Automation of measurements and data evaluation in biomolecular NMR screening

Alfred Ross and Hans Senn

This article reviews the equipment required for biomolecular screening applications in the automated preparation of samples and the acquisition of a large number of NMR data sets. New hardware connecting lab-bench and NMR spectrometers is introduced. In addition, the article focuses on software used for the automated processing of data and the calculation of similarity between spectra – a prerequisite for the identification of test compounds interacting with a target molecule.

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▼ The use of NMR to probe interactions of small test molecules with biological target molecules of high MW has been of practical interest since the early 1960s (Refs 1–3). A description of the fundamentals of ligand–target interactions can be found in the literature[4]. Recently, NMR binding studies have gained new momentum in the search for small molecular structures that bind to target proteins and have attractive pharmacological properties[5,6]. These molecules do not have to be tight ligands (i.e. they can have sub-micromolar affinity), it is sufficient that they bind specifically to a predefined site on the target with relatively weak affinity (μM < K_d < mM). Furthermore, the binding structure(s) should be amenable to chemical modification for consecutive hit-to-lead development.

Because of the dynamic averaging of NMR-detectable parameters, binding studies are ideally performed on systems with rapid exchange of the ligand molecules between a bound state in the active site(s) of the macromolecular target and a free state in the bulk of the aqueous solution. Two fundamentally different screening approaches are in use: first, methods based on spectra of low-MW test compounds use one-dimensional (1D) ^{1}H-NMR experiments; and, second, methods based on spectra of the high-MW target use heteronuclear two-dimensional (2D) NMR experiments.

The first class of experiments is based on the analysis of 1D ^{1}H spectra of test compounds measured in aqueous solution at a concentration of 50–200 μM in the presence of the biological target (concentration <5 μM). Here, the interaction between target and test compound is manifested in the chemical shift position or line broadening of ligand signals, the observation of intermolecular nuclear Overhauser effects (NOEs)[7] and/or saturation transfer, as well as the reduction of translational diffusion[8]. Changes of dynamic parameters, which differ for the bound and the free state of a ligand by several orders of magnitude, can be measured with high sensitivity. It is sufficient that only a very low fraction of the test compound is bound to the target. As a consequence, the concentration of the costly biological target can be as low as 100 nM. Current technology development aims at a further reduction of the concentration of target molecules needed by the use of electron-spin labels[9], miniaturization of the detection volume[10] and the introduction of cryogenic probe technology[11]. Disadvantages of the 1D affinity NMR approach are the need for dynamic averaging and the lack of topological information about the binding site(s). The binding site on the target surface can only be inferred indirectly by displacement experiments using a tight binding ligand with a well-defined binding location. All 1D ^{1}H-NMR spectra to be analyzed are intrinsically different; therefore, data interpretation is achieved only at a low level of automation at present (e.g. subtraction of pairs of spectra acquired in the presence and absence of target molecule).

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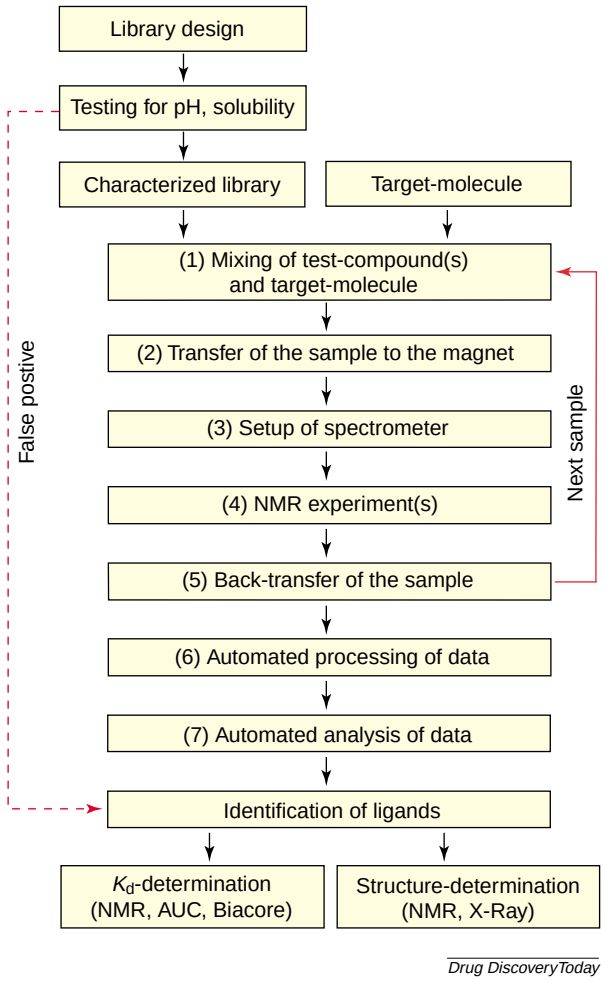

Figure 1. Strategy of NMR-based screening to identify ligand–target interactions.

In the second class of experiments, which circumvents dynamical averaging as a prerequisite, the structural information obtained can be greatly improved by isotope labeling of the target molecule[2]. Here, the molecular interaction is experimentally monitored by the analyses of heteronuclear correlation spectra acquired on ^{15}N- and/or ^{13}C-labeled target molecules[12–14]. It has been shown that perturbation of ^{1}H, ^{13}C and/or ^{15}N chemical shifts is a sensitive parameter with which to monitor the formation of specific ligand–target complexes. The analysis of changes of chemical shifts upon titration of ligands can help to determine affinities over a broad range (mM > K_d > nM)[15]. In contrast to 1D techniques, the binding site of the ligand can be localized on the surface of the target if the shifted signals of the spectrum are assigned, or if they coincide with shifts induced from a tight ligand whose binding interaction is structurally well characterized (e.g. by X-ray crystallography). In addition, these methods allow for a variety of automated data-analysis protocols, as the spectrum of free target can always be used as a reference that is only perturbed if a ligand–target interaction occurs. As NMR experimental methods are beyond the scope of this article, the reader is referred to recent review articles[16,17].

