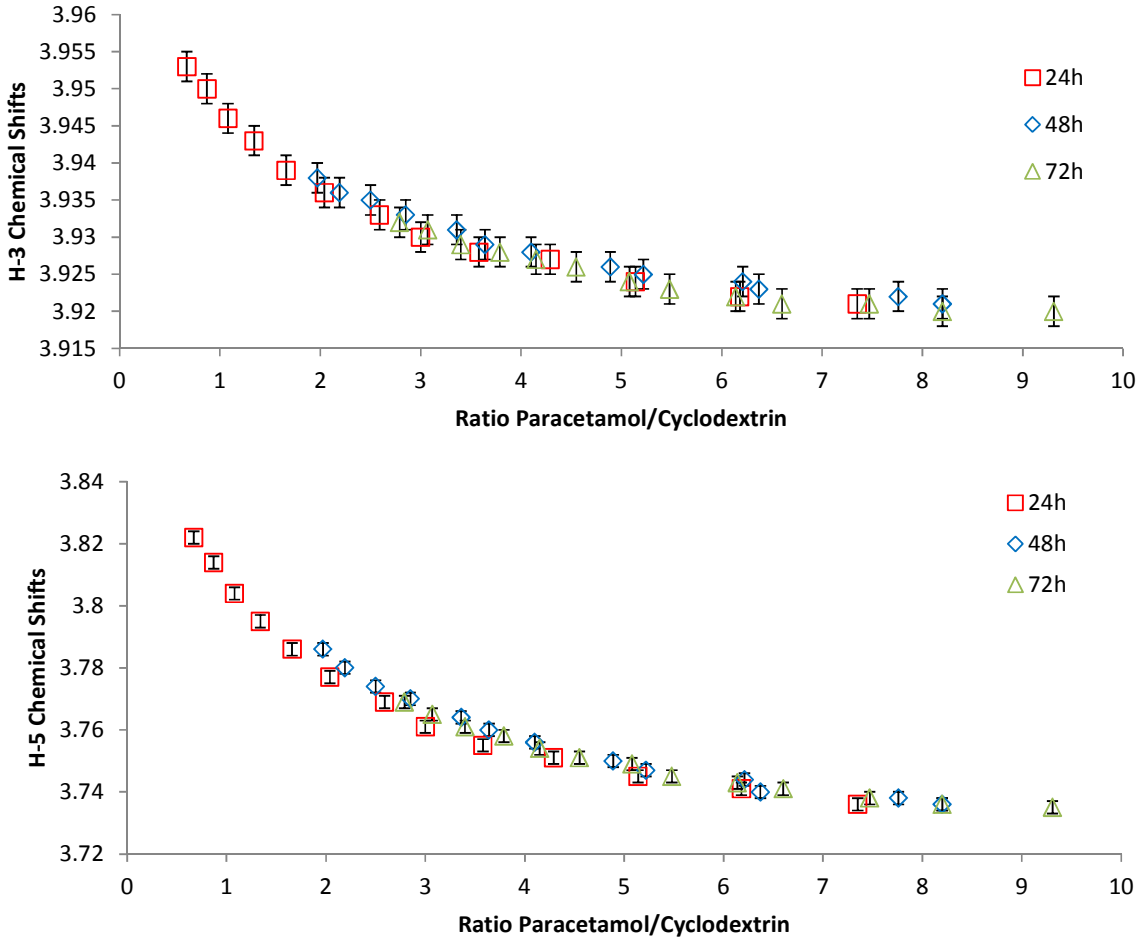

Fig. S5. Titration curves for H-C(3) and H-C(5), obtained after 24, 48 and 72 hours. The concentration gradient was developed at room temperature outside the magnet and the sample was inserted prior to the measurements. Note the excellent agreement between the titration curves, proving the stability of the gel system. The error bars represent 0.002 ppm (1Hz at 500 MHz).

Despite the large differences in the molecular weights, diffusion of cyclodextrin from the gel in the titrant solution was also observed (Figures S6-S8). To account for the presence of this "back-flow", the concentration of both species were calculated separately for each slice and the optimized values were used in the calculation of the association parameters.

