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Advances in NMR-based biofluid analysis and metabolite profiling

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Abstract

Significant improvements in NMR technology and methods have propelled NMR studies to play an important role in a rapidly expanding number of applications involving the profiling of metabolites in biofluids. This review discusses recent technical advances in NMR spectroscopy based metabolite profiling methods, data processing and analysis over the last three years.

Introduction

An analysis of the metabolite spectrum in a biological system provides a detailed and specific view into cellular metabolic processes under normal and altered (*i.e*. disease-related) conditions.[1–6] The collective measurement of the entire or even a large subset of the metabolome is a challenging task and relies on the use of reproducible and dependable technologies which provide sufficiently high sensitivity, high resolution and wide dynamic range. The information-rich analytical techniques of mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the primary analytical methods employed in metabolite profiling based on their high sensitivity and/or resolution that are necessary to both identify and quantify known and unknown metabolites. While a complete characterization of the entire metabolome is currently out of reach, efforts are underway to improve the ability to access and measure a greater number of metabolites.[7] MS is extremely attractive in metabolite studies due to its exquisite sensitivity, experimental flexibility and ability to determine unknown molecules. While MS is more sensitive compared to NMR, the data from NMR are often more easily quantitated and highly reproducible. In particular, the same nuclei detected (*i.e*. all ^{1}H) in an NMR experiment have the same sensitivity, independent of the properties of metabolite molecules. Therefore, the absolute quantities of different metabolites can be measured with a single internal or external standard. In addition, NMR requires minimal or no sample preparation or separation, and is nondestructive. A primary advantage of this approach is that the metabolite profile of a biological sample can be acquired rapidly (1–15 min) with sufficient sensitivity to differentiate even subtle biological differences. The application of NMR to the analysis of biofluids dates back to early 1980s for the study of multiparametric metabolite compositions.[8–13] These studies have been expanded, driven to a large extent by the continuous improvement of NMR techniques in sensitivity, resolution, and especially after computer-based pattern recognition and statistical prediction approaches were introduced to help to interpret the NMR metabolic

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data in early 1990s.[14,15] Nicholson and co-workers coined the term “metabonomics” in 1999, in analogy to genomics and proteomics;[16] although in a sense, the developments in NMR-based multivariate metabolite analysis have been very much concurrent with those in the other OMIC’s fields. Since the late 1990s, such NMR-based studies have undergone an explosive growth and this trend is still continuing, with 286 papers published in 2009 (statistics obtained from Web of Science). NMR-based metabolomics (the terms “metabolomics” and “metabolite profiling” are more widely used now than “metabonomics”) is currently applied to a variety of areas such as disease diagnosis, drug discovery, microbiology, nutrition, toxicology, plant and environmental sciences.[7,17–26] In this review, we present a number of significant advancements in NMR-based methods for the analysis of small molecule metabolites in complex biological samples that have been reported primarily during the past three years. These advances include new methods in data acquisition, processing and analysis, and are summarized thematically in Fig. 1. This review focuses mostly on methodology developments as there have appeared a number of fine reviews that discuss a wide variety of applications.[5,7,18,21,22,24–29]

Samples and their preparation

Urine and blood serum or plasma are the most commonly used biofluids for metabolomics-based studies for the simple reasons that they both contain hundreds to thousands of detectable metabolites and can be obtained non- or minimally invasively.[2] A number of other fluids such as tissue extracts,[30,31] cerebrospinal fluid,[32] bile,[33–39] seminal fluid,[40] amniotic fluid,[41,42] synovial fluid, gut aspirate and saliva have also been studied. Metabolic profiling of intact tissue or its lipid and aqueous metabolite extracts is gaining more importance for biomarker detection,[7] based on the fact that changes in biological state are often more concentrated in the tissue of origin.

Detailed procedures to collect, store and prepare biofluids (*e.g.* urine, serum and plasma) or tissue samples for NMR analysis have been provided as guidelines for metabolomics applications.[2] Briefly, urine, serum and plasma usually require minimal pretreatment such as the addition of sodium azide to control bacterial growth, phosphate buffer to control pH, deuterated water to lock the magnetic field, TSP (3-(trimethylsilyl)-propionate, sodium salt), DSS (2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt) or DFTMP (1,1-difluoro-1-trimethylsilanyl methyl-phosphonic acid)[43] for chemical shift calibration and quantitation. Tissue samples, which can be directly analyzed by high resolution magic angle spinning (HRMAS) NMR, are often subjected to solvent extraction followed by analysis by liquid-state NMR. Chemical derivatization/isotope tagging can be used to improve the sensitivity and resolution in detection of metabolites containing specific functionalities.[44–46]

Advances in instrumentation

The large concentration range and high number of metabolites present in bio-samples pose technical challenges for NMR detection. Improving both sensitivity and resolution is the key to the successful application of NMR spectroscopy to metabolic profiling since quantitation and reliability are inherently less challenging issues. The advances in NMR technology,

such as the development of stronger magnets, cryogenic probes, microcoil probes, advanced pulse sequences, and isotope labeling, have dramatically enhanced NMR performance.