For all of the concepts described above, automation is a prerequisite to monitor the interaction of the large number of test compounds needed to ensure that a rich variety of structural motives can be systematically tested. Automated spectrometer operation and sample changers[18,19] are widespread, and can be found today in most analytical NMR laboratories that handle large numbers of samples. Automated sample preparation was achieved in the mid-1990s by the introduction of robots connected to the sample changer of the NMR spectrometer (AutoPrep™; Bruker, Karlsruhe, Germany)[20].

Recently, the introduction of flow-through NMR probeheads[21–23] has further simplified automated sample handling. However, the commercially available hardware can only be used to a limited extent for NMR-based biomolecular screening.

A second prerequisite to achieve high-throughput analysis is an automated similarity analysis of spectra. In analytical chemistry, this analysis is restricted to a comparison of measured spectra to reference spectra in a data bank[24,25], or to simulated spectra calculated by the use of chemical shift prediction tools[26]. Recently, methods based on pattern recognition and statistical data analysis have been added to the toolbox of NMR software for spectral comparison[27]. These algorithms have found widespread application in toxicology and nutritional chemistry[28].

The purpose of this article is to describe the methods, hardware and software available for NMR screening in order to support the NMR spectroscopist in automating biomolecular NMR. In addition, some highlighting case studies are presented, and the article concludes with an outlook on miniaturization.

Automation in biomolecular NMR

NMR-based screening methods, if applied to identify ligand–target interactions on a large number of samples, have to be automated as far as possible. The screening protocol can conceptually be subdivided into seven individual steps (summarized in Fig. 1):

(1) Just-in-time sample preparation.
(2) Transfer of the sample to the magnet.
(3) Setup of the NMR apparatus including locking of the field and shimming.
(4) Measurement of an appropriate composite NMR experiment comprising a set of selected 1D and 2D techniques.

(5) Back transfer of the sample to a park position outside the magnet, and storage.

The following two steps can be performed off-line after all the NMR raw data have been acquired:

(6) Automated data processing, including all phasing and baseline correction steps as needed

(7) In test compound spectra, the spectra taken in the presence of the macromolecular target are compared with reference data of the test compounds in the absence of the target, or application of affinity NMR methods (discussed later). In target molecule spectra, a comparison is made of the spectrum of the free target (reference) with data taken after addition of the test compound.

It is clear that all of these tasks have to be performed repeatedly with as few manual interactions as possible. Computerized book-keeping is compulsory to avoid losing track of hundreds or thousands of NMR experimental runs. The need for high-throughput and reliability for all steps involved cannot be over-emphasized.

The careful selection of the compound library to be subjected to NMR screening is a formidable task on its own and is not covered in this article. Recent reviews on this topic can be found in this journal[29] and others. The NMR screening library can either be universal or dedicated to a particular target and/or target family. Storage of the compounds is ideally done in deep-well plate (DWP) format in deuterated solvent in a deep freezer. To prevent false-positive findings and to simplify the final data analyses, two tests are needed. First, each compound in the library is characterized with respect to solubility and its potential to induce pH changes. Theory shows that changes in pH can only be reduced by use of strong buffering (approx. ± 0.2 units) but can never be excluded completely (Fig. 2)[30]. Second, pH-sensitive target resonances are identified directly in titration experiments using diluted HCl and NaOH. This knowledge helps to identify false-positive hits: observed changes in the target spectrum can be separated into effects resulting from ligand binding and effects resulting from pH drifts, respectively. In addition, recording spectra at different concentrations of the library solvent identifies solvent-induced shifts.

All of these data are carefully consulted in a final step to identify binding ligands positively. It has to be noted that insensitivity against pH drift and different concentration of the solvent is, in general, not a problem in the compound-spectra-based approach. Here, addition of the small aliquots of target solution can hardly induce a significant change of sample conditions. By contrast, in the second class of experiments, where the macromolecular target is observed, the addition of concentrated ligand solution might introduce shifts of resonances because of pH drifts