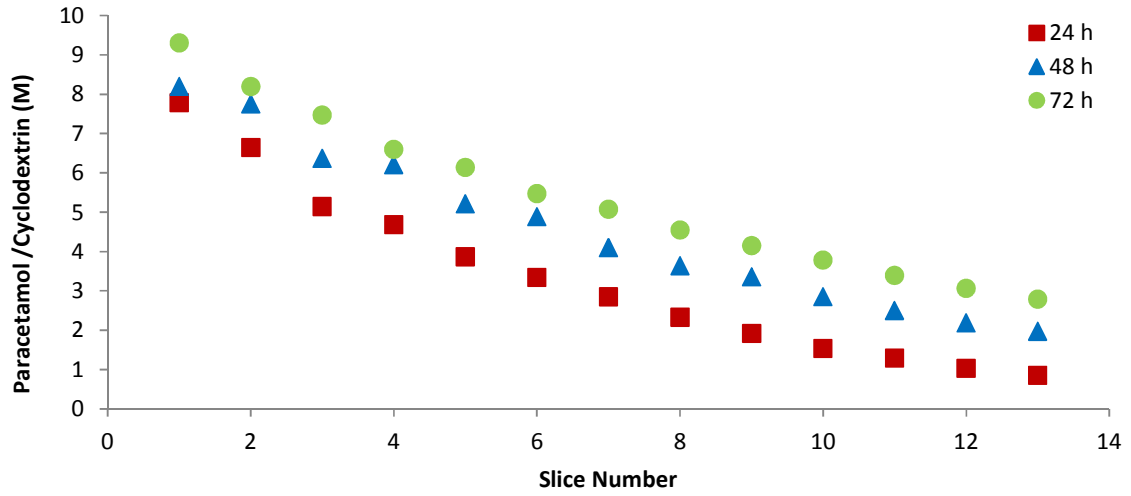

Fig. S6. Paracetamol/cylcodextrin ratios observed after 24, 48 and 72 hours.

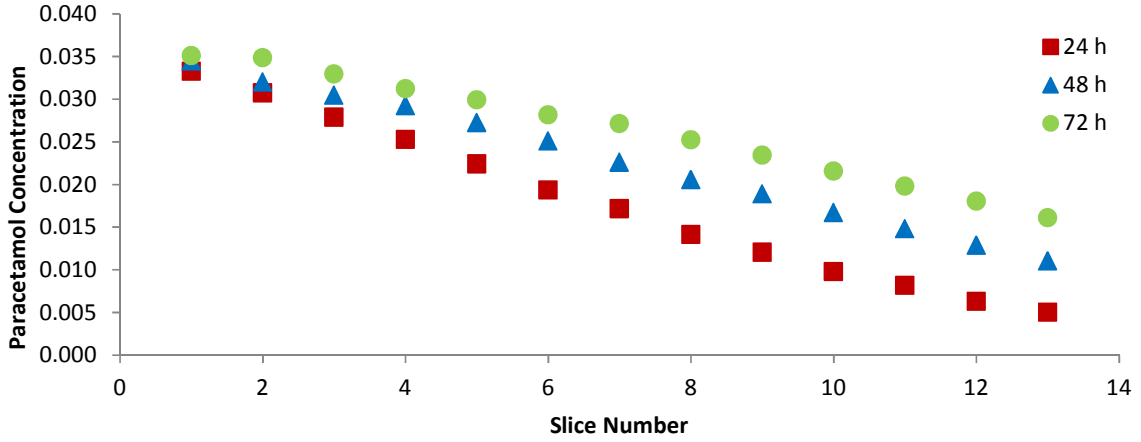

Fig. S7. Paracetamol concentration profiles at different times. Note the constant concentration, equal to half of the initial titrant concentration near the solution-gel interface (slice 1).

