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Development of a dual cell, flow-injection sample holder, and NMR probe for comparative ligand-binding studies ☆

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Abstract

NMR based ligand screening is becoming increasingly important for the very early stages of drug discovery. We have proposed a method that makes highly efficient use of a single sample of a scarce target, or one with poor or limited solubility, to screen an entire compound library. This comparative method is based on immobilizing the target for the screening procedure. In order to support the method, a dual cell, flow injection probe with a single receiver coil has been constructed. The flow injection probe has been mated to a single high performance pump and sample handling system to enable the automated analysis of large numbers of compound mixes for binding to the target. The probe, having an 8 mm $^{1}H/^{2}H$ dual tuned coil and triple axis gradients, is easily shimmed and yields NMR spectra of comparable quality to a standard 5 mm high-resolution probe. The lineshape in the presence of a solid support is identical to that in glass NMR tubes in a 5 mm probe. Control spectra of each cell are identical and well separated, while ligand binding in a complex mixture can be readily detected in 20–30 min, thus paving the way for use of the probe for actual drug discovery efforts.

Keywords: Fragment based drug discovery; Ligand screening; TINS; Flow-injection NMR probe

1. Introduction

Biophysical methods such as NMR, mass spectroscopy and surface plasmon resonance, are playing an increasingly important role in all aspects of drug discovery from pre-clinical stages to clinical trials [1]. Much of this success has been driven by advances in hardware that have allowed increased sensitivity and throughput. One particular area, the hit to lead stage of drug discovery, is witnessing substantial growth. Biophysical techniques, sensitive to even weak intermolecular interactions, provide an alternative to high throughput screening which has well documented limitations. In this area NMR plays a particularly important role. Originally described as SAR by NMR [2], screening for simple, low affinity ligands as starting points for drug discovery is now recognised as the "fragment based approach" (FBA). The FBA has been successfully employed to generate small molecule inhibitors of pharmaceutical targets that have proven intractable for so-called, high throughput screening (HTS) approaches. These successes include developing new chemotypes for targets that yielded limited or no chemistry from HTS [3], triaging hits from HTS campaigns to remove artefacts [4] and *de novo* generation of small molecule inhibitors of protein–protein interactions [5]. Further, the FBA is generating excitement due to its intrinsic ability to find ligands that lie well within the limits of Lipinski's "rule of fives" [6]. Thus in principle, the FBA should deliver compounds with higher oral

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bioavailability and lower toxicity, thereby increasing the likelihood of successful completion of the clinical trials stage of drug development.

In addition to SAR by NMR, which focuses on changes in the spectrum of the target upon ligand binding, a number of other approaches have been developed to screen for fragments that bind a pharmacological target. These methods, which focus on changes in the NMR spectrum of the compounds to be screened (''small molecule methods''), include DOSY [7] and linebroadening techniques [8], as well as observation of intermolecular magnetisation transfer by NOEs [9] or by saturation transfer difference spectroscopy (STD, [10]). Despite efficient use of the target by the small molecule methods, they still require significant quantities (10's of mg) of a soluble target. We have developed a method called TINS, for Target Immobilized NMR Screening, that has the potential for higher throughput while using less target than other compound based screening methodologies [11]. Further, TINS may allow the application of the FBA to proteins that aggregate or are unstable or potentially even membrane proteins, an important class of pharmaceutical targets that have proven particularly challenging. TINS is a comparative methodology where the target to be screened is immobilized on a solid support that is compatible with high-resolution, non-spinning NMR spectroscopy. In order to detect binding, a 1D ^{1}H spectrum of the dissolved compounds acquired in the presence of the immobilized target, is compared to a spectrum recorded in the presence of a reference sample which may have an empty solid support or more advantageously, a solid support on which a reference protein or a mutated version of the target or a protein closely related to the target has been immobilized. The feasibility of TINS was well documented for a number of proteins and nucleic acids in batch mode i.e., where the reference and experimental samples were in separate 5 mm NMR tubes [11]. In particular it was shown that a single sample of the target could be used to screen an entire fragment library of at least 2000 compounds. However, to implement TINS for drug discovery, it is necessary to have a flow-injection probe where both of the samples can be held simultaneously in the magnet in order to minimize differences due to artefacts, maximize the throughput and best take advantage of the immobilized target.

In recent years there has been a significant effort to increase the throughput of NMR spectroscopy. In addition to flow-injection and cryoprobes, a number of research groups have demonstrated the feasibility of multi-sample probes [12–16]. Ross and co-workers demonstrated an arrangement of eight capillary tubes inside a standard 5 mm NMR tube [17]. In this work chemical shift imaging [18] was employed to simultaneously record separate spectra of each sample. More recently the group of Webb have constructed an eight sample probe with eight independent receiver coils [19] allowing independent acquisition of NMR spectra of each sample using standard high-resolution pulse programs. However, neither of these hardware arrangements used flow-injection to load samples (although in principle the hardware of Webb could be adapted to flow-injection). The group of Raftery has demonstrated a four sample, four coil, flow-injection high-throughput NMR spectrometer [20]. While this impressive achievement allows very high throughput, the active volumes in the receiver coils are small requiring highly concentrated samples. Other applications of multi-sample, multicoil probes include their use as a detector for fast chemical reactions [21], to reduce line-broadening when coupled to electrophoretic separations [22] or for solvent suppression techniques [23]. Moreover, recently micro-imaging applications using multiple detection coils [24–27] have been reported, as well as applications to achieve time reduction for two-dimensional data acquisition [28].