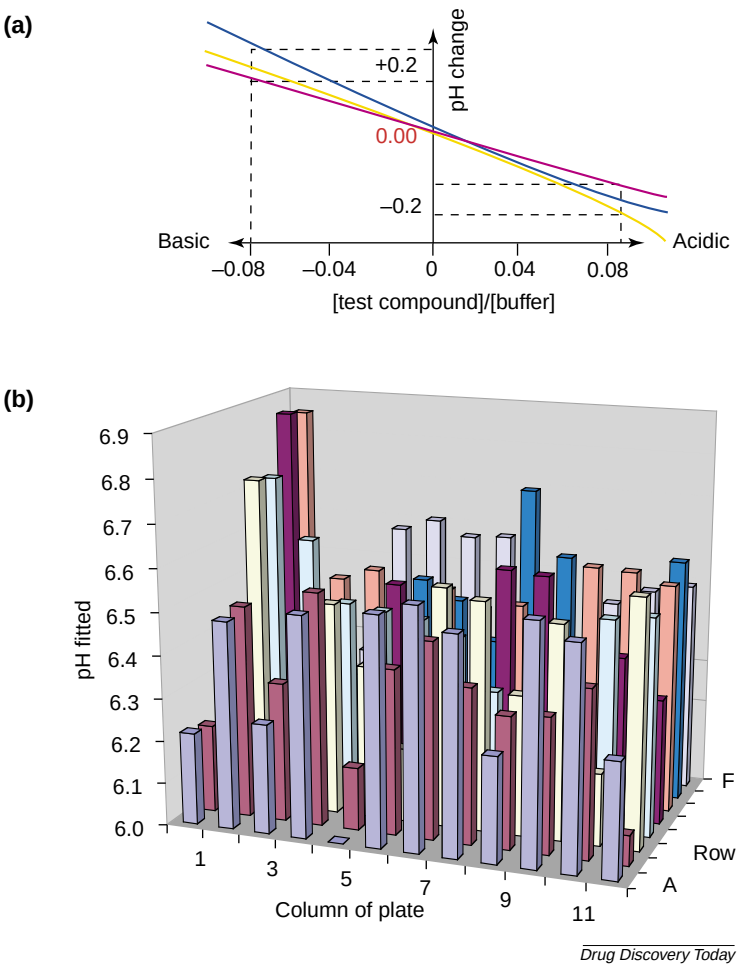

Figure 2. The pH change in a buffered aqueous solution upon addition of a strongly acidic or basic test compound. **(a)** Result of a calculation based on the Henderson–Hasselbalch equation; x-axis: ratio of concentrations of test compound to buffer (positive values for acidic compounds, negative values for basic compounds); y-axis: induced pH change. The pink line shows the result under optimal buffering condition ($pKa_{buffer} = pH_{buffer}$). The blue and yellow lines are calculated for pK_a values with +0.5 and –0.5 units off, respectively. The pH drift for an actual sample (8 mM compound in 100 mM buffer) can be as large as 0.2 pH units. **(b)** Experimentally observed pH changes for a DWP library of 96 test compounds dissolved at 8 mM in a 100 mM buffer of pH 6.4. The pH was determined by reading the optical absorption at 620 nm after the addition of bromthymol blue (pI~6.8). The experimentally determined pH change can be larger than the calculated value. This is probably due to additional solvent effects influencing pK_a of the buffer. pH measurements for 1000 samples per day are possible by an optical reading system for DWPs.

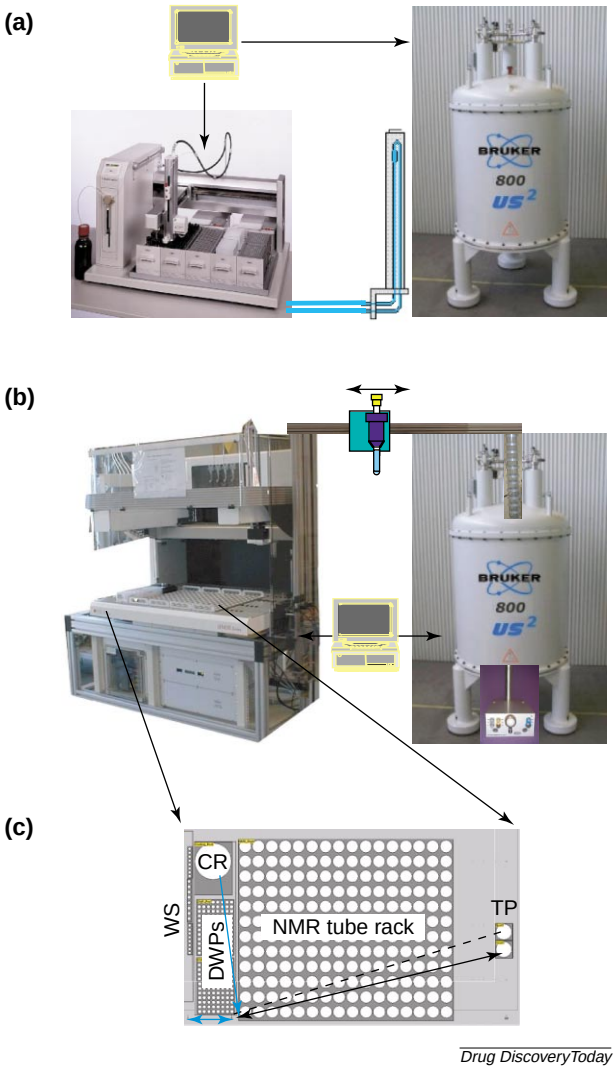

Figure 3. Automated 'just-in-time' sample preparation for NMR screening. The communication between the sample preparation robot and the NMR spectrometer is coordinated and controlled by PC software. **(a)** A Gilson 215 liquid-handling robot is connected via a capillary to a flow-through NMR probe head [e.g. the 'Bruker Efficient Sample Transfer' (BEST) setup]. **(b)** Samples are prepared in discrete NMR sample tubes by a Tecan Genesis robot. The system is connected via Bruker SampleRail to the NMR spectrometer, ideally equipped with a cryogenic probe for highest sensitivity. **(c)** View on the worktable of the Genesis. The blue arrows indicate liquid transfer steps by the pipetting arm; the black arrows indicate the forth and back (dotted line) transfer of the NMR samples to TP by the robot arm. Abbreviations: CR, cooling rack for storage of macromolecular target; DWPs, deep well plates for library storage; TP, sample transfer port to SampleRail; WS, washing station.

and solvent effects. In the following sections, details will be discussed of the general procedure as outlined above and summarized in Fig. 1.

Just-in-time sample preparation

For automated screening purposes based on the analysis of target spectra, the established procedure is as follows: small aliquots (1–10 µl) of single compounds or mixtures thereof[5] at high concentration (100–800 µM) in deuterated solvent [e.g. d6-dimethylsulfoxide (d6-DMSO)] are mixed thoroughly with the target molecule. In principle, many commercially available pipetting robots can do this task, but special requirements on the system emerge for the following reason: to prevent time-dependent processes in the sample (e.g. aggregation, oxidation and chemical reactions), the test compound(s) and target molecule have to be mixed immediately before the NMR screening experiment.