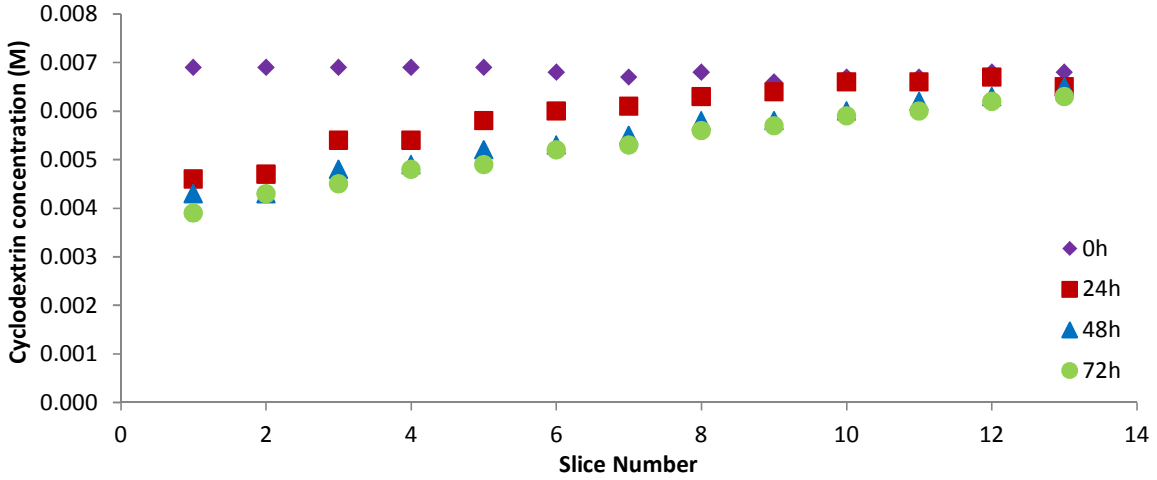

Fig. S8. Cyclodextrin concentration profiles over time, showing the presence of a back-diffusion from the gel to the titrant solution.

The calculation of the association constant and complexation induced shifts was done by nonlinear least-square global fitting according to 1:1 complexation model. [2] The concentration of the complex in each slice was calculated as a function of the association constant (K) and the measured concentrations of paracetamol (G_o) and cyclodextrin (H_o)

$$[C] = \frac{\left(Ho + Go + \frac{1}{K}\right) - \sqrt{(Ho + Go + \frac{1}{K})^2 \; - \; 4HoGo}}{2}$$

The observed chemical shifts (δ_{obs}) were calculated according to the fast exchange condition

$$\delta_{\text{obs}} = F_{\text{free}} * \delta_{\text{free}} + F_{\text{bound}} * \delta_{\text{bound}}$$

where F denotes mole fractions of the free and bound cyclodextrin. The obtained values were fitted to the experimental data by minimizing the sum of squared deviations (ssd).

$$ssd = \sum (\delta_{\text{obs}} - \delta_{\text{calc.}})^2$$

Table S2 summarizes the results from the iterative data fitting. In all cases excellent agreement between the values, obtained by the newly proposed method and other techniques is observed. This is especially true for data, obtained during the first 24 hours, where complexation in the favorable interval between 20 and 80 % is present.[3]

Table S2. Association parameters, obtained by the gel titration method, conventional NMR titration and literature data.

Analytical method	% complexation		Ka (M^{-1})	H-C(5) CIS[a]	H-C(3) CIS[a]	ΔG (kJ/mol)
Gel titration (24h)	0.30	0.84	170	-0.061	-0.167	-12.7
Gel titration (48h)	0.62	0.87	200	-0.062	-0.157	-13.1
Gel titration (72h)	0.60	0.79	107	-0.072	-0.182	-11.6
Gel titration (all data)	0.24	0.79	109	-0.067	-0.182	-11.6
NMR - conventional	0.21	0.82	170	-0.062	-0.159	-12.7
Fluorescence	-	-	184[b]	n.a.	n.a.	-12.7
	-	-	148[c]	n.a.	n.a.	-12.4

[a] CIS – complexation induced shifts, $\Delta\delta = \delta_{bound} - \delta_{free}$. [b] data from reference 4, recorded at 293K. [c] data from reference 5, recorded at 298 K.

Diffusion dynamics

The development of the concentration gradients with time can be described using Fick's second law.[6]

$$C = \frac{Co}{2} * \mathrm{erfc}\left(\frac{X}{\sqrt[2]{4Dt}}\right)$$

where C_o is the initial concentration of the titrant, X is the diffusing distance (mm), D is the diffusion coefficient of the titrant (mm^2/s) and t is the time (s).

Two conclusions, concerning the initial diffusion stages can immediately be drawn on the basis of the equation:

- The concentration at the top of the gel (slice 1) will be constant and equal to half of the initial concentration of the titrant (which was indeedobserved during the titration – Figure S7).

- The concentration profile along the sample does not depend on the volume of the titrant – only on its concentration.