To best achieve the goal of a reliable instrument capable of analysing hundreds of mixes of compounds for ligand binding to an immobilized target in a fully automated manner, we set a number of design principles. First, we desired a flow-injection probe to capitalize on the fact that the target and reference samples are immobilized on a solid support enabling high flow rates. This would allow an experimental setup in which one mixture of compounds could be applied to each sample on the solid support in a parallel flow. Once fully equilibrated, flow would be stopped and the appropriate NMR experiment recorded, whereupon the mixture of compounds would be washed out and the next mix applied. Second, in order to obtain maximal sensitivity, the largest possible active volume was needed. Since it was clear that triple axis gradients would be necessary to experimentally separate the NMR spectra of each sample and since we desired to use as much ''off the shelf'' hardware as possible, the practical limit for the coil was an 8 mm design. Third, we choose a single coil design to minimize spectroscopic differences between the two samples. In TINS, it is extremely important to have minimal signal contamination from one sample in the second and a single coil design eliminates coupling artefacts. Finally, the flow path should be as smooth as possible so that a minimal volume would be needed to equilibrate and wash the mixes.

Here, we present a description of the hardware that has been developed to meet these requirements and initial data demonstrating the level of its performance.

2. Results and discussion

2.1. NMR performance of the probe

A probe was developed and designed to be used with the TINS method but that would also exhibit features typical of high-resolution NMR. The probe was built for a 500 MHz spectrometer as an 8 mm selective-probe with one RF coil. The coil is double tuned for proton (^{1}H) observation and decoupling with a deuterium (^{2}H) lock channel. The probe is optimised for an 8 mm sample using a saddle coil geometry to take advantage of the vertical

cryomagnet for inserting and ejecting the sample. As designed, the probe has a Q factor of 247 on the ^{1}H channel. Although the RF circuitry is chosen equivalently to high-resolution NMR probe construction principles, special care was taken in selecting low-susceptibility and/or susceptibility compensated materials, particularly for the coil itself, as well as material close by. Thus, the shim properties of the probe are similar to those of a 500 MHz high-resolution probe. The probe is optimized for nitrogen atmosphere for the cooling-gas and has triple axis, *XYZ*-gradients. The NMR performance of the probe was initially assessed using a suite of tests with standard samples in an 8 mm sample tube (Fig. 1). The lineshape test (Fig. 1A) suggests that the overall performance of the 8 mm probe is very comparable to a high-resolution 5 mm probe and, as expected, the sensitivity is considerably better (Fig. 1B). Finally, the water suppression test, which is a very sensitive measure of the real world performance of a probe, particularly for biological samples, gave excellent results (Fig. 1C).

2.2. Fluid flow path

We chose to use a single pump that was compatible with the available controlling software (HyStar, version 3.1). This decision dictated the rest of the flow path (see Fig. 2A). An LC22 HPLC pump (with LC225 gradient former) was used that could operate comfortably at the moderate flow rate (up to 2 ml/min) and backpressure intended (60–90 bar). A simple flow splitter was placed downstream of the HPLC pump. In order to minimize potential differences in flow over the two cells of the sample holder, we used PEEK tubing with an inner diameter of 90 μm. At a flow rate of 0.5 ml/min this tubing generates about 59 bar of back pressure while the sample holder filled with resin generates less than 1 bar. In this way, even if one of the cells is poorly packed resulting in a twofold increase in back pressure, there is less than 2% difference in flow over the cells. Given the width of the peak of mixtures flowing through the system, this difference is easily accommodated, thereby ensuring that each cell is optimally equilibrated for every experiment. We placed two sample loops of 400 μl each after the flow splitter. The loops are controlled by a 10 position, two-way valve that alternately places the loops in serial or parallel flow configuration. When configured for serial flow, the loops are overfilled using 0.95 ml of the compound mixture using a standard BEST liquid handler (Gilson 215 autosampler). Automated valve switching returns the loops to parallel flow for sample injection. Timing of the delivery of the sample to the cells is easily calibrated by following the amplitude of e.g., the water signal in each cell using a 1D imaging type experiment. Once the sample has been delivered, flow is halted and the NMR data are acquired (see below). Finally the sample is washed with 900 μl of buffer per cell (4.5 × the cell volume). In contrast to typical flow-injection applications, it is very important to keep air out of the system since this could lead to various problems with the resin in the cells including cracking or increased susceptibility mismatch leading to line broadening. Once the wash is complete the cells are then ready to repeat the cycle. The entire process is under control of HyStar software which can interface with ICON NMR (TOPSPIN v.1.3) to automate the complete process of sample injection and NMR acquisition.

The heart of the system is formed by the dual-cell sample holder. A number of points had to be considered when designing the sample holder. The geometry of the cells should not generate unduly broad resonances while at the same time maximizing the volume of each sample. The geometry of the cells should also contribute to minimal signal cross-talk using only standard triple axis gradient hardware (50 G/cm). Finally, the sample holder must be open able in order to fill/empty the cells with the solid support. While glass is the ideal material for a flow cell, in the case where the cell needed to be opened and closed this was not practical. Instead we machined a cylinder 8 mm in diameter out of KelF (see Fig. 2B). Two cylindrical holes of 3.2 mm were then bored out to form the individual cells that hold the solid support. Each cell has volume of approximately 190 μl of which 135 μl are within the coil when placed inside the probe (the active volume). To insure smooth fluid flow, the cells taper towards the end which is formed by a porous frit designed to retain beads of at least 50 μm in diameter. The sample holder uses standard threads for a 1/32″ headless PEEK fittings and tubing (Upchurch). A similar arrangement forms a cap for the sample holder that can be screwed in place for flow-injection and removed to fill the cells. To aid in filling the cells with the solid support, we designed a reservoir that mounts onto the sample holder in place of the cap and provides 120% of the volume of each cell. A 50% slurry of the solid support is easily pipetted into the sample holder/reservoir combination. After settling, the reservoir can be attached to an HPLC pump and the resin in each cell simultaneously packed under pressure using a flow splitter.