This 'just-in-time' preparation of samples, also of importance for screening based on test compound spectra, is guaranteed by using a setup comprising a pipetting robot connected via a capillary line to a flow-through NMR probe head. Alternatively, a setup has been developed for preparing discrete samples. It consists of a Genesis sample-handling robot (Tecan, Hombrechtikon, Switzerland), which mixes the sample in an NMR tube immediately before it is transferred to the spectrometer by a Bruker SampleRail system (discussed later). Both setups are shown schematically in Fig. 3.

The liquid-handling equipment needed is part of the work-table of the Genesis system as shown in Fig. 3: the target molecule is stored in a container adequate to prepare enough samples to run the automation for several days (up to 192 × 500 µl, which approximates to 100 ml for a 1D screening). Storage conditions are selected to ensure stability of the target molecule over the same period of time by cooling (4°C) and by an inert argon atmosphere in the container. Two DWPs filled with test compounds are positioned on the robot working area, where up to 192 pre-mounted disposable NMR tubes are parked in a dedicated rack. The robot has the flexibility to perform additional sample preparation or testing steps (e.g. to mix selected test compounds from the DWPs; to include various incubation periods; and to perform a titration experiment with one sample).

Each sample is prepared in an individual container (e.g. a single well of a DWP for flow-through setup or an NMR tube for discrete sampling). All equipment in contact with the compounds is rinsed between any dispensing step. It should be noted that precipitation in the robot needle can only be excluded if the solvent of the target molecule is used as the system liquid of the robot. Knowledge on acidity and basicity of the test compounds could be used for automatic readjustment of the pH during the sample preparation by the addition of properly calculated aliquots of acid or base[31]. Automation of this pH adjustment is conceivable given the flexibility of the Genesis robot.

Transfer of the sample to the magnet

To take full advantage of 'just-in-time' sample preparation, it is mandatory to connect the sample-preparation robot to the NMR spectrometer. Two different technical solutions are commercially available (Fig. 3): the flow-through probe head technology and the SampleRail system for discrete samples.

Flow-through probe head technology

A pipetting robot (e.g. 215 System; Gilson, Middleton, WI, USA) prepares samples in DWP format. Each sample is subsequently injected via a port to a capillary line connecting the robot and the spectrometer. Immediately after the end of the NMR experiment(s), the sample is pumped back into the mixing container or into waste. This setup is available as 'Bruker Efficient Sample Transfer' (BEST; Bruker, Karlsruhe, Germany) or 'Versatile Automated Sample Transport' (VAST; Varian, Palo Alto, CA, USA) and has several disadvantages. First, carryover of test compounds between different samples (because of coating of long capillary lines) cannot be excluded. The possibility of clot formation in capillary lines and in the NMR probe head makes prescreening for compound solubility mandatory. However, such a selection might exclude potential ligands with high solubility in the protein (because of binding) but low aqueous solubility. Second, all recovered samples experience dilution by the system liquid. Third, the NMR technology offering highest sensitivity (i.e. the cryogenic probe) is currently not available in flow-through design.

SampleRail system for discrete samples

The samples are prepared in individual NMR tubes moved by a robot arm to the pneumatically driven sample-lift system connecting the preparation station and the NMR spectrometer. After completion of the NMR experiment(s), the tube is back-transferred to its initial position for storage. This setup is currently only available as a joint platform comprising a Genesis robot connected via the SampleRail to a spectrometer. All problems related with flow-through technology as discussed above can be circumvented by use of this more-complex hardware.

Setup of the NMR apparatus

After the sample is transferred (either by flow-through technique or as sample tube) to the magnet, the usual setup of the spectrometer including locking and shimming (ideally, gradient-shimming) of the field has to be done automatically. Automation as provided by all vendors supports this step.

In our experience, it has proved helpful to use the deuterated solvent of the compound library for locking purposes. Thus, no locking agent (D_2O) has to be added to the target fraction. Furthermore, if any failure occurs during liquid handling, the faulty sample cannot be locked because of lack of deuterated solvent, and is immediately replaced by the next freshly prepared sample in the queue. To date, the addition of DMSO at a concentration below 5% has never caused irreversible modification (aggregation) of the proteins investigated (Ross and Senn, unpublished observation).

Measurement of NMR experiment(s)

Depending on the information sought and the availability of isotope-labeled target molecules, a set of NMR experiments (i.e. a composite experiment) as defined by the operator has to be measured for each sample. This task can easily be achieved by automation.

In 2D heteronuclear single quantum concerence spectroscopy (HSQC)-based screening, we include a 1D ^{1}H-NMR experiment(s) to estimate the concentration and aggregation state of the test compounds. If a pH-dependentmarker substance is included in the samples (e.g. imidazole or phosphate buffer), the pH of the sample can be monitored by proton chemical shift or by ^{31}P-NMR. The reproducibility of the automated sample preparation and the homogeneity of the magnetic field can be checked by integration of the residual signal of the small partially deuterated fraction of the solvent added by the robot.

In favorable cases, inspection of resolved signals in the 1D spectrum of the target molecule might help to indicate binding interaction. In contrast to 1D techniques that rely on the analysis of ligand spectra, this target-focused 1D screening can, as for its 2D counterpart, easily be automated. An example is shown in Fig. 4.

Back-transfer and storage of the sample

All sample back-transfer steps (in a capillary or an NMR tube) have to be performed in a fully integrated way. This feature is implemented in both commercially available platforms. Storage of the test compound/target mixture under stabilizing conditions (i.e. a cooling, non-oxidative atmosphere) would be helpful for additional experiments to be performed later on. Cooling of recovered samples is possible using water-cooled racks for DWPs.

The Tecan/Bruker platform is currently being modified to include a titration protocol for the stepwise addition of aliquots of the test compound followed by NMR experiment(s). This will allow the fully automated determination of ligand–target affinities (K_d values) for a large number of test compounds using known NMR methodology[15].