Generally, 500 or 600 MHz NMR instruments are used in metabolomics, as these fields are cost-effective and easily accessed, although the use of 800 and 900 MHz fields has been reported.[47–49] The use of lower field instruments can be contemplated for targeted studies. As the interface between the sample and spectrometer, the NMR probe characteristics and performance crucially determine the data quality. Major efforts have improved the performance and flexibility of NMR probes. Introduction of cryoprobes to cool the probe electronics to 20 K to reduce the thermal noise and miniaturized sample detection coils for measuring limited samples have had a large impact on sensitivity. Such technologies benefit high throughput analysis and hyphenated technologies. The sensitivity enhancement obtainable from cryoprobes can be as high as a four-fold and allows smaller quantities of metabolites to be measured.[50] Microcoil probes further enhance the ability of NMR to measure mass-limited biosamples. The signal-to-noise ratio (SNR) is increased by the use of small diameter coils since the coil efficiency is inversely proportional to the diameter of the coil. The use of microcoils with solenoidal geometry improves the SNR further as they capture more magnetic flux than Helmholtz geometry coils. Commercially available micro-coil probes can analyze samples with volume of a few microlitres (Bruker BioSpin, Billerica MA), and nanolitre detection volumes have been reported.[51] These methods are beneficial particularly when sample analytes can be concentrated into small volumes. A dual sample micro-coil probe was recently developed and can be used to acquire 1D and 2D spectra of metabolites using as little as 400 nl sample volume and sub-nanomole analyte quantities.[52] A mid-volume micro-coil probe designed to improve the coupling of LC to the NMR detection with high sensitivity has also been reported.[53] A recent innovation is the use of strip-line detection[54] which has been incorporated into a microfluidic NMR flow probe.[55]

Pulse sequence methods to suppress interfering signals

Solvent suppression methods

NMR analysis of biological samples invariably requires solvent (water) signal suppression. Sensitive detection of small molecules of interest in biofluids requires effective water suppression. The pursuit for optimal solvent suppression has continued over the past several decades. There are two basic concepts in water suppression: (1) signals of interest (for example, metabolites) can be selected by following the desired coherence transfer pathway, examples include WATERGATE-type solvent suppression including excitation sculpting;[56] (2) the interesting magnetization can be stored along the *z*-axis while the solvent is suppressed, examples include weak RF irradiation type methods such as Pre-SAT, 1D NOESY, Presaturation utilizing relaxation gradients and echoes (PURGE)[57] and combinations of gradient and weak RF pulses such as the WET sequence. Several of these sequences have been compared.[58] The exponentially expanding use of NMR for metabolic profiling is increasingly dependent on advances in automation for high throughput analysis and requires solvent suppression methods that tolerate imperfect pulse calibration and slight changes in shimming, tuning and matching. Solvent suppression should also be optimized to detect metabolites maximally and quantitatively. The new Solvent-Optimized Double

Gradient Spectroscopy (SOGGY) sequence[59] was developed to suppress unwanted magnetization based on the excitation sculpting template, which utilizes arbitrary waveforms and pulsed field gradients. Another sequence, Pre-SAT180,[60] incorporates an adiabatic toggling of an 180° pulse inversion with pre-saturation, and is aimed at canceling water distant from the detection coil which experience reduced *B1* fields. A similar approach was used to develop WET180,[61] which combines the advantage of the 180 inversion concept with the B_1 insensitive nature of WET and is robust and simple to implement, allowing for automation of the analysis.