2.3. Characterization of the fluid flow

Using the hardware described above, a sample holder can be routinely packed in about 1.5 h. It is of course extremely important that both the volume of packed material and the back pressure of each cell is the same in order to minimize artefacts during ligand screening. Back pressure differences are easily detected by simply observing the pressure generated when flow is directed through one or the other of the two cells. In the more than ten different sample holders that have been packed, pressure differences between the two cells have been typically 0.1–0.2 bar (not shown). This observation is further supported by careful measurement of the volume of parallel flow (using a flow splitter) through each cell in a defined time period, which is typically less than a 1% difference. The volume of solid support in each cell is readily detected using NMR.

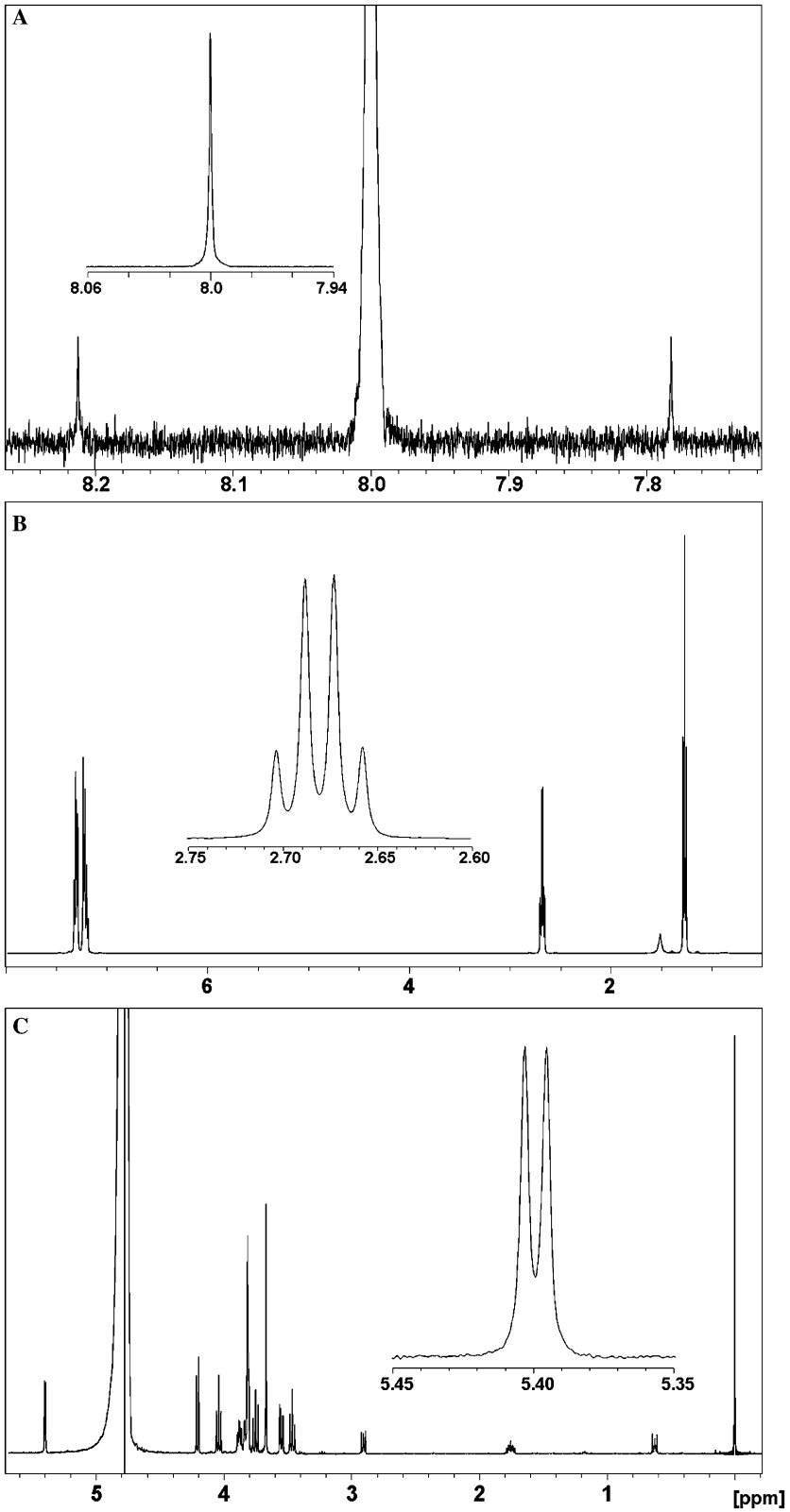


Fig. 1. Characterisation of the NMR performance of the 8 mm probe. Standard high-resolution test samples were used to determine the linewidth and sensitivity of the probe. (A) Lineshape test with 1% $CHCl_3$ in d_6-acetone. Lineshape: 5.4/14.1 Hz; resolution: 0.4 Hz; ^{1}H-pulse: 8.5 μs (+2 dB). (B) Sensitivity test with 0.1% ethyl benzene in $CDCl_3$: S/N = 1205:1 (noise = 200 Hz). (C) Water suppression with 2 mM sucrose and 0.5 mM DSS in 10% D_2O and 90% H_2O: hump: 55.2/81.1 Hz; resolution: 20%; sensitivity: S/N = 252:1.