Automated data processing

The automation features for data processing are summarized in Table 1 for several available software packages. All

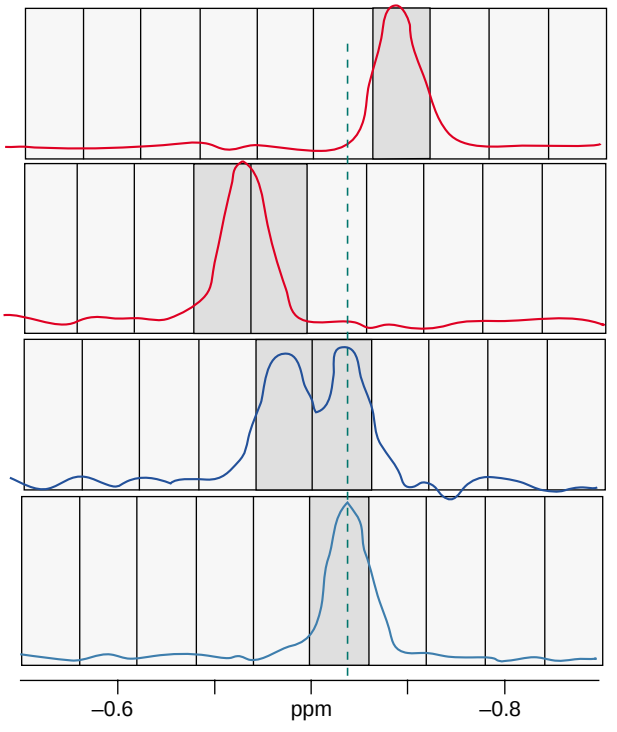

Figure 4. Binding interaction of the ligand molecule can be observed in favorable cases in the one-dimensional (1D) ^{1}H-NMR spectrum of the macromolecular target. Here, only the resolved high-field methyl resonance of an isoleucine residue close to the active site is shown. From bottom to top: free uncomplexed target protein (trace 1); as the ligand is present in excess concentration, the duplication of the signal is indicative of a covalent modification of the target protein (trace 2); non-covalent binding interactions of the added ligand is reported by the shifted signal of the protein (traces 3 and 4). The individual spectra can be described as data vectors, $\vec{v}_i$, with components defined by the integrated intensity of the individual buckets colored in gray (see text).

automated spectral analysis algorithms in use rely at one stage or another on integration of spectral areas or peak picking. The availability of accurate automated correction of phases and baseline is essential for data processing without manual intervention. In our experience, all tested packages work reasonably well for 2D ^{1}H-^{15}N HSQC-type spectra. As ^{1}H spectra acquired in aqueous solution are prone to a huge solvent signal, the performance of automated processing is not satisfactory for automated data analysis without intervention by the operator. Visual inspection and manual re-adjustment of phases is always required. All software vendors should improve on this situation.

Comparison of spectra – spectral similarity

All NMR-based screening rests on the fact that spectra experience changes with respect to positions or intensities of signals upon binding of a ligand to the target. As a comparative analysis of hundreds of spectra (1D or 2D) is a laborious task, ideally it has to be left to tailored software.

For compound-spectrum-focused approaches, automation is hampered by the fact that the signals of the test compounds are intrinsically all dissimilar. Applying 1D-affinity NMR methods where a reference and a test spectrum are acquired for one and the same sample (test compound mixed to the target) can circumvent the problem. Here, calculated difference spectra only contain signals if the test compound binds to the macromolecular target. In our experience, the recently published saturation transfer difference (STD) method[32] and the reverse NOE pumping method[33] fulfill this requirement most reliably.

Because of the availability of a single reference spectrum acquired on the free target, the variety of analysis tools is much broader for target-spectrum-focused approaches. Some NMR software packages include analysis tools for spectral comparison. There are also stand-alone software

Table 1. Features of software for automated processing and analysis of a large number of NMR spectra

Software platform	Batch-wise processing	Automated correction of phases	Automated correction of baseline	Automated data analysis
XwinNMR/Amix	Yes	Works well for 2D	Works well for 2D	Bucketing, single parameter similarity, peak matching
Felix[a]	Yes	Yes	Yes	Several single-parameter and peak matching algorithms
NMRPipe	Yes	Works well for 2D	Works well for 2D	PCA tool providing visualization, peak matching

[a]This package is not in use in our laboratory, therefore performance cannot be rated. Abbreviations: 2D, two-dimensional; PCA, principal component analysis.

packages providing more-flexible tools working on extracted numerical integrals of spectral regions. As there is no clear-cut superiority of any software over the others, selecting the most convenient platform is left to the experience and preference of the scientist.

Any numerical analysis of data can only be performed if the original spectra are transformed to a table of numbers. This can be done by two methods summarized in Figs 4 and 5. Any spectrum (1D or 2D) can be converted to a table of 'n' numbers (represented by a data vector $\vec{v}_i$ in an n-dimensional space) by integration of 'n' equal-sized areas distributed as a 'grid' (bucket) over a defined region of the spectrum. In the 1D case, this results in a picket-fence-like pattern as shown in Fig. 4; for the 2D case, the resulting mesh-like distribution is shown in Fig. 5. For obvious reasons, this procedure is called 'bucketing'. The procedure uses information from all regions of the spectra, thus appearance (e.g. by freezing-out flexible regions of the target molecule upon ligand binding) and disappearance of signals is taken into account.