Suppression of other interfering signals

The most commonly used method for suppressing the broad signals from large molecules (*e.g.* proteins in tissue or serum samples) is the multiple pulse spin-echo experiment Carr–Purcell–Mieboom–Gill (CPMG).[7] This sequence is generally robust, and has widely been used in a large number of (if not most) NMR metabolomics studies to date. Recently, a mathematical transformation of the standard 1D NMR spectrum was used to suppress broad signals effectively and enhance small molecule signals. This algorithm, referred to as relaxation-edited spectroscopy (RESY), is based on the calculation of the first derivative of the NMR spectrum (D-RESY) and Gaussian shaping of the free induction decay (G-RESY) and provides similar spectra to those from CPMG spin-echo experimental data.[62] The transformation can be performed using commercially available software. Another method, the Diffusion Ordered SpectroscopY (DOSY) takes the advantage of the large difference between the molecular weights of small molecules and macro molecules and provides a spectral separation of these different molecular weight groups along a diffusion coefficient dimension. The DOSY spectroscopy has been recently applied to the analysis of natural and biological media.[56,63,64]

Methods to enhance resolution and sensitivity

Isotope labeling

Generally, the complex and severely overlapped ^{1}H NMR biofluid spectra make it difficult to detect many low-concentration metabolites. Heteronuclei such as ^{13}C and ^{15}N exhibit broader chemical shift dispersions and fewer couplings, and hence provide simpler spectra than ^{1}H; thus, utilization of such nuclei potentially promises high utility in metabolomics. However, neither ^{13}C nor ^{15}N is naturally highly abundant. Hence, NMR experiments involving such low abundant nuclei have poor sensitivity. To circumvent this problem, a method to convert amino acid metabolites in body fluids to ^{13}C-acetamides by reacting with ^{13}C-acetic anhydride was reported.[44] The resulting ^{13}C-tagged metabolites were detected with ^{1}H–^{13}C 2D HSQC experiments. Due to the chemoselectivity of the *in vitro* chemical ^{13}C labeling and the low natural abundance of ^{13}C, only compounds reacting with ^{13}C-acetic anhydride were selectively detected by the 2D NMR experiments. Therefore, ^{1}H–^{13}C 2D NMR spectra with high resolution and clean backgrounds were obtained. More recently, ^{15}N-ethanolamine was used to tag a much larger class of metabolites, carboxylic acid containing species, in body fluids and convert them into ^{15}N-ethanolamides for ^{1}H–^{15}N 2D NMR analysis.[45] About 200 signals were found in either human urine or serum samples, indicating that over 150 different metabolites with carboxyl

groups are detectable by this approach, assuming most metabolites contain only 1 or 2 distinct acid groups. The relatively strong $^{1}J_{NH}$ coupling made the 2D NMR detection highly linear and unbiased to different metabolites. The concentration limit of detection was estimated to be a few μM, representing a great improvement from more conventional NMR methods. Continuing these efforts, fingerprinting lipid molecules was achieved by ^{31}P labeling using the derivatizing agent 2-chloro-4,4,5,5-tetramethyldioxa-phospholane.[65] These metabolite isotope labeling approaches have a distinct advantage for biomarker discovery utilizing biofluids that contain diverse low molecular weight metabolites. Metabolites in serum, urine, or even tissue extracts can be quantitatively analyzed with up to two orders of increased sensitivity and improved resolution in the 1D/2D NMR spectra. This *in vitro* labeling approach is of course complemented by the *in vivo* isotope labeling, which has a long and successful history. For examples, cells can be incubated in a medium containing labeled nutrients (*e.g.* [$^{13}C_6$] glucose), or even invertebrate animals can be fed a labeled diet (*e.g.* [$^{13}C_6$] glucose and [^{13}C, ^{15}N] amino acids) prior to 2D NMR analysis.[66,67] The advantage of this *in vivo* approach is again that hundreds of metabolites can be analyzed rapidly and identified ambiguously *via* heteronuclear multidimensional NMR spectroscopy or mass spectrometry.[66]

Hyperpolarizaiton methods

Dynamic Nuclear Polarization (DNP) has been shown to increase the sensitivity of NMR dramatically, as much as 10 000-fold, to enable the detection of low concentration compounds that would otherwise be unobservable by NMR.[68–70] A number of studies have been performed to expand the applications of this promising method. Several other polarization techniques such as Parahydrogen and Synthesis Allow Dramatically Enhanced Nuclear Alignment (PASADENA),[71–73] magnetization transfer from polarized helium or xenon, and chemically induced dynamic nuclear polarization (CIDNP) have proven useful for sensitivity enhancement. Among the polarization transfer techniques, DNP and PASADENA appear to hold the greatest promise for enhanced metabolite analysis. They have been successfully applied to several metabolically relevant compounds such as pyruvic acid[74] and succinic acid,[71–73] although the applicability for mixtures analysis remains to be proven. DNP and isotope labeling have recently been combined using prepolarized [1,1-^{13}C] acetic anhydride. This approach, termed “secondary hyperpolarization,” has demonstrated success in providing high SNR NMR spectra of amino acids derivatives and other biomolecules for imaging applications.[75]