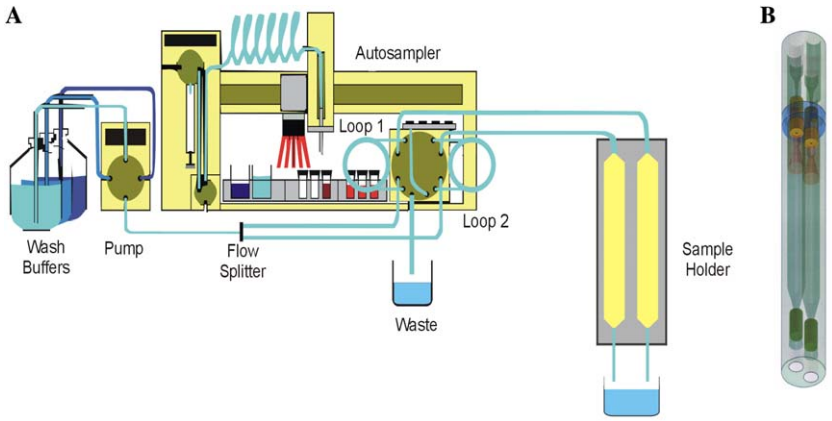


Fig. 2. Liquid path for the TINS ligand screening apparatus. (A) Fluid flow diagram. The automation system includes an HPLC pump from which the flow is split in two. An autosampler loads the mixes of compounds to be tested for binding into two loops that alternate between serial and parallel flow via a 10 position, 2 way valve. Each mix of compounds to be tested for binding is injected in serial into the two loops. The valve is then switched and the contents of each loop are injected in parallel into the dual-cell sample holder, which is placed in the NMR probe for the duration of the screening process. (B) Schematic view of the dual-cell sample holder. See text for details.

The cells are first equilibrated with a biocompatible buffer such as phosphate buffered saline (PBS) in H_2O. Then a 1D imaging experiment is recorded to separate the ^1H signal in each cell. Integration of the two peaks generally indicates a signal difference of at most 1–2%, which is well within the noise regime of the system (Fig. 3A). If the hardware is to be capable of screening hundreds of mixtures of compounds for ligands, it is vital that the back pressure remains stable and that system is not prone to clogging. Fig. 3B presents pressure profiles from two different mixture applications to the sample holder. Trace A is from the first application and trace B is the 17th application of mixes from 3 to 10 compounds derived from our compound library. While this is still a limited number of applications, the very good stability of the back pressure in combination with the apparent effectiveness of the washing step (see below), suggests that the system will be capable of handling large numbers of compounds without clogging.

2.4. NMR characterization of the probe/sample holder in the presence of solid support

In order to test the NMR performance of the probe/sample holder combination under the conditions used for ligand screening we packed each cell with a solid support (Actigel ALD, Sterogene, USA) to which no target molecule had been immobilized. Both cells were equilibrated with a mixture of eight compounds at 0.5 mM each in D_2O and a 1D, non-spatially selective ^1H spectrum was acquired (Fig. 4A). Despite the use of deuterated solvent, the residual water signal still requires suppression in order to maximize the sensitivity of the experiment. Due to the inhomogeneity induced broadening of resonances in the heterogeneous system, simple presaturation is insufficient for solvent suppression. Therefore, the residual water resonance was suppressed using the WATERGATE method [29]. The linewidths of the resonances of the compounds in the presence of this solid support in the dual-cell sample holder are identical to those of a similar sample measured in a 5 mm glass NMR tube (about 27 Hz). The high quality of the NMR spectrum recorded in the dual-cell sample holder should allow for reliable detection of ligand binding even in relatively complex mixtures (see below). Broad residual signals from the solid support can also be seen in the spectrum in Fig. 4A at around 3.7 ppm from the sugar ^1H's of the Sepharose and upfield around 1 ppm. Use of a simple T2 filter effectively removes these signals from the spectrum (see below).

Since one of the main goals of having both samples in the magnet at the same time was to minimize artificial differences between them, we sought to determine whether the hardware described above actually accomplished this goal. The two cells were simultaneously equilibrated with a solution of phosphotyrosine and arginine at 0.5 mM and a 2D chemical shift imaging (CSI, [17]) experiment was used to record independent spectra from each cell (Fig. 4B and C). As can be seen by the projection along the vertical spatial axis, the signal from the two cells is reasonably well resolved using this experiment. The two overlaid 1D spectra result from adding the slices in the two different cells. Clearly the spectra are highly similar with the only differences resulting from noise. The difference spectrum highlights the similarity and suggests that the goal of achieving identical conditions in the two cells has been met. Note that the peaks derived from the resin have been strongly attenuated by incorporation of a CPMG T2 filter (total duration ~20 ms) while the residual H_2O signal has been mostly suppressed using WATERGATE. To test for contamination of compounds from one cycle to the next, each cell was equilibrated with the 8 compound mix described in 4A and subsequently washed with 900 μl of PBS buffer. Flow was then stopped and the CSI experiment was recorded for 5.3 h to detect any remaining compound.