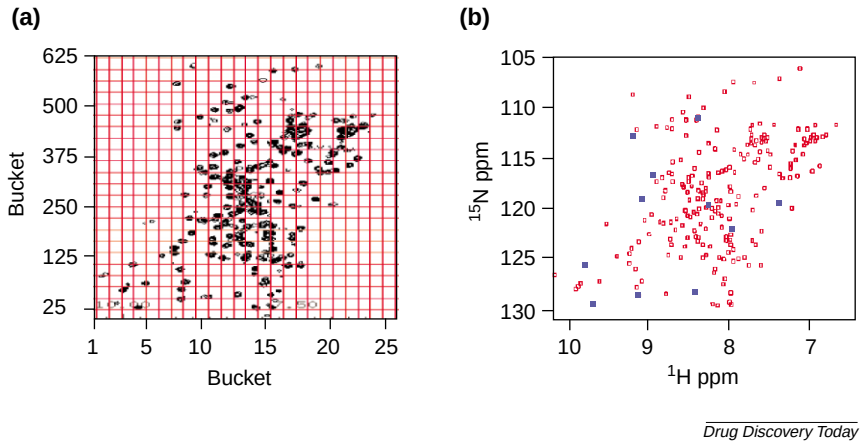

Figure 5. Preparation of experimental raw data for automatic spectral comparison. **(a)** A grid is superimposed on the two-dimensional (2D) ^{1}H-^{15}N spectrum to define a data vector with components given by the signal intensity integrated over each bucket. **(b)** A subset of the cross-peaks (blue) or, alternatively, a subset of the vector components, is selected for data description. These reduced data may represent for example a putative binding site of the macromolecular target.

If signals related to atoms in the binding pocket are known, this information can be included in a second method using selected areas (pattern) that are integrated in all spectra. An example is shown for a 2D approach in Fig. 5. This biased integration results in data vectors $\vec{v}_i$ of much lower dimensionality compared with those obtained by unbiased bucketing. In our experience, a combined approach resting on different methods of similarity calculation and data extraction (e.g. statistical versus non-statistical analysis) helps to judge the quality and reliability of the results obtained, and to remove false-positive ligands from the list.

In the following sections, three different analysis algorithms for similarity calculation as applied to 1D and 2D techniques are explained, and are highlighted using examples obtained in our laboratory.

Non-statistical approach based on 'scalar products' of data vectors

A similarity coefficient $S_{i,ref}$, describing the likeness between a spectrum 'i' and a reference 'ref' (represented by two data vectors $\vec{v}_i$ and $\vec{v}_{ref}$) can be calculated by use of the normalized scalar product:

$$S_{i,ref} = \frac{\vec{v}_i \cdot \vec{v}_{ref}}{|\vec{v}_i| \cdot |\vec{v}_{ref}|} \quad (1)$$

In Equation 1, $|\vec{v}_i|$ is defined as the 'length' of the data vector $\vec{v}_i$ in its n-dimensional space. The similarities of a set of spectra can thus be calculated, resulting in a list of numbers $S_{i,ref}$ in the range {0,1}. Finding test compounds that induce changes in the spectrum of the target means finding low-similarity coefficients in this list. This procedure works if a single reference spectrum (e.g. the spectrum of the free protein) is available, against which the similarity can be ranked for all test spectra. Whether the approach is performed using an NMR software platform (e.g. AMIX; Ref. 34) or 'stand-alone' software (e.g. MS Excel) to analyse the buckets is left to the individual. An advantage of this algorithm is that a single parameter is obtained that rates the similarity of a spectrum to the reference spectrum. However, a single parameter can only describe global similarity: no classification with respect to cause or quality of dissimilarity is possible. A change of sample condition (e.g. pH), a spectrometer failure, or an interaction between the molecules in the sample could result in a similar 'number'. An application is shown in Fig. 6.

Statistical approach based on principal component analysis (PCA)[35]

As we have shown[36], PCA can be applied successfully to document the differences between spectra in terms of a few variables. For NMR-based screening data, we have shown that typically less than five principal components (PCs)