Selective TOCSY

The 1D selective TOCSY provides another approach for alleviating the sensitivity and resolution limitations of NMR. The greatest advantage of the TOCSY approach is that it reduces overlapping signals to thereby improve resolution and sensitivity simultaneously. The method is highly useful in detecting metabolites at concentrations 10–100 times below those of the major components.[76] This approach has been shown to be useful for detecting targeted metabolites in biological samples.[76,77]

Covariance NMR

Covariance NMR spectroscopy combines normal Fourier transformation along the t_2 dimension with a covariance calculation along t_1. This approach provides a method to obtain high resolution along the indirect dimension using a reduced number of t_1 increments, thus saving significant time in acquiring 2D NMR spectra. The covariance processing provides an excellent example of applying statistic methods on large amount of spectroscopic data available in the NMR spectrum. It provides enhanced resolution and sensitivity which are very valuable for the analysis of natural product and biological macromolecule structures. It is particularly useful in complex mixture analysis and has been extended to metabolomics.[78,79] The method has been applied to multidimensional NMR, up to four dimensions, and to the analysis of the individual components of a mixture.[80,81] The idea of using covariance to process NMR data was first introduced by Brüschweiler and Zhang in 2004[82] and had been further developed by Martin and co-workers to obtain the equivalent of 2D or hyphenated 2D spectra from reduced 2D NMR datasets.[83–85] One caveat, however, is that in general correlation methods cannot distinguish true cross-peaks from artifacts. To alleviate this sensitivity to artifacts, more spectra can be obtained and used in the calculation. Freely available code, covNMR, can be obtained running under Linux with or without NMRpipe software (NMR Science Inc., North Potomac, MD). The covariance NMR approach has already been implemented in a commercial software package, MestReNova (Mestrelab Research, Escondido, CA).

Hyphenation of chromatography and NMR

The resolving power of NMR increases substantially when coupled with chromatographic methods. Liquid chromatography (LC)-NMR offers great capabilities for biofluid analysis due to its ability to separate complex mixtures into individual components and provide the information necessary for structural elucidation of the components. In recent years, a number of applications have been published dealing with the use of LC-NMR in detecting drug metabolites or elucidating unknown compounds.[86] Real-time 2D NMR spectra of amino acids mixtures and urine samples were also recorded on a commercial on-flow HPLC system based on the Hardamard matrix for 2D-NMR frequency encoding.[87] Nevertheless, the use of LC-NMR for metabolic profiling of biofluids has not become routine due to the high complexity of biofluids and the limited mass sensitivity of NMR. LC-NMR also has to address practical issues related to sample coupling between the LC and the NMR detection coil. Sample coupling is usually not a problem for off-line LC-NMR because the chromatographic fractions eluted from the LC can be loaded into regular NMR tubes (*e.g.* 5 mm) and then detected using conventional NMR probes. The on-line LC-NMR mode requires the use of a continuous-flow probe with a suitably matched sample detection volume to provide sufficient sensitivity for low concentration analyte detection.[53] Pre-concentration systems prior to NMR detection including SPE and column trapping[88] offer clear advantages in signal enhancement. Further, incorporation of a cryo-flow probe for such applications not only increases the sensitivity but also allows for rapid characterization of metabolites.[89]

Data analysis

Preprocessing

Metabolomic studies often involve comparing dozens to hundreds of samples in order to determine the statistical variations between diseased and healthy groups and between different physiological states. Extreme care should be taken since non-biological variations such as various experimental artifacts can potentially introduce some systematic and random variations in the NMR spectra. For example, unwanted macro-molecule signals may cause phase and baseline distortions; factors such as solvent, pH and ion strength can introduce variations in the peak locations thus increasing the need for peak alignment and spectral binning; water suppression, shimming and acquisition parameters, as well as sample handling prior to the measurements can induce additional variations. The effects of these artifacts are often exacerbated when spectra under comparison are acquired in multiple batches or labs, although in general NMR suffers much less from these issues than other analytical methods. Various data processing steps are sought to account for the variations irrelevant to biological interests: phasing, baseline correction, peak alignment, binning, scaling and normalization.[90] During the past three years continued efforts have made to improve the robustness and reliability of data binning, alignment and normalization algorithms for 1D and 2D NMR spectra.[74–76] Commercial software especially designed for NMR-based metabolomics has been developed to provide the functions described above. Examples include AMIX (Bruker Biospin), KnowItAll (Bio-Rad, Life Science, Hercules, CA) and MestReNova. Recently, a freely available software, MetaboMiner, was reported that is capable of identifying metabolites from 2D TOCSY and HSQC-spectra of biofluids.[91]