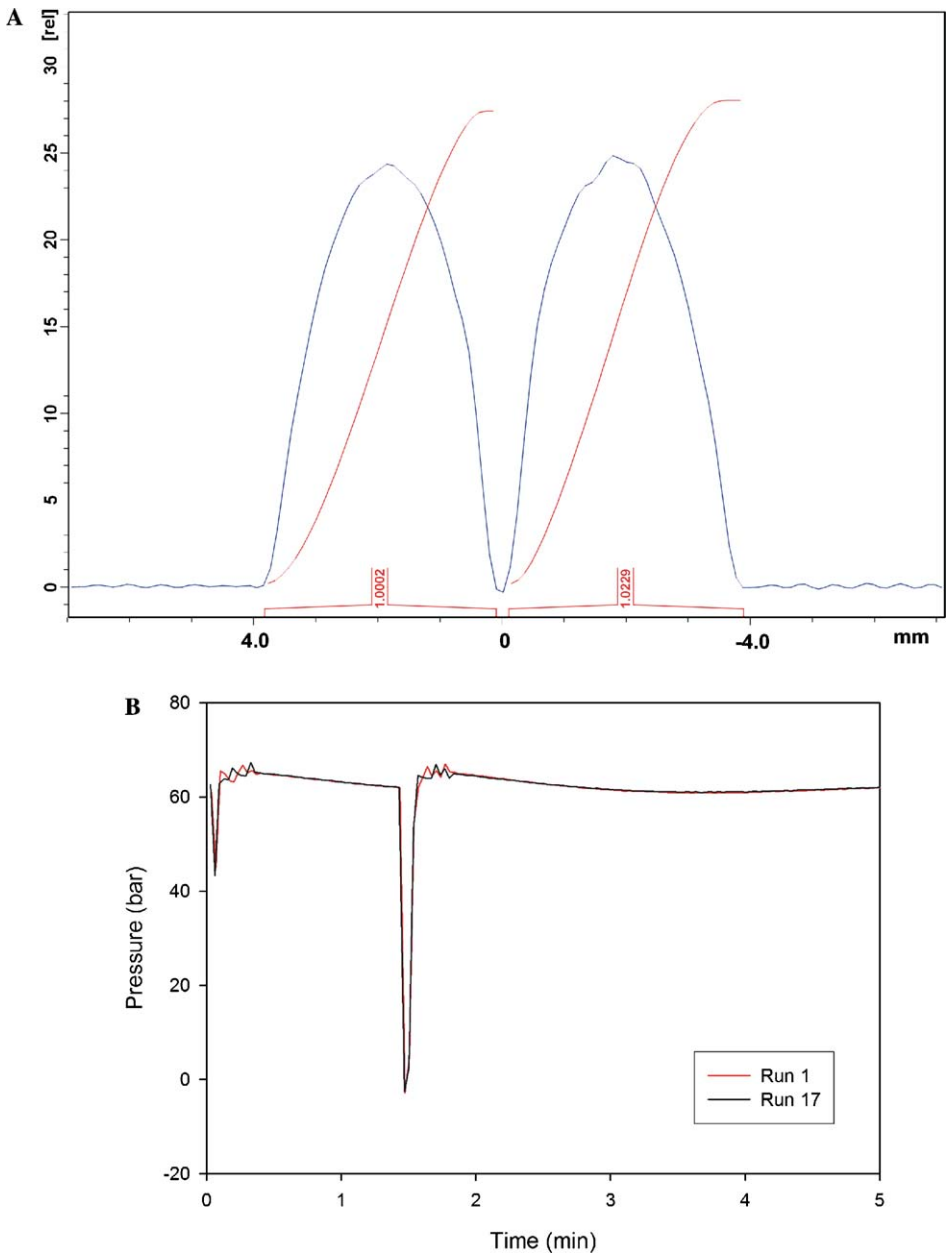
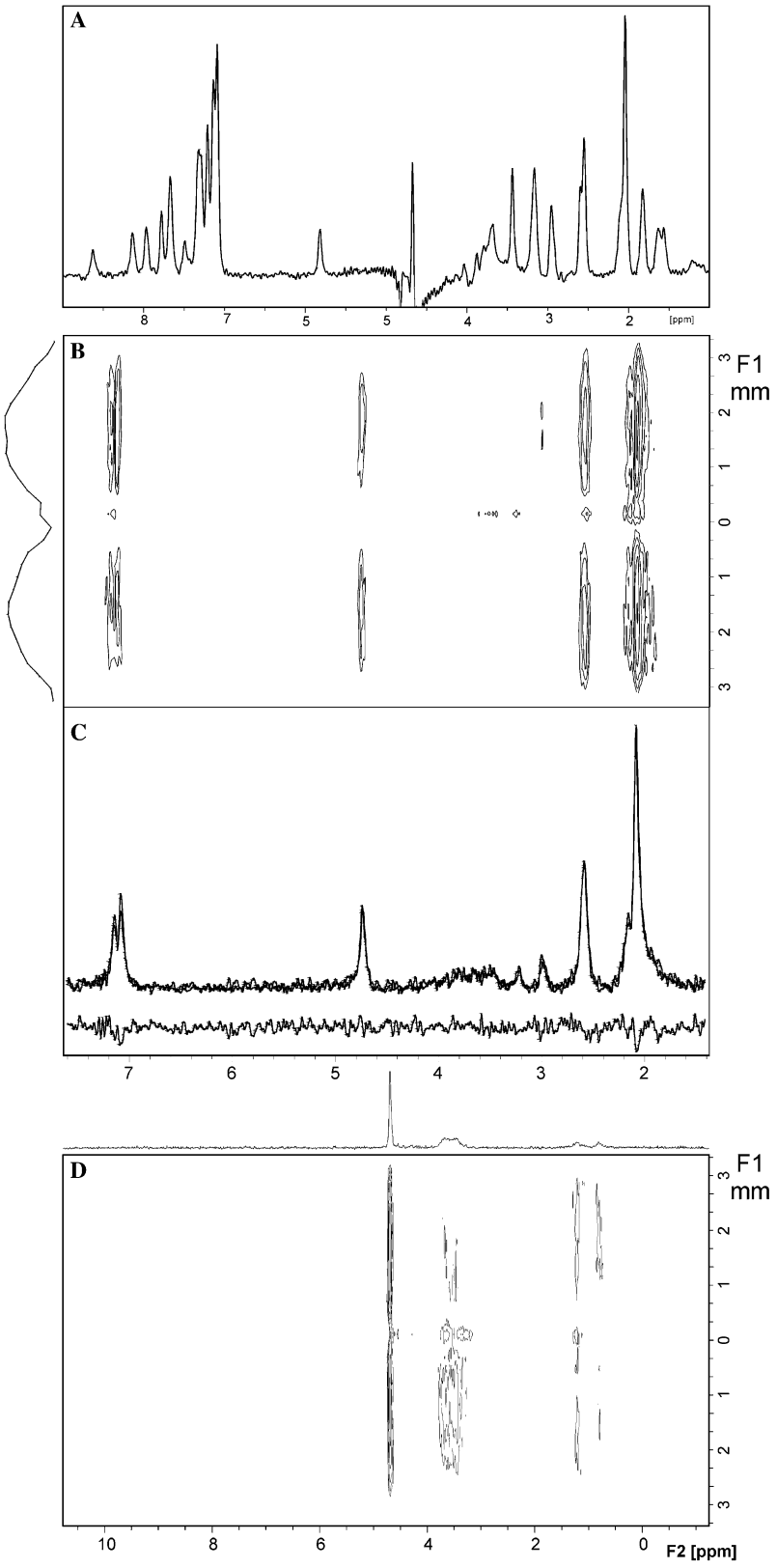


