The Future of NMR-Based Metabolomics

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Abstract

The two leading analytical approaches to metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Although currently overshadowed by MS in terms of numbers of compounds resolved, NMR spectroscopy offers advantages both on its own and coupled with MS. NMR data are highly reproducible and quantitative over a wide dynamic range and are unmatched for determining structures of unknowns. NMR is adept at tracing metabolic pathways and fluxes using isotope labels. Moreover, NMR is non-destructive and can be utilized *in vivo*. NMR results have a proven track record of translating *in vitro* findings to *in vivo* clinical applications.

Graphical Abstract

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Introduction

The metabolic state of an organism depends on its genome, transcriptome, proteome, epigenome, microbiome, and exposome (environment). Thus, metabolomics, the study of small molecules (< 1,500 Da) in living systems, provides information with a high potential for accurately describing the physiological state of an organism. The two most successful approaches to determining the metabolic state of an organism have been mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Several years ago, the number of publications utilizing the two approaches were comparable; more recently, however, MS-based metabolomics has clearly overtaken NMR-based metabolomics. This state of affairs prompted the organization of a workshop to review the current state of NMR-based metabolomics, to assess its strengths and weaknesses, and to envision its future potential. As reported here, this workshop ("NMR-Based Metabolomics," held in the Discovery Building, Morgridge Institute for Research, Madison, Wisconsin, USA, on June 10, 2016) highlighted a number of benefits of NMR-based metabolomics that appear to be currently underappreciated. MS and NMR offer different strengths, which can be used synergistically. The workshop stressed the need for more extensive small molecule databases and improved standards at each step of a metabolomics study.

The metabolome

The two major fields of chemical research on biological small molecules, metabolomics and natural product discovery, have the similar goals of identifying and characterizing small molecules, either in their isolated active state (natural product chemistry) or as mixtures (metabolomics) [1]. The number of small molecules of importance to humans is far greater than those currently represented on metabolic charts, with the excess constituting current "metabolic dark matter" (Figure 1). The swapping of metabolites between pathways in humans and those of organisms in the human microbiome increases the network of relevant reactions by a staggering amount. The HMDB [2] lists 42,000 metabolites and the number of lipid variants is on the order of 100,000; thus, a lower limit of expected endogenous and exogenous human metabolites is around 150,000, but the actual number of metabolites could be much higher. Of this vast number of metabolites, only 1,500 may be identified from global profiling, 200–500 from targeted profiling, and far fewer are routinely subjected to quantitative analysis.

NMR and its advantages

Despite its lower sensitivity, NMR spectroscopy offers many unparalleled advantages over MS [3,4]. NMR offers a window into observing and rigorously quantifying all of the more abundant compounds present in biological fluids, cell extracts, and tissues without the need for elaborate sample preparation or fractionation. NMR offers advantages for compounds that are difficult to ionize or require derivatization for MS. NMR allows the identification of compounds with identical masses, including those with different isotopomer distributions. NMR is the mainstay for determining structures of unknown compounds. Through the use of stable isotope labels, NMR can be used to elucidate the dynamics and mechanisms of metabolite transformations and to explore the compartmentalization of metabolic pathways. NMR has advantages in drug screening [5]. Finally, site-specific NMR imaging and spectroscopy offer approaches for metabolic studies in living organisms.

Strategies for the identification of metabolites in complex mixtures from NMR data have been reviewed recently [6]. The most important nuclei in biomolecular NMR studies are ^{1}H (proton), ^{13}C, ^{15}N, and ^{31}P. Of these, ^{1}H is the most sensitive followed by ^{31}P; both are present at near 100% natural abundance. ^{31}P NMR is useful for studies of cellular energy states *in vivo* and *ex vivo*, but a limitation is that the ^{31}P signals from most phosphorylated compounds overlap. One-dimensional (1D) ^{1}H NMR is the most widely used NMR approach in metabolomics. Signals are either binned and then analyzed or fitted to patterns of signals corresponding to the metabolites expected to be present in the mixture. The latter approach can be problematic in that many ^{1}H signals overlap in ways that offer alternative fitting solutions, a problem that can be overcome by standardizing the analysis in terms of biofluid, solution conditions, data collection protocol, and by employing probabilistic fitting (Bayesil) [7]. ^{13}C NMR signals cover a 200 ppm range compared with 10 ppm for ^{1}H and as a consequence are better resolved; however, the low sensitivity of ^{13}C (less by a factor of 8 or more) is compounded by its low natural abundance (1.1 %). Two dimensional (2D) NMR methods offer improved approaches for unambiguous identification of metabolites in mixtures. These 2D methods include ^{1}H-^{1}H COSY (correlated spectroscopy), ^{1}H-^{1}H TOCSY (total correlation spectroscopy), and ^{1}H-^{13}C HSQC (heteronuclear single-quantum correlation). A widely used software package (rNMR) matches regions of interest in spectra of standards to those in experimental mixtures for compound identifications [8]. Software is available for automating metabolite identification from combined TOCSY and HSQC data [9,10]. By setting tolerances for the matching of ^{1}H and ^{13}C signals, one can maximize compound identification while minimizing false positives [11]. This approach has been extended to a calculated confidence level for compound identifications from NMR data [12]. Another approach for connecting signals from individual compounds in mixtures is based on searching for statistical correlations among the intensities of NMR signals from various samples [13]. Nuclei present at low natural abundance ^{2}H (deuteron), ^{13}C, and ^{15}N serve as ideal metabolic tracers [14].