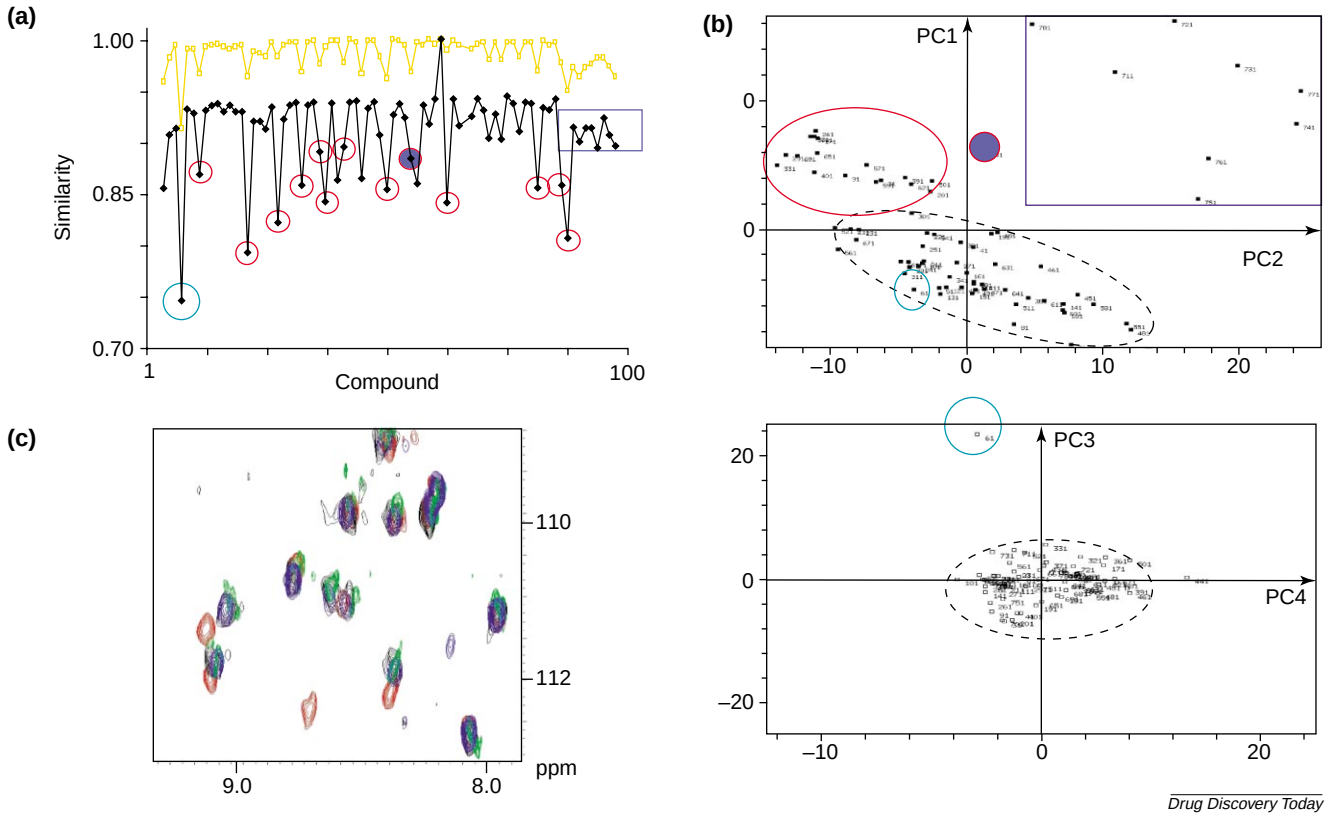

Figure 6. Comparison of two different data evaluation methods for binding analysis of 100 test compounds. These compounds were subjected to a two-dimensional (2D) ^{1}H-^{15}N NMR screen. **(a)** The spectral similarity coefficient, $S_{i,ref}$, as defined in the text, is plotted for all tested compounds. Results are shown for two different preparations of the experimental data: automatic peak picking and integration of all cross-peaks (yellow), and bucketing approach (see Fig. 5a). **(b)** Two score plots (PC1 versus PC2, and PC3 versus PC4) obtained from principal component analysis (PCA) of the same experimental data. **(c)** Four selected 2D spectra of the target in the presence of different test compounds are superimposed. Spectral similarity analysis based on $S_{i,ref}$ allows the clear distinction between two classes of compounds [class I: substantially lowered $S_{i,ref}$, marked individually by red and green circles in **(a)** and enclosed in shapes of the same color in **(b)**; class II: relatively high $S_{i,ref}$ values shown in black]. The statistical PCA-based analysis in **(b)** is able to cluster the screened compounds in four different clouds (highlighted by dotted black, red, blue and green colors). By consulting the 2D spectra in **(c)**, the target binding compounds can be assigned to the red cloud, and the non-binders to the black-dotted cloud. Effects due to spectrometer failure could be resolved (one compound, green circle), and effects due to partial digestion of the target protein in blue. Note that in one case (red/blue), a test compound is binding to the partially degraded form of the protein. Analysis that relies only on non-statistical methods creates false-positive hits. Nevertheless, this technique is helpful to decide on the combination of principal components to be used for clustering. On the other hand, PCA analysis did not find 'hits' that would not have been found only by non-statistical procedures.

explain >90% of the observed differences in the spectra. Numerically, PCA is performed by diagonalization of the correlation matrix S with matrix elements S_{ij} (Eqn. 1) calculated for all pairs of spectra. For visualization, the data vectors $\vec{v}_i$ are projected in the 2D space spanned by pairs of PCs (see Fig. 7). An application is shown in Fig. 6: each point in this figure represents a complete spectrum of the target exposed to one test compound. Different clusters of points can clearly be distinguished. Visual inspection of a few typical spectra in each cluster reveals different types of spectral changes caused by different physical effects: samples with no ligand interaction (black) can clearly be distinguished from those that show specific interaction (red). In a third cloud (dark blue), spectra of samples that contain a small percentage of digested protein are found. It is to be noted that a single combination of PCs (e.g. PC1 versus PC2) cannot explain the total variance; for instance, the single distorted spectrum obtained as a result of spectrometer failure shows up only in the score plot of PC3 versus PC4. PCA analysis can thus reveal different types of distortion. This important result of the data analysis allows one to distinguish between specific binding effects of test compound and artifacts.

PCA analysis can be performed either integrated in an NMR software package[37] or off-line using the result of any bucketing or integration procedure[38] (e.g. UNSCRAMBLER

Version 6.0, Camo, Oslo, Norway, and Tsar 3.3, Molecular Simulations, Cambridge, UK).

Analysis based on peak matching

The methods described above lose all information on the sizes of the chemical shifts induced upon molecular interaction. To what extent these induced changes reflect the strength of molecular interaction is still a topic of ongoing discussion. To obtain this information, every peak in a free-state spectrum has to be matched to the corresponding peak in the bound-state spectrum. This can be done manually for selected cases. Automation is possible by use of a nearest neighbor algorithm, tree-search or simulated annealing protocols (Refs 34,37; C. Peng and S. Szalma, unpublished), taking into account the intensities and lineshapes of signals. As a result, a measure of similarity can be defined by adding up all induced chemical shifts (see for example Felix software package; C. Peng and S. Szalma, unpublished). The information on the induced chemical shift of each macromolecular target peak can be displayed graphically or mapped onto the surface of the target structure (Fig. 8). Different causes of spectral distortion can thus be distinguished visually or by use of statistical analysis. Chemical shift differences induced on target spectra for structurally similar test compounds can be used to extract information on the binding mode of the ligand[39]. For instance, analysis of shifts induced on the ligand spectra has been used for epitope mapping[32] to identify substructures of the ligand interacting with the target.