Fig. 3. Characterisation of the fluid flow through the dual-cell sample holder. (A) Quantitation of the excluded volume in cell. The cells were filled with a solid support to which protein was immobilized at a solution equivalent of 0.5 mM and equilibrated with buffer in H_2O. A 1D imaging experiment (single scan) was performed and the profile of each cell was integrated. The integral values are shown below each peak. (B) Chromatogram showing the pressure profile during the 1st and 17th application of compound mixtures to the sample holder as described in (A). The dip at 1.4 min is due to flow stoppage to acquire the NMR data.

Fig. 4. Performance of the dual-cell sample holder/probe combination. The sample holder was packed with unmodified resin in each cell. (A) Lineshape in the presence of a solid support. Both cells were equilibrated with a mix of eight compounds (see Supplementary Material for details) at 500 μM each in D_2O. A 1D ^1H spectrum was recorded of both cells using 32 transients and WATERGATE to suppress the residual H_2O signal. Resonances in this spectrum are between 25 and 30 Hz wide at half height, precisely the same as measured in a 5 mm glass NMR tube. Control experiments to detect inherent differences between the cells. The cells were equilibrated with a mix of phosphotyrosine and arginine at 500 μM each in D_2O. (B) A 2D chemical shift imaging experiment employing the WATERGATE sequence for solvent suppression was recorded in 20′ using 32 ''T1'' increments and 32 transients per increment. The projection along the distance axis (left) clearly shows the profile of the two cells. (C) The signal from each cell was added and the two spectra are overlaid. The difference spectrum of the two overlaid spectra is presented below. (D) Removal of the compounds from the dual cell sample holder. The compounds from panel A were removed by washing with 900 μl of D_2O per cell. The CSI experiment was then recorded for 5.3 h. The projection along the chemical shift axis is shown above the spectrum.

As shown in Fig. 4D, only the broad peaks from the resin itself were visible indicating effective washing. In routine use we do observe discoloration of the solid support after multiple rounds of application of compound mixes. However, the backpressure remains constant (Fig. 3B) and we have previously shown that this low level of accumulation does not effect the ligand-binding capacity of an immobilized protein [11].



2.5. Detection of ligand binding in the dual-cell sample holder

We next filled a sample holder with unmodified resin in one cell and resin to which the protein FKBP12 [2] had been immobilized at 5.5 mg/ml of settled resin as described previously [11], a solution equivalent of 0.5 mM protein. Both cells were then equilibrated with a simple mixture of 5 compounds (see Supplementary Material) at 0.5 mM each in deuterated PBS. The mix included a known ligand (compound **4**) with a K_D of 0.6 mM [30]. We used the CSI experiment to detect binding of the known ligand to the immobilised FKBP. Fig. 5A shows the 1D slices from each cell overlaid. All four resonances (indicated) from this moderate affinity binder are clearly reduced in intensity while those of the non-binding compounds are unchanged (within the noise limits of the experiment). We then used a somewhat more complex mixture of eight compounds at 0.5 mM each, again including a known ligand for FKBP12 (compound **9**, Supplementary Material) with a K_D of 60 μM [30]. Again, a clear reduction in the height of all of peaks derived from compound **9** can be seen while the height of all peaks from non-binding compounds is similar in both cells. This result is identical to that obtained in batch mode using separate samples in standard NMR tubes in a 5 mm high-resolution probe [11].