Need for standards in NMR metabolomics

Standard NMR spectra and associated information on small biological molecules are available from freely-accessible databases, including HMDB [2], BMRB [15], TOCCATA

[9], and COLMAR [10], but they still cover only a fraction of relevant compounds. A repository has been established for results of metabolomics studies from the NIH Common Fund Centers [16]. The Coordination of Standards in Metabolomics (COSMOS) Initiative is developing a robust data infrastructure and new data exchange standards (http://nmrml.org) for metabolomics data and metadata to support workflows metabolomics applications [17]. One of the COSMOS projects is a website (http://metabolomexchange.org) that federates data available from the leading metabolomics data repositories. Best practices and standards have been published for metabolic phenotyping of biological fluids [18,19]. An open-source platform for complete NMR metabolomics data handling (MVAPACK) has been developed as a step toward establishing best practices for the analysis of metabolic fingerprinting data [20].

Sample preparation

Certain biofluids, e.g. cerebrospinal fluid, require little or no preparation for NMR. Others, such as plasma contain proteins and lipids that interfere with NMR spectral quality. Treatment with methanol at solvent-to-serum ratio of 2:1 (v/v) has been shown to remove lipoproteins and minimize the loss of metabolites [21] enabling the detection of about 67 different compounds [22]. Another promising protocol utilizes the removal of protein by added silica nanoparticles [23].

Tagging

An approach for compounds with overlapping ^{1}H signals or present at lower concentration is to tag them with an NMR-active label. Nitrogen-15 with attached hydrogen is an attractive tag because 2D ^{1}H-^{15}N signals can be acquired at high sensitivity without interference from signals from unlabeled compounds owing to the low natural abundance of ^{15}N [24,25]. Such tags also provide a permanent positive charge for MS analysis.

Combining NMR and MS

As reviewed recently [26], advances in NMR- and MS-based metabolomics, including the combination of the two approaches, promise to greatly improve the identification and quantitation of compounds in mixtures. One example is the simultaneous analysis of DI-ESI-MS and 1D ^{1}H NMR spectral data to yield accurate mass measurements and class separation scores [27]. Other approaches filter data from one approach against the other to increase the number of compounds confidently identified [28,29]. Another method identifies compounds by exploiting the principle that abundance/intensity ratios are relatively constant for the same metabolite in different samples [30]. Combined NMR and MS has advantages for isotope tracing experiments and metabolic flux analysis. MS generally quantifies isotopic labeling distributions but even with MS/MS often does not give the specific labeling position, which is available from NMR.

Quantification

If 1D ^{1}H NMR peaks from a compound are well resolved with acceptable signal-to-noise, their intensities correlate linearly with its relative concentration. To determine absolute

concentrations, one adds a standard of known concentration. The cross peak intensities of 2D ^{1}H-^{13}C HSQC spectra, however, do not correlate linearly with concentration. One can collect spectra of mixtures with known concentrations bracketing those of the unknowns and use these to determine factors that translate peak intensity to concentration [31]. Peak intensities in 2D ^{1}H-^{13}C HSQC spectra can also be converted to concentration by from the slopes generated by spectra utilizing different replicates of the pulse sequence module; linear extrapolation back to zero time of the peak intensities following the delays from one and two modules yields the "HSQC$_0$ spectrum" whose peak intensities are proportional to concentration [32]. Spectral overlaps can be accounted for by methods such as FMLR (fast maximum-likelihood reconstruction) [33]. A new experiment (^{1}H-^{13}C QUIPU HSQC) aims to quantify in one map a complex mixture composed of low concentrated metabolites [34]. Another approach, one that requires full ^{13}C labeling, achieves quantification through the collection of ^{13}C-^{13}C CT-TOCSY spectra and the application of analytical approximations based on the known carbon-backbone topologies [35].