In order to verify the validity of these assumptions, 50 uL of a 20 mM paracetamol solution was layered on the top of 1% agar gel and slice selective spectra were recorded every 3 hours. Figure S9 presents the result from data fitting, using eq. 1 and the literature value for diffusion of paracetamol in D_2O.[6]

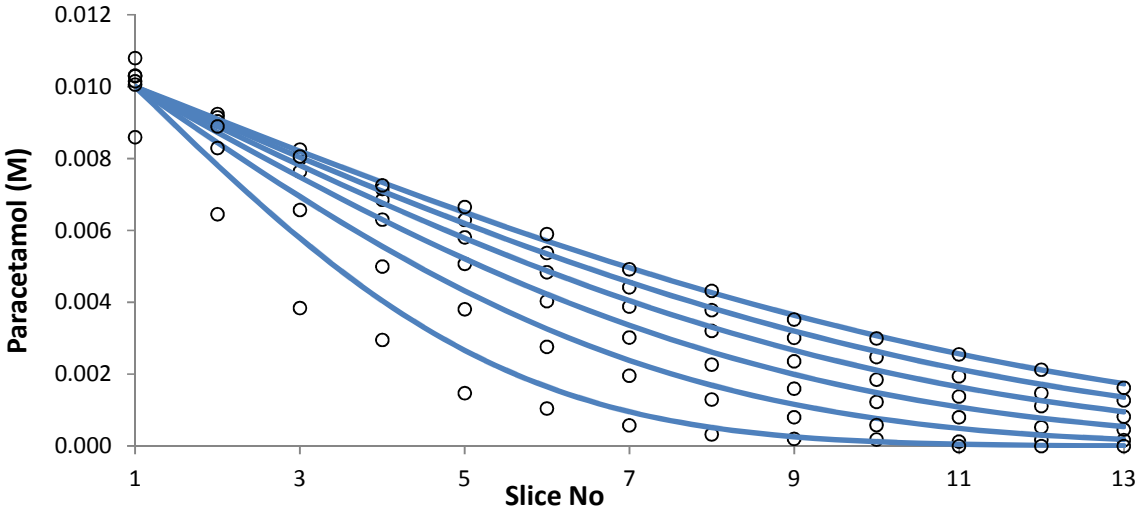

Fig. S9. Calculated (blue line) and experimentally measured concentrations (circles) for the diffusion of paracetamol in 1% agar gel. The diffusion is monitored for 18 hours, with separate curves every 3 hours. The fitting is according to Eq. 1, with C_o = 20 mM, X = 13 mm and D = 0.00062 mm^2/s

Despite the differences between the calculated and observed concentrations in the first 3 hours, good agreement between calculated and measured concentrations was observed, especially at the bottom of the observed volume. This suggests that the model can be used to evaluate the

optimal gradient development time, using solution state diffusion data. However, more experimental data are needed to determine the overall reliability of the model, it has to be used as an estimate, due to the approximated nature of the model itself.

Sensitivity of slice-selective experiments

The sensitivity of slice-selective NMR experiments is proportional to the volume of the selected slice. Each spectrum of a series of 1-mm slices therefore show *ca.* 1/20 of the sensitivity of a spectrum recorded using the classical titration. Narrow slices are desirable because they increase the spatial resolution and limit signal averaging over the concentration gradient (see next paragraph). The lower limit is about 1 mm. Bellow, the imperfections of the gel (small meniscus at the interface with the titrant) make the radial concentration distribution over the selected cylinder dominate the longitudinal distribution.

Signal enlargement due to spectral averaging within the slice thickness

When compared to classical titration, a disadvantage of slice-selective approach is the broadening of signal caused by the spectral averaging over the slice thickness (see Figure S10). When the total shift is up to *ca.* 0.1 ppm (or 50 Hz at 500 MHz), this broadening is negligible, but for shift > 0.5 ppm, signals broaden significantly and may be difficult to observe. Note that when determining affinity constants, the lowest precision in the chemical shifts of broad signals is compensated by the advantage of a large total shift. Difficulties should therefore only occur when signals become so broad that they cannot be observed. A solution to the problems of large changes in chemical shifts consists in recording two additional sets of slice-selective spectra. The first should be recorded shortly after the addition of the titrant so that its concentration is low and the changes in chemical shifts of sensitive protons are still limited. The second set is acquired after appropriate time when the concentration along the NMR tube is not varying significantly.

