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Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry: Complementary Approaches to Analyze the Metabolome

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Abstract

Over the last decade, the rapid advancement of analytical technologies has made it feasible for researchers to target a wider area of any given biological sample. Metabolomics, an emerging field of scientific research, involves studying the endogenously synthesized small molecules within the biological system. This recently developed 'omics' platform has been used for the discovery of disease-specific biomarkers; and for providing deep insights into the etiology and progression of a variety of endocrine disorders, including type 2 diabetes, polycystic ovarian syndrome, Addison's disease, etc. Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) are the two most powerful and information-rich analytical platforms that have currently been employed in metabolomics studies worldwide. The unique properties of NMR, including a high degree of reproducibility, relative ease of sample preparation, highly quantitative nature, and inherently non-destructive nature, have made it an eminent technique useful in several disciplines of metabolomics. However, a major drawback of this approach is its low sensitivity (≥ 1 μM) when compared with MS. Conversely, MS has the potential to detect the metabolites in the femtomolar to the attomolar range and has a higher resolution (~10^3-10^4) relative to NMR, but quantification and sample preparation are a little cumbersome. This mini-review discusses the assets and limitations of NMR and MS approaches for metabolomic studies and the latest emerging technological developments that are being used to cope with these limitations in metabolic applications.

Keywords: Cryoprobe, Metabolic Imaging, Metabolomics, Signal-to-Noise, Time-of-Flight

1. Introduction

Metabolomics – the youngest kid in the block of different 'omics' platforms - deals with the characterization of the complete set of small molecules (molecular weight <1500 Daltons) at a given instant of time and its variation due to a variety of stress factors. Since the metabolome has been often linked with the physiology of the organism, it has increasingly been integrated into systems biology along with genomics, transcriptomics, and proteomics. Indeed, metabolomics has been helpful for not only providing disease-specific biomarkers; but has also been useful in providing deep insights into the etiology and progression of a variety of complex disorders.

The endocrine system is the major controller of body metabolism that regulates a spectrum of functions such as growth and development, sexual function and reproduction, metabolism, appetite, etc.[1]. Endocrine-related diseases thus influence a number of metabolic traits or pathways, which are affected by multiple genetic and environmental factors and cannot be characterized by a single measurement. Also, the clinical characterization of endocrine-related diseases generally includes the measurement of single effector hormone and their major

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pituitary regulators. However, the main limitation of the current laboratory characterization of the endocrine disorders is the moderate coefficient of variation of the most relevant immunoassays, moderate specificity, and substantial inter-laboratory differences. Therefore, metabolic profiles are considered as the ideal approach to obtain the information which can be used not only for understanding the disease mechanism and biomarker discovery but also can be useful for determining the effects of therapeutic interventions on metabolic pathways affected in the disease. The integration of metabolomics into clinical studies has grown substantially over the last few years due to its effectiveness in discriminating various pathophysiological states. Indeed, over the past few years, metabolomics has been widely used for identifying potential biomarker candidates and for delineating the mechanisms influencing endocrine-related diseases, including type 2 diabetes[2-4], polycystic ovarian syndrome[5-7], thyroid disorders[8-10], Addison's disease[11], endometriosis[12], and osteoporosis[13-16].

Metabolomics is a technology-driven approach, and application-driven emerging science, wherein the recent advancements in computational methods, analytical tools, algorithms, software, and statistical tools propel the field forward. Although various analytical platforms have been used, such as Mass Spectrometry (MS), Gas Chromatography (GC), Liquid Chromatography (LC), infrared (IR) and ultraviolet and visible (UV–Vis) spectroscopy, and Nuclear Magnetic Resonance (NMR) spectroscopy; the most important analytical tools commonly used in metabolomics research are NMR spectroscopy and Mass spectrometry[17-19]. Both techniques have their advantages and disadvantages; thus, there is no single analytical technique that is fully suited for performing metabolomics studies. Indeed, NMR and MS have been demonstrated to be complementary and powerful analytical approaches for the complete characterization of the metabolome[20]. While the choice largely depends on the objective of the research study and the nature of the samples, in this review, we will list the positive and negative aspects and thus would draw a direct comparison between these two analytical approaches.