Direct approaches for correcting small frequency shifts include peak alignment and frequency binning. First, a global alignment is done using a signal reference peak such as TSP or alanine to correct any small systematic shifts due to sample matrix effects. A fine alignment on individual peaks can then be performed either manually or using special algorithms such as the recursive segment-wise peak alignment method.[92] Data binning (spectral averaging or bucketing) is still popular because of its simplicity and is often used following spectral alignment. Binning can partially account for any small frequency shifts in peak locations by reducing spectral resolution.[90] Analysis is seldom adversely affected unless severe binning is performed. Considering the inaccuracy in quantitation induced from assigning neighboring peaks to the same bin, variable binning is now preferred over fixed binning. The variable binning is implemented in software such as KnowItAll or MestReNova through which variable bins are automatically assigned based on spectral peaks, while still allowing the users to manually perform fine adjustments. Adaptive binning,[93] using the undecimated wavelet transform, has been demonstrated to have advantages over conventional binning on both theoretical and experimental metabolomics-related NMR spectra. Other binning methods such as Gaussian binning[94] and adaptive intelligent binning[95] have also been developed for NMR spectral data.

Data scaling, which allows an emphasis to be placed on signals from low concentration metabolites, is often used. A number of scaling methods are popular, including variance

scaling (division by the standard deviations of peak intensities across the set of spectra) and Pareto scaling (division by the square root of the standard deviations). Log scaling has been used to reduce the size of very large and dominant peaks. The data are then typically mean centered by subtracting the average of all the spectra. The most widely used normalization methods are total of sum normalization and creatinine normalization.[90] However, these approaches have been frequently questioned due to their quantitative inaccuracy, especially when dealing with spectra dominated by strong signals (such as glucose signals) in blood or those from highly disturbed systems (such as diabetes).[96] New normalization algorithms such as probabilistic quotient normalization[97] and histogram matching normalization[98] appear to show unique advantages.

Statistical analysis

The approaches used for the statistical analysis of NMR data are conceptually the same as those for other analytical data, and include two steps: exploratory analysis and/or confirmatory analysis. Exploratory analysis aims at finding patterns in the data using methods such as hierarchical clustering analysis (HCA) and principal component analysis (PCA). Confirmatory data analysis, on the other hand, makes an explicit use of the group labels (*i.e*. supervised analyses), and allows for statistical inference regarding features and groups. Examples are the partial least square-discriminant analysis (PLS-DA), orthogonal signal correction-PLS-DA (O-PLS-DA), *t*-test and supervised classification procedures such as logistic regression and soft independent modeling by class analogy (SIMCA). Commonly used chemometric software packages include R (http://cran.r-project.org/), SAS (SAS Institute Inc., Cary, NC, USA), PLS toolbox (Eigenvector Research Inc., Wenatchee, WA, USA), Matlab (The MathWorks Inc., Natick, MA, USA), Pirouette (Infometrix Inc., WA, USA), Minitab (Minitab Inc., PA, USA) and SIMCA-P+ (Umetrics, Umea/Malmo, Sweden). Certain software packages such as KnowItAll (BioRad, Philadelphia, PA) and AMIX (Bruker, Billerica, MA) are designed to be user friendly for NMR focused metabolomics studies and typically start from raw NMR data, perform the necessary data preprocessing and ultimately various multivariate statistical analyses.[7,99]

Statistical TOCSY (STOCSY) and Heterospectroscopy (SHY)

STOCSY was developed to identify latent biomarkers from complex NMR spectra of biofluids.[100] In STOCSY, the correlation analysis is performed on a set of 1D NMR spectra from different samples (as opposed to covariance NMR, which requires multiple spectra from the same sample) to construct a pseudo-2D spectrum that is highly useful in identifying metabolites from heavily overlapped NMR spectra.[100] Peaks from the same metabolites are intrinsically correlated (ideally $R = 1$) and thus produce pseudo cross-peaks as represented in the STOCSY spectrum. STOCSY may potentially contain artifacts induced by nonstructural correlation (*e.g.* biological correction) under diverse biological conditions and sample size. Recently, it was shown that STOCSY can be an effective and robust tool in the routine assignment of complex spectra.[101] The STOCSY strategy was successfully used to process a series of 2D DOSY spectra under different pulsed field gradient conditions that demonstrated its ability to characterize urinary biomarkers and recover additional information from plasma NMR spectra.[64] An important extension of the correlation approach is SHY, which was introduced to enhance the coanalysis of NMR and other

spectroscopic datasets (*e.g.* MS data) acquired on the same samples.[102,103] The SHY approach is of general applicability in biofluids analysis and has unique advantages for exploring biomarkers and/or correlated biological events as well as producing highly reliable results through its ability to perform multi-spectroscopic cross-validation.