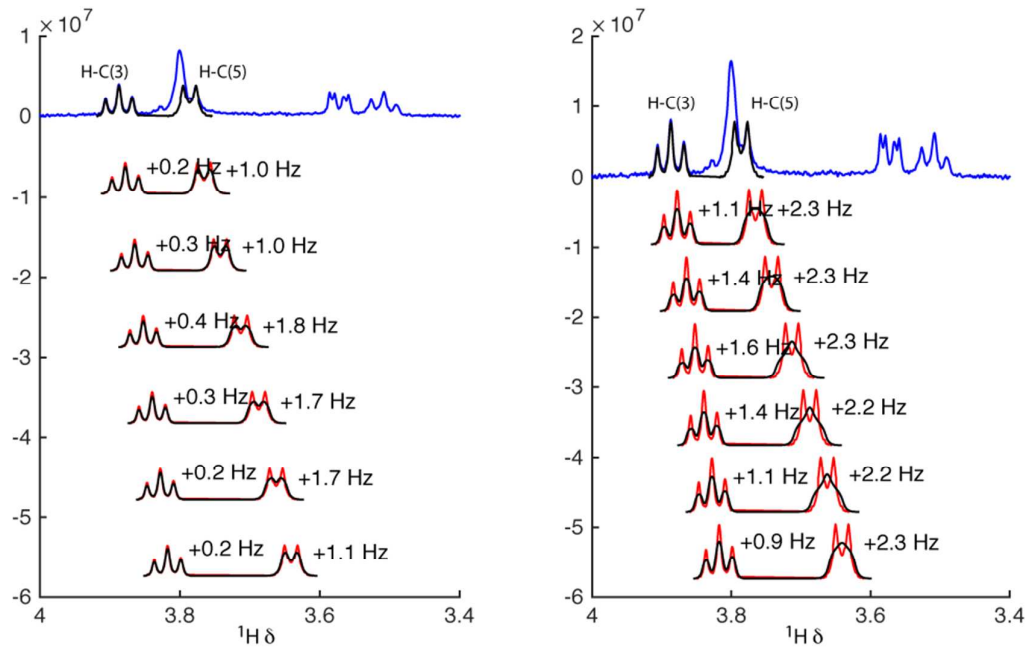

Fig. S10. Simulations of the signal shapes of H-C(3) and H-C(5) of cyclodextrin in classical multiple-tubes titration with paracetamol (red) and taking into account the spectral averaging observed in slice-selective experiments through the agar concentration gradient (black). Slice thickness of 1 mm (left) and 2 mm (right) were considered. The signal amplitudes of normal signals (in red) were scaled down to facilitate the comparison of the line shapes. Only spectra 1, 3, 5, 7, 9, 11 and 13 were shown for the 1 mm series. The chemical shifts used for the simulations originate from the data of Fig. S11. The signal enlargements (black v/s red) were measured at half-height of the respective multiplets.

Analysis in the presence of signal overlap

When the shifting signals under investigation overlap with the fixed signals of inactive compounds, subtracting the spectra of two consecutive slices cancels the latter and the symmetry properties of the result of the subtracted signals allows, in favorable cases, to determine the position of moving signals. This method is illustrated in Figure S11 where chemical shifts obtained from normal spectra ("x") and from the differences between pairs of spectra ("+" in the center of *Fig. S11*) are compared for the titration in the absence (top, black labels) and presence (bottom, green labels) of the numerous signals of maltotriose. In the presence of maltotriose the "difference" method provided quite satisfactory chemical shift data for H-C(3) (see the green "+" in the right subplot of Fig. S11), but its lower reliability is obvious from the missing data and the lower precision of the chemical shifts obtained for H-C(5). Obviously, the determination of the affinity constants using the "difference" method requires an analytical approach for which the quantification of signals is not necessary. (See for example: C. L. Perrin, M. A. Fabian, *Anal.*

Chem., 1996, **68**, 2127–2134 and C. L. Perrin, Y. Dong, *J. Am. Chem. Soc.,* 2007, **129**, 4490–4497.)