2. NMR Spectroscopy

A basic NMR experiment for spin-half nuclei can be understood as follows[21]: quantization of the nuclear spin angular momentum aligns the tiny nuclear magnets either parallel (α state) or antiparallel (β state) relative to the applied magnetic field. Depending on the nucleus under study, alignment of one of the spin-states (e.g., α or β in case of proton or carbon) is energetically more favored, and a net bulk magnetization over an ensemble of nuclei builds up with a population difference between favored and unfavored spin-state. Electromagnetic radiation in the range of radiofrequency is then used to realign this bulk magnetization along a direction perpendicular to the magnetic field. The bulk magnetization then relaxes back to the equilibrium position while precessing about the applied magnetic field. Each nucleus, depending on the amount of electron density present around that nucleus, precesses at a characteristic NMR frequency called the 'chemical shift' and gives rise to a detectable oscillating magnetic field. The time-domain oscillations are then Fourier-transformed to yield the standard frequency-domain NMR spectrum, in which different nuclei give signals at characteristic chemical shift frequencies. For metabolomics applications, one is typically interested in the NMR-active nuclei ^{1}H and ^{13}C.

NMR spectroscopy is a compelling and versatile analytical technique used in metabolomics since it allows the qualitative and quantitative analysis of chemical compounds from complex mixtures and the structural elucidation of unknown compounds[22,23]. The versatility of NMR spectroscopy to different sampling morphologies (liquid samples, solids, gases, and tissue samples) and range of volumes (μL to nL) has vastly enhanced the applications of NMR in the field of metabolomics[23-31]. Further, NMR spectroscopy-based metabolomics has been used in various research fields, including medical research, toxicology, nutrition, drug metabolism, food science, metabolic diseases, etc.[17,22,32-37]. NMR has an important role in metabolomics owing to the use of easy and rapid sample preparation methods, full recovery of precious samples after the data recording, lack of dependence on chromatographic separation, and a high degree of reproducibility[38,39]. It is non-selective (i.e., it is not biased towards detecting specific metabolites present in a biological sample) and can simultaneously analyze all abundant aqueous metabolites present in a biological sample containing a mixture of metabolites. NMR experiments generally require minimal sample preparation, often consisting only of pH adjustments and the addition of an internal standard.

Further, one of the greatest strengths of NMR lies in its utility for the absolute quantification of metabolites

as the integral of a peak in the NMR spectrum is directly proportional to the molar concentration of the corresponding metabolites[40,41]. The metabolite concentrations can be precisely calculated by comparing the area under each peak corresponding to a particular metabolite with that of the internal standard of known concentration, such as 3-trimethylsilylpropionic acid (TPS) or 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS). In view of this, a direct comparison of different metabolites is possible without using a calibration curve for each metabolite identified from the analysis.

Besides, NMR can be used in *in vivo* studies conducted in animals and humans, referred to as Magnetic Resonance Spectroscopy (MRS)[42]. MRS enables the identification of small molecules, typically in concentrations of 0.5–10 mM, with sufficiently high flexibility within cells or in extra-cellular spaces[43]. The acquired MR spectra provide information on the metabolic pathways and alterations therein, making MRS a very appropriate technique for tracking and monitoring disease-related metabolome. The other *in vivo* application of NMR is Magnetic Resonance Imaging (MRI), used to obtain detailed anatomical images throughout the human body[42,44]. Because of the large concentration of water, the images provided by conventional MRI come predominantly from the water protons, and thus limited information of physiological relevance is contained in these images. The recently developed Hyper-Polarised MR (HP-MR) significantly increases the signal-to-noise ratio (10^4 to 10^5) of conventional MRI and enables the imaging of nuclei other than protons[45-47]. Using non-radioactive ^{13}C and ^{15}N-labeled substrates, HP-MR can detect endogenous metabolites substrates in real-time to monitor *in vivo* metabolic fluxes through imaging probe metabolism[33,48,49].