Metabolite quantitation

For a number of studies, quantitative analysis of the metabolite profile is crucial. The acquired 1D proton NMR spectra that incorporate an internal quantitative standard are often used for the determination of metabolite concentrations. The most popular internal standards are water soluble salts such as TSP or DSS, which are variants of TMS (tetramethylsilane). The use of TSP or DSS is advantageous in offsetting experimental variations in amplitude (in addition to providing a chemical shift reference) and can provide high quantitative accuracy, with errors below 2%. However, for samples containing binding factors such as proteins in blood or tissue extracts, these ionic chemicals are not desirable for internal calibration.

An artificial signal termed ERETIC™ (Electronic REference To access *In vivo* Concentrations) was developed to serve as a concentration reference.[104,105] In this technique, an external signal is generated by the rf electronics and provides a reference to calculate analyte concentrations. The replacement of the spiked reference with this electronically synthesized reference makes the measurement of absolute concentration easier and more reliable since it is free of sample contamination. It is particularly useful in the situations where internal standard cannot be used as the case with blood and tissue samples. The utility of the ERETIC™ method was evaluated on biological specimens such as tissue and was proven to be a more accurate and stable reference than TSP.[104,105] Traditional implementation of ERETIC™ requires either an additional spectrometer channel or a high frequency waveform generator clocked with the NMR instrument. Successful achievement using a capacitive coupling between a secondary RF channel and the gradient coil allows the quick integration of ERETIC™ into a standard spectrometer with minimal hardware modification.[106] However, one downside is that the accuracy of the method is susceptible to probe tuning changes. A new alternative is to use the solvent (mostly water in biological samples) signal as a quantitative reference, which also results in high accuracy and precision (2% or better) over a wide range of concentrations (>6 orders of magnitude). This solvent referencing method is robust and simple, and only requires the acquisition of a separate spectrum for the solvent signal.[107]

Often, the accuracy of 1D NMR-based quantification is affected by the high peak overlap due to the complexity of biofluids. 2D NMR spectroscopy alleviates peak overlap issues substantially and presents an accurate method for metabolite concentration determination. 2D NMR methods are generally used to determine the connectivity between the nuclei to make assignments and identify metabolites. The 2D ^{1}H-^{13}C HSQC experiment was recently shown to have high utility in quantifying metabolites from tissue extracts and urine with accuracy higher than 1D NMR,[108] GC-MS or LC-MS.[109] Intensity losses suffering from the low natural abundance of ^{13}C may also be recovered by tagging metabolite classes of interest with isotope-labeled tags.[44,46] Other 2D methods such as 2D–J spectroscopy,

correlation spectroscopy (COSY), and total correlation spectroscopy (TOCSY) may also be useful in assisting metabolite quantitation.

Databases to assist metabolite identification

A number of NMR database resources have been developed to facilitate the identification of metabolites in biological fluids. Publically available electronic databases include the Human Metabolome Database (HMDB),[110,111] the NMR metabolomics database of Linkoping, Sweden (MDL) (www.metabolomics.bioc.cam.ac.uk), and The Magnetic Resonance Metabolomics Database (www.liu.se/hu/mdl/main/). These databases are designed to contain or link chemical data, clinical data, and molecular biology/biochemistry data; thus they not only provide efficiency in metabolite identification but also facilitate the elucidation of relevant biological pathways. Other databases with potential utility for metabolite identification from NMR spectra include the Spectral Database for Organic Compounds (SDBS) (www.riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi?lang=eng), NMRShiftDB (www.ebi.ac.uk/NMRshiftdb/) and The BioMagResBank (www.bmrb.wisc.edu/). A number of companies have developed NMR databases, including Bruker, BioRad, ACD, Chenomx and others.

Trends in metabolomics applications

NMR-based metabolomics is witnessing an exponentially increasing number of studies in a large variety of applications. Many of these studies have been reviewed especially in the areas of disease diagnostics,[7,112] drug discovery,[4,17,18,29,113] nutrition,[21,22] plants,[25,114–116] toxicology,[24,117] environmental science,[26,118,119] and microbiology,[19] which highlight the increasing impact of the metabolomics field. In light of these numerous reviews, we have focused specifically on technical advances and kept the discussion of applications concise.