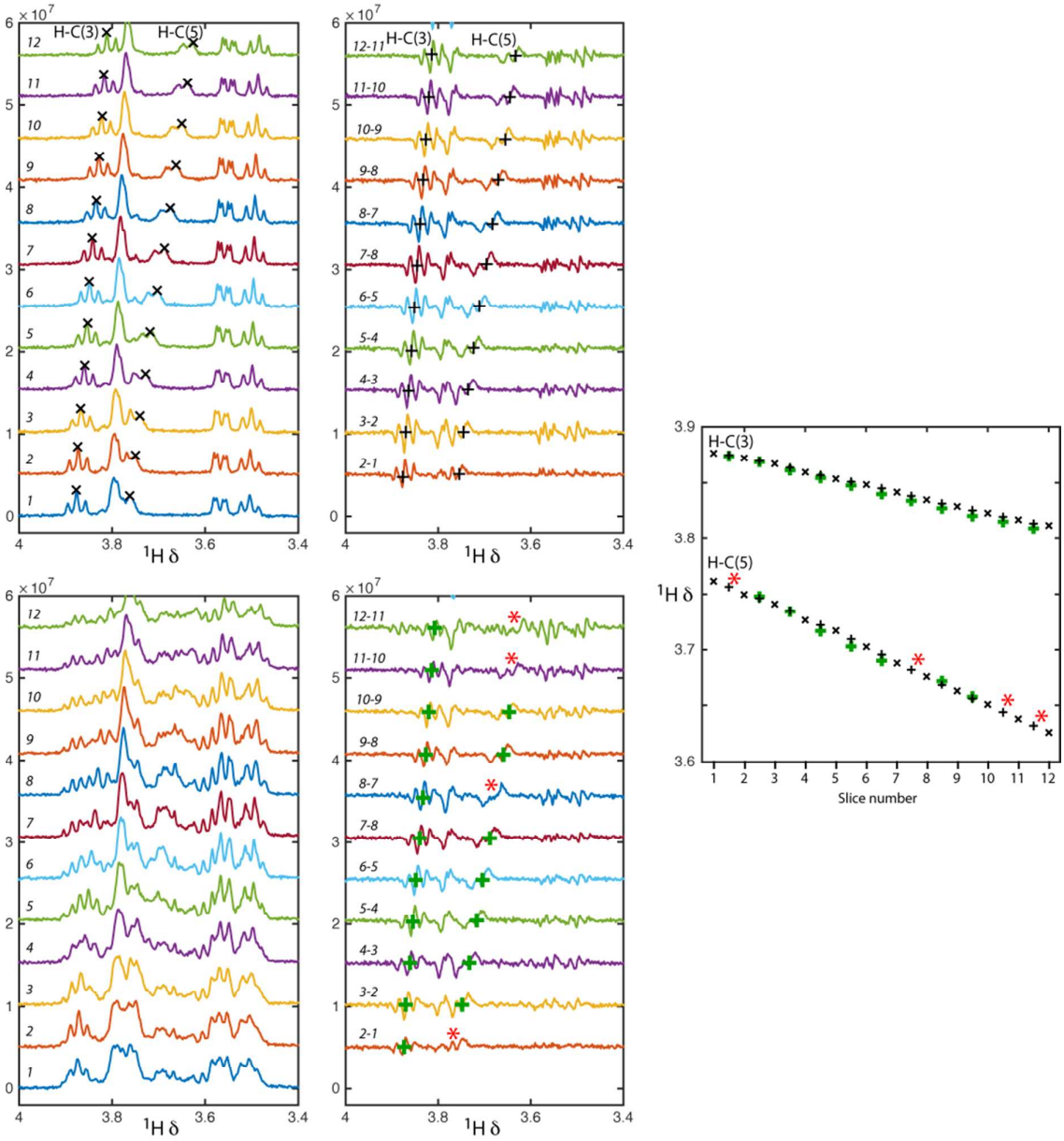

Fig. S11. Comparison of the chemical shifts obtained from the top amplitude of the signals ("x", left) and on the passage through zero of the difference of pairs of consecutive spectra ("+" symbols, center). The red stars indicate missing data. The spectra correspond to the titration of 400 µl of 7 mM CD and the absence (top) and presence (bottom) of 40 mM of maltotriose dissolved in 1% agar gels. The titrant were 200 µl solutions containing 70 mM of paracetamol.