Apart from liquid and solid samples, NMR can also be used to evaluate metabolic profiles of intact tissues with high spectral resolution using High-Resolution Magic Angle Spinning (HRMAS) NMR spectroscopy[50-52]. Line-broadening due to the anisotropic interaction can be eliminated by spinning the samples at high rates at an angle of 54.74° (the 'magic angle') with respect to the external magnetic field[53-56]. This particular technique requires less sample preparation, and a bulk sample mass from 5 to 10 mg is needed to obtain the metabolite information. However, recent developments in the micro-MAS (μMAS) probe for investigating microscopic specimens (<500 μg) has demonstrated the possibility of metabolic profiling with μg-scale samples[57-59].

3. Overcoming the Limitations of NMR in Metabolomics

Techniques based on Magnetic Resonance, however, face a lot of challenges due to their inherent low sensitivity. Thus, it can only detect abundant metabolites, typically anything in or above the micromolar range (≥ 1 μM)[20]. Increased Signal-to-noise (S/N) ratio and spectral resolution can be achieved by the technological advances in the NMR hardware, including the application of a higher magnetic field, cryoprobe to reduce thermal noise, small volume probes, and hyperpolarization techniques[60,91]. The sensitivity and resolution of an NMR experiment depend strongly on the applied magnetic field – B0, which determines both the initial Boltzmann population of the nuclear spin levels and their Larmor frequency. The Signal-to-Noise ratio (S/N) increases with the strength of the magnetic field by a factor of $\sim B_0^{3/2}$ [62]. In addition, the NMR spectral resolution in an n-dimension NMR experiment increases as the n^{th} power of B_0, thereby explaining the quest for considerable efforts for the development of higher and higher field NMR spectrometers (maximum magnetic field available in commercial NMR magnets is 25.9 Tesla).

Apart from magnetic field strength, the use of cryogen-cooled Radio-Frequency (RF) coils, Cryoprobes from Bruker, and Ultracool probes from Jeol, has drastically enhanced the sensitivity of NMR experiments. The enhanced sensitivity or S/N in the cryogenic probe is due to the reduction of thermal noise (Johnson-Nyquist noise)[63] in the signal transmit/receive coils and in tuning and matching circuitry[64]. Typically, up to a 5-fold sensitivity gain compared to conventional room temperature probe heads can be accomplished using cryogen-cooled probes[61,65].

Next, the use of small volume probes (1 mm and 1.7 mm tube probes) requires much less sample volumes (less than 30 μL) and can provide several-fold increased sensitivity when compared to the conventionally used probes (3 mm or 5 mm cryoprobes)[66-68]. This is due to the fact that for a given mass of analyte, a reduction in the diameter of the RF coil increases the S/N ratio. The 1 mm TXI MicroProbe (Bruker) presents the maximum ^1H mass sensitivity for Room Temperature (RT) probes,

which results in a mass sensitivity up to 4 times greater than 5 mm conventional probes (for a given mass of analyte). Similarly, a newly designed commercial 1.7 mm ^{1}H-^{13}C-^{15}N CPTCI cryoprobe (Bruker) operating at 600 MHz attains an S/N of 1000:1 for ^{1}H with a sample fill volume of 30 µL when compared to 9000:1 for a 5 mm TXI probe with a sample fill volume of 750 µL at the same field strength, suggesting a mass-sensitivity improvement of >10-fold relative to a conventional 5 mm RT probe[25].

Hyperpolarization methods such as Para-Hydrogen-Induced Polarization (PHIP) and dynamic Dynamic Nuclear Polarization (DNP) are powerful tools that dramatically enhance the sensitivity of NMR measurements[62,69,70]. The prominent way of creating a hyperpolarized state is DNP, which involves transferring polarization from electrons (created by microwave irradiation close to electron Larmor frequency) to nuclear spins[71,72]. By hyperpolarizing the nuclear spins, the S/N can be enhanced over 10,000 times[73].

In addition to this, isotopic enrichment of the metabolites through chemical reactions can be commonly applied to enhance the sensitivity of heteronuclear 2D NMR experiments. Other limitations of NMR experiments include the heavy overlap of spectral resonances as several endogenous metabolites may contribute to a signal; variation in chemical shift of resonances due to changes in pH, concentration, and ionic strength due to alterations in the acid-base equilibrium and solute-solute interactions; poor water suppression in case of dilute samples; baseline distortions; and chemical exchange between metabolites, particularly with water. To overcome these limitations, several approaches such as the implementation of multi-dimensional NMR experiments: two dimensional (2D) NMR experiments (JRES, TOCSY, HSQC, etc.)[17] that provide additional dimensions can be carried out in order to alleviate the congestion of spectral resonances. Also, the use of appropriate buffered conditions and water-suppression pulse programs has substantially reduced the problems due to pH and poor water suppression in biological samples.