In medicine, driven by the potential for earlier disease detection, therapy or recurrence monitoring, there has been an explosive growth in the application of NMR-based metabolomics.[7] The noninvasive approach is highly useful in identifying the presence and severity of diseases from urine and blood samples that may improve testing accuracy and reduce follow-on testing including the use of highly invasive biopsy. To date, a variety of diseases including cancers,[5] diabetes,[120–123] inborn errors of metabolism,[44,109,124,125] Parkinson's disease,[126] gastrointestinal disease,[27] and heart disease[28] have been distinguished based on the NMR metabolic profiles. However, most of these studies are still at an early biomarker discovery stage, and therefore it will be crucial to perform careful cross-validation and ultimately external validation studies on separate sample sets in order to verify the reliability of the discovered biomarkers. Significant progress has been made in the area of drug toxicity evaluation for preclinical drug discovery[23,24,26,117,127,128] and therapy (drug and nutrition) monitoring.[20,29,113,129] A major goal of these studies is to achieve more personalized medical treatments.[130]

NMR-based metabolomics has made a large impact in the area of environmental science[26,118,119,131] and has been applied to a number of aqueous animals and microbes.[19,132] Such studies promise excellent means to widen the understanding of the environmental impact on metabolism. Studies comparing the data on fish from different

sources have demonstrated that NMR-based metabolomics can produce data that are consistent between laboratories, a necessary step to support the use of metabolomics for regulatory environmental applications.[47] The approaches developed thus far may lead to adoption into environmental monitoring and chemical risk assessment.[29]

Conclusions

Technological developments in NMR have enabled the rapid detection of a sizable number of low concentration metabolites, and the analysis of complex biological spectra through the development of metabolomics-based methods. These developments are in part a response to the demand for applications in a number of important areas with potentially large societal impact. Efforts are continuing to further improve both resolution and sensitivity to allow lower concentration species to be detected, and to turn these data into actionable information. The number of NMR-based studies in metabolomics for a variety of applications including early disease diagnosis, prognosis and therapeutic monitoring, natural, environmental, *etc.* is growing enormously. Significant efforts are also being made to understand and alleviate the effects from a large number of confounding factors such as age, gender, diet and environment that interfere in the detection of biomarkers. Current trends indicate that NMR continues to play a central role in metabolomics. Combinations of NMR with MS, the other mostly commonly used technique, along with further efforts to allow NMR to probe even lower concentrated metabolites, and separation techniques such as liquid chromatography will further enhance the capabilities of NMR in metabolite profiling. NMR's future in metabolite profiling is indeed very bright.

Biographies

Shucha Zhang

Shucha Zhang graduated from the School of Chemistry and Chemical Engineering at Shanghai Jiao Tong University with her BS and MS in 2002. She received her MS in environmental engineering from University of Delaware in 2004. Shucha Zhang finished her PhD study in the Chemistry Department at Purdue University. Her dissertation is focused on biomarker discovery in diabetes using NMR and MS-based metabolomics.

G. A. Nagana Gowda

G. A. Nagana Gowda is presently working as a Research Scientist. He received BSc degree (Physics, Chemistry, and Mathematics) in 1983 and MSc degree (Analytical Chemistry) in 1985 from Mysore University, India and PhD degree (NMR Spectroscopy) in 1999 from Bangalore University, India. He did his Postdoctoral work at the State University of New York, Buffalo, USA (1996–1998). He worked at the NMR Research Centre, Indian Institute Science, Bangalore, India (1986–2001). During 2001–2006, he was a faculty member at the Centre of Biomedical Magnetic Resonance, Lucknow, India, before moving to Purdue.

Tao Ye

Tao Ye received his BS in Applied Chemistry from Peking University, Beijing in 2001. After graduation he went to the University of Oklahoma, Norman and received his MS in Organic Chemistry in 2004. He is now pursuing his PhD in Analytical Chemistry under the direction of Professor Daniel Raftery at Purdue University. His current research focuses on developing new methods to improve the performances of NMR and MS-based metabolic profiling.

Daniel Raftery

Professor Raftery has been a member of the Chemistry Department at Purdue University since 1994. He received his AB degree from Harvard, and his PhD from U.C. Berkeley. Before joining Purdue, he was an NSF Postdoctoral Fellow at the University of Pennsylvania. Dr Raftery has published over 100 peer reviewed journal articles, several book chapters, and has 4 issued patents to date, as well as 6 more under review. Professor Raftery leads a research group of 25 graduate students, undergraduates and postdoctoral scientists who are focused on developing new methods and applications in metabolomics and bioanalytical chemistry.