Bruker pulse program for slice-selective excitation

```
;selzg.yavor
;avance-version (02/05/31)
;1D sequence
;slice selective excitation using a shaped pulse and gradient
;original pp: selzg (Bruker), modified by Yavor Mitrev
;$CLASS=HighRes
;$DIM=1D
;$TYPE=
;$SUBTYPE=
;$COMMENT=

#include <Avance.incl>
#include <Grad.incl>

"p17=p11"
"acqt0=-d16-50u"

1 ze
2 30m
  d1
  50u UNBLKGRAD
  4u pl0:f1
  10u
  (center (p11:sp1 ph1:r):f1 (p17:gp0))
  d16
  50u BLKGRAD
  go=2 ph31
  30m mc #0 to 2 F0(zd)
exit

ph1=0 2 2 0 1 3 3 1
ph31=0 2 2 0 1 3 3 1

;pl0 : 120dB
;pl1 : f1 channel - power level for pulse (default)
;sp1: f1 channel - shaped pulse
;p11: f1 channel -  90 (or 270) degree shaped pulse
;d16 : gradient recovery time (200us)
;gp0 : slice selection gradient
;d1 : relaxation delay; 1-5 * T1
;NS: 1 * n, total number of scans: NS * TD0
;GPZ0 : RECT.1
;choose p11 according to desired selectivity
;the flip-angle is determined by the amplitude
;set O1 on resonance on the multiplet to be excited or use spoffs
```

Bruker "au" programs for serial acquisition of slice selective 1D spectra

Non-interleaved version

```
/*      multi_fq.yavor                  10.02.2015              */
/*****************************************************************************/
/*      Short Description :                                     */
/*      Performs multiple slice selective acquisitions on increasing expnos.    */
/*****************************************************************************/
/*      Keywords :                                              */
/*      serial acquisitions                                     */
/*****************************************************************************/
/*      Author : Yavor Mitrev*/
/* This program sets up and runs multiple slice selective experiments with     */
/* fixed increment of the shaped pulse offset in different expno.              */
/* The user is asked to input the number of spectra,                           */
/* for each spectrum and the shaped pulse offset increment                     */
/*The shaped pulse increment value should be negative, when starting from the top slice  */
/*and positive, when starting from the bottom.                                 */


GETCURDATA
GETINT("Enter total number of spectra",i1)
GETINT("Enter the number of scans for each spectrum",i2)
GETINT("Shaped pulse increment (Hz)", f1)
STOREPAR("ns",i2)
Proc_err(0,"multiFq run in progress");
RGA
ZG
TIMES(i1-1)
IEXPNO
SETCURDATA
FETCHPAR("SPOFFS 1",&f2)
STOREPAR("SPOFFS 1",f1+f2)
STOREPAR("ns",i2)
ZG
END
QUITMSG("Data Collection Finished")
```

Interleaved version

```
/*      multi_fq_inter.yavor            10.02.2015              */
/*****************************************************************************/
/*      Short Description :                                     */
/*      Performs interleaved multiple slice selective acquisitions on increasing expnos.    */
/*****************************************************************************/
/*      Keywords :                                              */
/*      serial acquisitions, fast aquisition,                   */
/*****************************************************************************/
/*      Author : Yavor Mitrev*/
/* This program sets up and runs multiple slice selective experiments in interleaved        */
/* mode of acquisition with fixed increment of the shaped pulse offset in different expno.*/
/* The user is asked to input the number of spectra, the number                              */
/* of scans for each spectrum and the shaped pulse offset increment                          */
/* The shaped pulse increment value should be negative, when starting from the top slice */
/* and positive, when starting from the bottom.                                              */
i3=expno;
/*prompts for user input*/
GETCURDATA
GETINT("Enter total number of spectra",i1)
GETINT("Enter the number of scans for each spectrum",i2)
GETINT("Shaped pulse increment (Hz)", f1)
/*start of the first loop - datasets creation and acquisition of the first scan*/
Proc_err(0,"multiFq run in progress");
STOREPAR("ns",1)
ZG
TIMES(i1-1)
IEXPNO
SETCURDATA
FETCHPAR("SPOFFS 1",&f2)
STOREPAR("SPOFFS 1",f1+f2)
ZG
END
/*start of the second loop - accumulation of scans*/
STOREPAR("DS",0)
TIMES(i2-1)
REXPNO(i3)
SETCURDATA
GO
TIMES2(i1-1)
IEXPNO
SETCURDATA
GO
END
END
STOREPAR("ns",i2)
QUITMSG("Data Collection Finished")
```

References

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