The spectra of Fig. 5 show good separation of the signal from the two cells but only moderate sensitivity. In addition, the data presented thus far simply recapitulate the literature. A much more stringent test of the performance of the sample holder/probe combination would be to use it to discover new ligands for a target. To address the first issue, sensitivity, we implemented a different NMR experiment referred to as slice selective spectroscopy [12]. Using this experiment we performed a limited screen of 40 compounds from a fragment library that we are currently developing, in order to find a novel ligand for FKBP12. The compounds were screened in mixes of 8–10 at 0.5 mM each in PBS plus 5% d_6-DMSO. To cancel out background signal from extremely weak, non-specific interaction of the com-

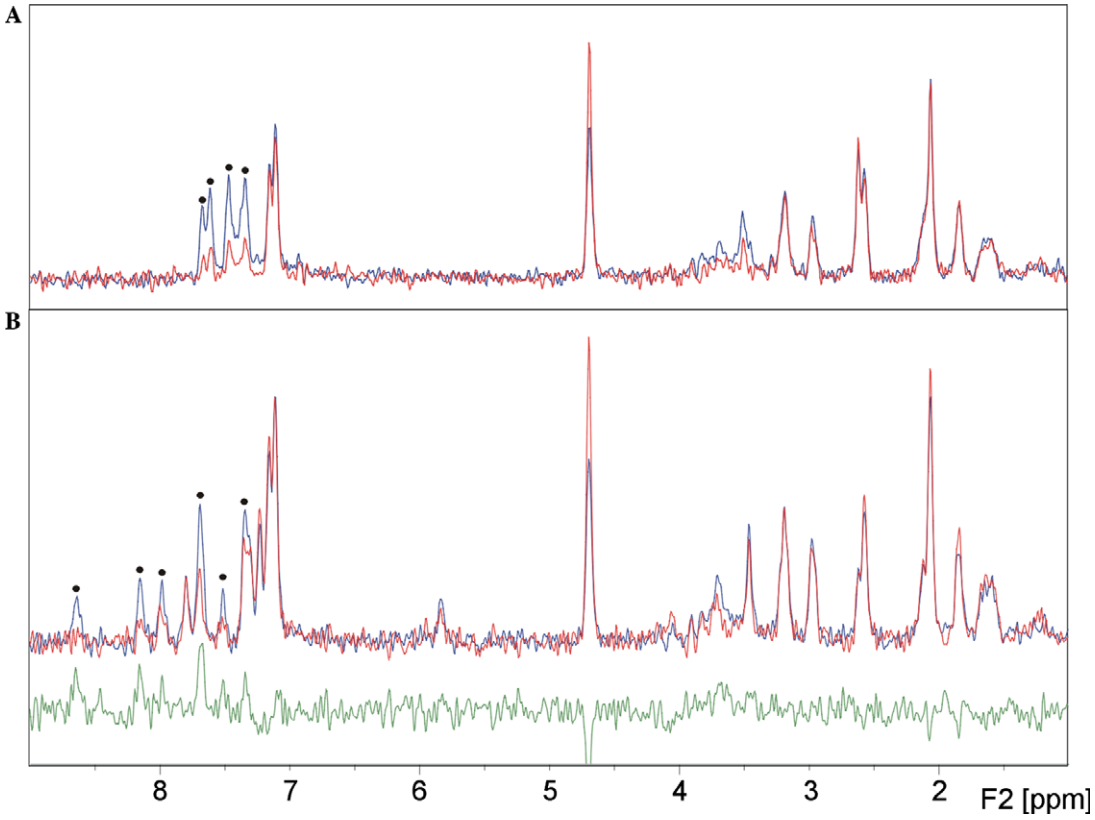


Fig. 5. Detection of ligand binding in the dual-cell sample holder. One cell of the sample holder was filled with a resin to which the protein FKBP12 was immobilized at a solution equivalent of 0.5 mM while the other was filled with a control resin to which no protein was immobilized. (A) A mixture of five compounds (see Supplementary Material for details) at 0.5 mM each was applied to each cell using the system described in Fig. 2. The mixture included a known binder with $K_D = 0.6$ mM (resonances marked by circles). The CSI experiment was used to detect binding of the ligand to the immobilized FKBP12 (Fig. 4B) and the traces from the individual cells are shown (blue control resin, red FKBP12 resin). All peaks from the known binder are reduced in intensity. (B) As for panel A but a mix of eight compounds was applied to the immobilized sample including a known binder with $K_D = 0.06$ mM (indicated by circles). As expected, only resonances from the ligand show reduced intensity. A difference spectrum, in which the primarily visible peaks all derive from the ligand, is shown in green.

pounds with the immobilised FKBP12, we immobilised a second protein in the other cell of the sample holder. We chose the PH domain of the protein AKT which had demonstrated extremely low hit rates in the SAR by NMR screening method [31]. The recombinant AKT PH domain (aa's 1–123) was purified as described [32] and immobilised using the same Actigel ALD resin procedure and at the same density as FKBP12. Fig. 6 shows the results of one mixture of compounds from this limited screen using the slice selective spectroscopy method. It is clear that a very significant improvement in sensitivity has been achieved. We have measured a difference of approximately 4.5-fold improvement in the signal to noise ratio of the slice selective vs. the CHI experiment. The NMR spectrum of each compound in the mix is shown below the spectra of the binding experiment. These spectra have been linebroadened to approximate the linewidth in the presence of the solid support. Inspection of Fig. 6 indicates that at least one peak from every compound in the mix is clearly visible and that no data have been lost. Six peaks in the region between 0.5 and 2.5 ppm are reduced in intensity in the FKBP12 cell with respect to the AKT cell, indicating that at least one of the compounds in the mixture binds. The peaks with reduced intensity correspond well with all six peaks in the spectrum of the isolated compound labelled ZB235, strongly suggesting that this compound is a specific ligand of FKBP12. Peaks derived from compounds that do not bind generally cancel out rather well. The small remaining differences are not consistent with the spectrum of any of the other compounds and likely derive from imperfect cancellation of the non-specific interactions.