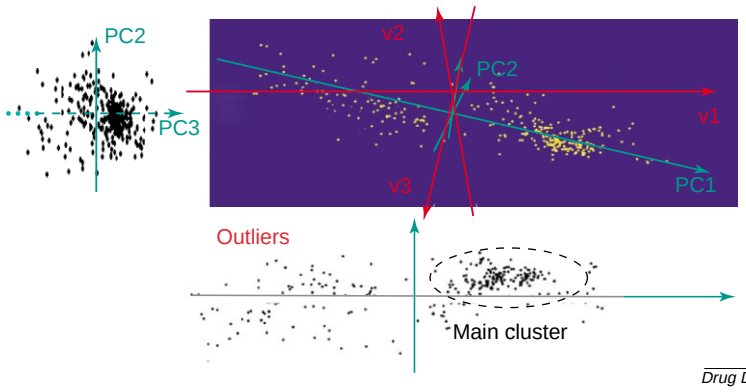

Figure 7. Illustration of principal component analysis (PCA). The experimental spectrum of an individual sample is described by a data vector (yellow dot) of three components v_1 to v_3 (e.g. integral values of three selected buckets in Fig. 5). PCA analysis of an entire NMR screening data set can reveal clustering of samples in clouds if, for example, binding interactions are observed in the spectra. The new coordinates (scores) of the sample (black dots) are linear combinations of the original data variables.

Summary and outlook

2D NMR techniques are well suited to obtaining detailed structural information on ligand–target interactions. Modern automated screening technologies allow moderately high sample throughput. Currently, 50–100 samples per day can be measured. With mixtures of ten or more test compounds per sample, the throughput is substantially increased[5,11]. The price to be paid for the high information content is the requirement for a relatively large amount of ^{15}N-labeled target protein (0.5–2 mg per sample for a 15 kDa protein depending on the probe head sensitivity).

1D techniques that observe the low-MW component in the sample allow for a much larger number of test

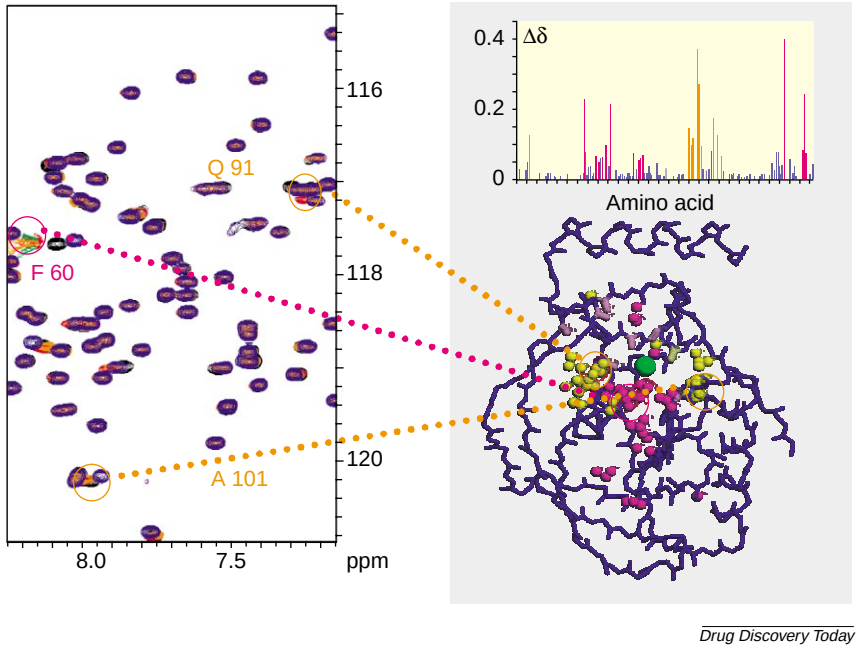

Figure 8. Detailed structural information is obtained from quantitation of the observed chemical shift changes in the target spectrum upon ligand binding (left). These shift changes can be mapped on the three-dimensional (3D) structure (if known), and/or can be plotted versus the corresponding residue in the amino acid sequence (right). In both cases, individual resonance assignment is a prerequisite.

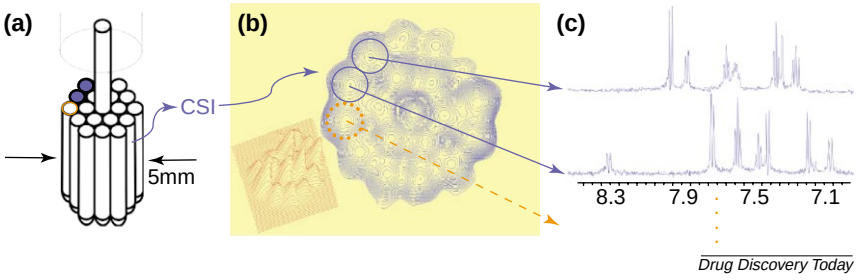

Figure 9. Outlook to future developments that have the potential to increase significantly the sample throughput in NMR screening. **(a)** A bundle of 1mm capillaries. In each capillary a test substance together with an aliquot of protein is filled using a pipetting robot. Data are taken in parallel by use of a chemical shift imaging-(CSI)-based method[41]. **(b)** The image of the bundle as obtained by Fourier transformation of the data set with respect to independently incremented x- and y-gradients. **(c)** Highly resolved spectra extracted orthogonal to this plane. If CSI is combined with affinity NMR methods[32,33], the resulting data can be readily interpreted to identify ligands. Thus, high throughput is possible without the need to revert to mixtures of test compounds.

compounds to be screened. Here, the throughput is limited by the time needed to replace the sample in the magnet. At present, the time needed to measure one sample is approximately 5 min. Furthermore, 1D techniques use much less target protein without the requirement of isotopic labeling. However, only limited structural information is obtained, and automation of data analysis is more demanding. To increase the throughput significantly (by at least one order of magnitude), we have implemented an NMR method that enables the simultaneous detection of 19 samples in parallel[40]. The samples are contained in individual capillaries, which form a bundle with an outer diameter of 5 mm. The principle of this new method is shown in Fig. 9.

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