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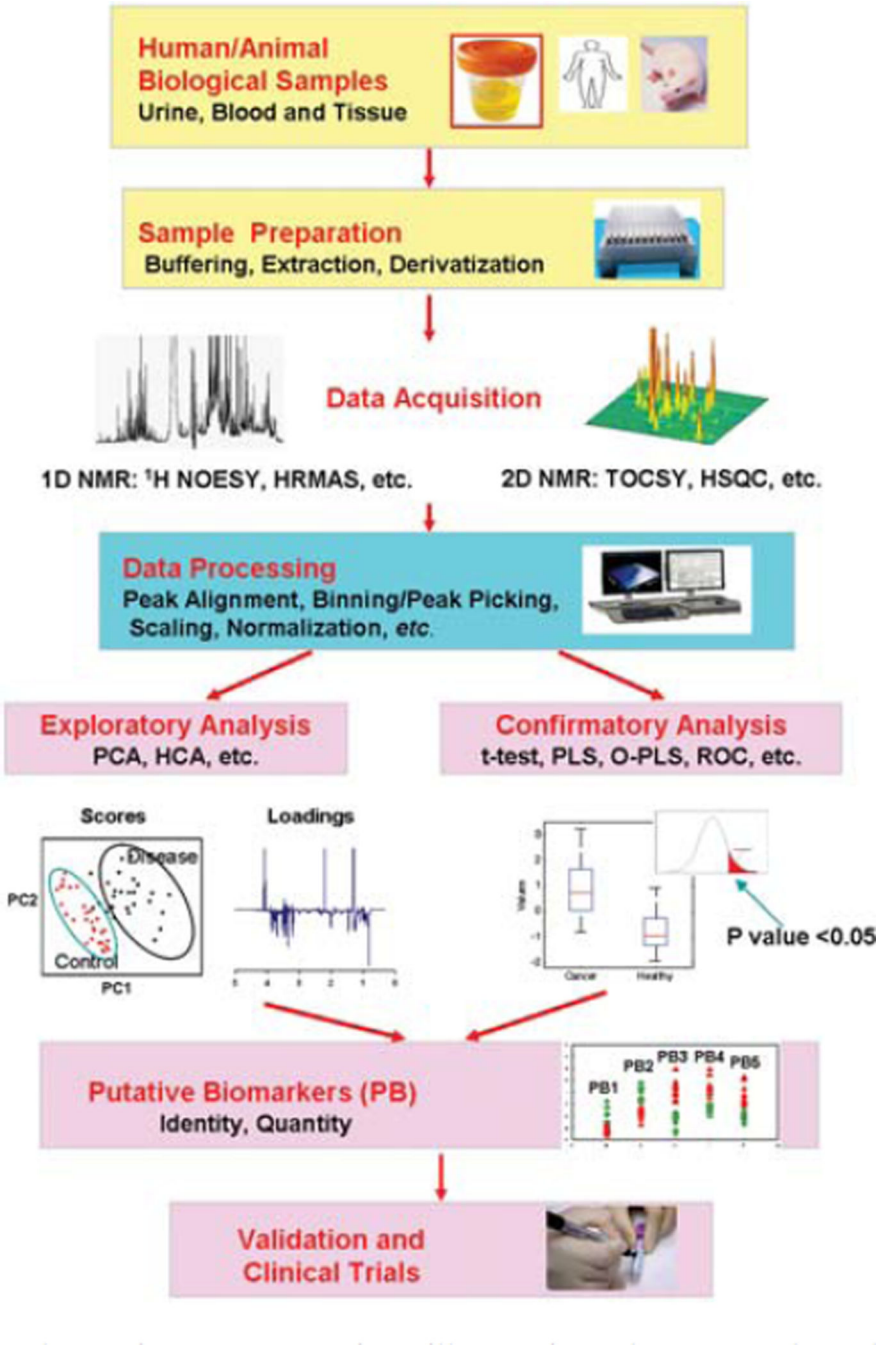

Fig. 1.

Schematic representation illustrating the NMR-based metabolomics workflow. DA: Discriminant analysis; HRMAS: High-resolution magic angle spinning; NOESY: Nuclear Overhauser effect spectroscopy; O-PLS: partial least square analysis with orthogonal signal correction; PCA: Principal component analysis; HCA: Hierarchical cluster analysis; ROC: Receiver operating characteristic; PLS: Partial least squares; TOCSY: Total correlation spectroscopy; HSQC: Hetero-nuclear single quantum correction spectroscopy.