The data shown in Figs. 5 and 6 were acquired in 20 or 30 min respectively with an additionally 5 min required to equilibrate and wash the cells of the sample holder. Assuming 10 compounds per mix, use of these conditions would result in a throughput of about 500 compounds per day. At this rate a library of 5000 compounds can be screened in about 10 days. We use a single injection of 950 μl of sample to load both loops. At 0.5 mM each, that corresponds to, on average, 125 μg of each compound per screen. At present we have immobilised the proteins at a solution equivalent of 0.5 mM and 200 μl of resin are required to fill the cell. By comparison, other small molecule methods such as STD or WATERLOGSY typically use samples of 500 μl of about 5 μM target. Assuming both methods screen mixes of 10 compounds at a time, the breakeven point for TINS would be screening more than 400 compounds. Fragment libraries that are screened by these methods often consist of 1000–2000 compounds suggesting that TINS screening could be accomplished using less

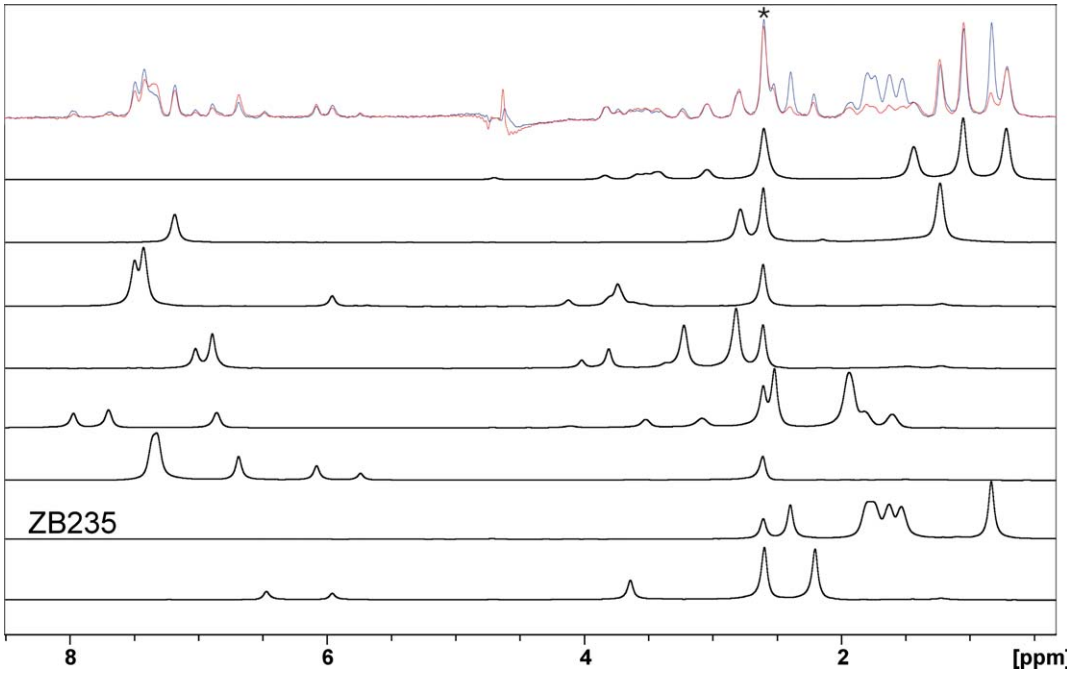


Fig. 6. Screening compound libraries using the dual-cell sample holder. A mix of eight different compounds at 0.5 mM each was simultaneously applied to both cells of the sample holder. One cell contained the immobilised protein FKBP12 (red trace) at 0.5 mM solution equivalent while the second contained the pH domain of the protein AKT (blue trace) at the same density. A slice selective spectroscopy experiment was performed using 1 k transients for each cell (total acquisition time 30′) and the spectra are overlaid at the top of the figure. A 1D ^{1}H spectrum was recorded of each compound in the mixture and line broadened to 25 Hz using an exponential function. The spectra of the individual compounds are presented below the overlaid spectra of the mixture in the cells. The peaks in the spectrum marked ZB235 match those with reduced intensity in the FKBP immobilised cell and therefore identify the compound that binds FKBP. The peak indicated by an asterisk derives from residual protonated DMSO.

protein. In addition, TINS can be carried out on proteins that have poor solution characteristics. For example, the purified AKT PH domain precipitates in buffers with salt concentration less than 300 mM (unpublished observation). Once immobilised however, this protein appears perfectly functional in PBS with a salt concentration of 140 mM as suggested by the absence of large non-specific interactions that one would expect if the protein were aggregated or unfolded. Finally, the method shows excellent sensitivity to weak binding, where fragments with K_D as low as 2.1 mM have been readily detected interacting with the immobilized target (see Supplementary Material).

In summary, we have developed a reliable system to deliver compounds for analysis to a two cell sample holder within an 8 mm coil, flow-injection NMR probe. The hardware has been used in conjunction with a limited screen of a commercial compound library to rapidly and clearly detect a new ligand for a known target protein. There are however, limitations and some areas which can still be improved. While the single, time-based pump is adequate to provide equal flow to each cell, a dual, volume based (syringe) pump would clearly be a more robust solution. At present we have only applied moderate numbers (less than 100) of compounds to the immobilized samples, we have not yet screened a full fragment library. Although our previous work suggests that clogging will not be a problem [11], a system in which a single sample is used for many analyses is always vulnerable to degradation. Therefore, it is very important that care is taken that all compound mixes remain in solution and do not precipitate spontaneously or in the presence of the target and that the condition of immobilised target be repeatedly probed by e.g., application of a known ligand.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2006.05.018.

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