Applications of metabolomics

Applications of metabolomics include disease diagnosis, monitoring the effects of medical interventions including drugs, detection of adulteration of food, and analysis of biochemical pathways and their perturbations resulting from mutations, aging, diet, exercise, or life style. A recent study showed how *ex vivo* 1D ^{1}H NMR spectroscopy can be used for the simultaneous identification of quantification of coenzymes that report on cellular function [36]. Another study used this approach to investigate alterations in the energy/redox-metabolome in dopaminergic cells exposed to environmental/mitochondrial toxins [37]. Studies of the metabolomics of model organisms are both timely and important for understanding of their different biology [38]. Protocols have been described for studies of the metabolomics of bacteria [39] and plants [40]. Metabolomics, along with activity-guided fractionation followed by structural analysis, constitutes a powerful approach for identifying biologically active compounds for studies in chemical ecology [41]. Metabolomics is used regularly in drug discovery programs to uncover the efficacy, specificity, or toxicity of lead compounds [42]. Metabolomics can provide information on the *in vivo* mechanism of action and to eliminate compounds likely to cause problems with side effects [43]. Recent studies have utilized metabolomics to search for biomarkers for colon cancer [44] and multiple sclerosis [45].

Future technology

All technologies that increase NMR sensitivity are of extreme importance as are improvements in sample preparation [46]. Approaches to high sensitivity include NMR spectrometers with ultra-high-field magnets operating at ^{1}H resonance frequencies of 1.2 GHz or higher. The first such systems are scheduled for delivery in 2017. Small high-temperature superconducting coils can maximize the signal per sample mass: a ^{13}C-optimized 1.5-mm high temperature superconducting NMR probe has enabled novel ^{13}C NMR studies of natural products [47], and this has been followed up with a ^{1}H-^{13}C dual-optimized NMR probe based on double-tuned high temperature superconducting resonators

[48]. These probes take advantage of the excellent peak dispersion of ^{13}C spectra [49], which can be augmented by further 2D ^{13}C-^{13}C experiments, such as INADEQUATE [50]

Hyperpolarization offers an approach for enhanced sensitivity with even higher potential. The underlying physics utilizes the magnetic moment of the unpaired electron, which is roughly 2800 times that of ^{13}C and 6900 times that of ^{15}N, to polarize nuclear spins. First demonstrated by Golman and coworkers [51], studies utilizing hyperpolarized ^{13}C to increase sensitivity are becoming routine. An exciting advance is the discovery [52] of an efficient and inexpensive method for hyperpolarizing ^{15}N spins at room temperature. Enhancements are on the order of >10,000 enabling the detection of NMR signals for over an hour [52]. It may become possible to use this approach to tag a range of compounds for metabolomics studies *in vivo*.

Conclusions

The workshop demonstrated that NMR-based metabolomics promises to continue to play an important role in the studies of complex mixtures of small biological molecules, their metabolic networks, and their interactions with biomacromolecules. Although the development of new and better methods continues to be an integral part, the field needs to focus on developing standardized, enlarged, and integrated databases of NMR data on small molecules as well as archives representing the NMR metabolic fingerprints of standard biological fluids and tissue extracts from humans and model organisms. Standardization of best practices for sample preparation, data collection and analysis should enhance the reproducibility of results within the metabolomics community, while at the same time avoiding the risk of adhering to methods and protocols that are suboptimal in a field that is very much in flux. In order to overcome the current skepticism in omics [53], it will be advisable for metabolomics to build the ideas of reproducibility and data sharing into every tool and database. Future work is expected to build upon core strengths of NMR spectroscopy, which includes its versatility and specificity in the form of 1D and higher dimensional spectra, its reproducibility, its quantitative ability, its capability for following chemical reactions and flux, its ability to identify compounds and deduce structures of unknowns, and its growing potential for collecting metabolomics data *in vivo*.

Acknowledgments

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Highlights

- NMR offers advantages for metabolomics that may be currently underappreciated

- In future, NMR-based metabolomics needs to focus on its inherent strengths

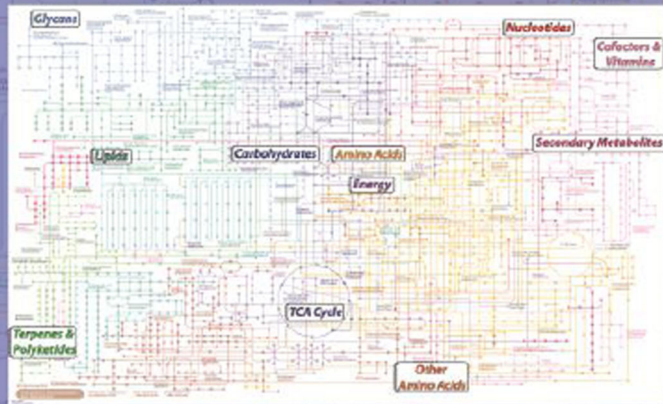- Reproducibility and data sharing are of key importance in technology development

Figure 1.
Schematic representation of metabolic "dark matter".