4. Mass Spectrometry

Mass Spectrometry (MS) is an analytical technique that separates the compounds in a biological sample based on their mass-to-charge ratios by taking into account the degree of deflection of charged particles in an electromagnetic field[74]. The higher sensitivity and selectivity of MS, with detection limits in the picomolar and nanomolar range and the ability to detect wide metabolome range, makes it an important technique for the metabolic profiling of complex biological samples (13). MS is often coupled with chromatographic techniques, as Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS), in order to reduce ion suppression, spectral complexity, and spectral overlap[75,76]. MS, when coupled to other chromatographic techniques, is advantageous over NMR spectroscopy in enabling the analysis of secondary metabolites as the detection limit ranges in picomole to femtomole scale[77,78].

In GC-MS analysis, the sample is passed through the gas chromatograph, which volatiles the sample and separates the components of the complex mixture in the gas phase. As the components emerge from the GC column, they enter the mass unit, where they get ionized by the Mass Spectrometer using different ionization approaches. The ionized molecules or ion fragments are then accelerated through the mass analyzer, which resolves these ions based on their mass-to-charge ratios. The peak heights in the mass spectrum directly correspond to the quantity of the metabolite. The metabolite identification can be carried out using well-established computer libraries of mass spectra such as the National Institute of Standards and Technology NIST[79] and Fiehn Metabolomics library[80]. Although GC-MS yields high sensitivity, high resolution, and prominent reproducibility, it is only limited to volatile compounds and is not suitable for non-volatile and thermally unstable compounds. LC-MS approach, which uses solvent as its mobile phase, overcomes the problem of sample derivatization and non-volatile samples and is the most powerful analytical tool for carrying out the global metabolite profiling studies in biological samples[81]. The LC-MS approach has many benefits over GC-MS due to its high sensitivity, simplified sample pre-treatment step (no chemical modification is required), and comprehensive metabolome coverage. In the LC-MS platform, the Reversed-Phase Liquid Chromatography (RPLC) and Hydrophilic Interaction Liquid Chromatography (HILIC) are commonly used chromatographic separation modes in metabolomics. The RPLC is extensively used to separate non-polar to medium polar analytes[82], while HILIC separation mode is the method of choice for polar molecules[83].

5. Overcoming the Limitations of MS in Metabolomics

Currently, MS-based metabolomics approaches are limited to the relative quantification of metabolites. In order to determine the absolute quantification of metabolites, analytical standards are required to construct calibration curves for each metabolite, which is an expensive affair. One of the main limitations of MS is that different classes of metabolites are detected by different ionization methods[76,84]. No single ionization method can cover all metabolite classes, such as hydrophilic, hydrophobic, ionic, etc. Thus, for a comprehensive metabolite profiling, more than one ionization method needs to be carried out independently to maximize the number and coverage of metabolites being profiled. Electro-spray ionization (ESI) and Atmospheric-Pressure Chemical Ionization (APCI) are the recommended ionization methods for performing MS-based metabolomics as they allow comprehensive metabolome coverage[85-87]. However, the sensitivity of the ESI method is highly reliant on the metabolites pK_a or hydrophobicity, which could be adversely influenced by the heterogeneous composition of metabolic samples. The overall throughput of this platform is further hampered by many unsolved problems such as a non-uniform detection caused by variable ionization efficiency, metabolite decomposition during derivatization or separation, lack of standardized protocols or procedures (as it requires optimization of separation conditions each time), a lack of a universal database due to different ionization mode[88], and metabolite ion-suppression due to co-eluting matrix compounds, etc.[88,89].

With the development of powerful MS technologies such as Quadrupole Time-Of-Flight (Q-TOF), triple quadrupole (QqQ), the mass range and the resolution have increased dramatically. However, sample preparation is still a critical concern in the MS approach. MS-based metabolomics typically requires complex pre-processing of samples that results in the loss of many non-derivatized chemical constituents. In order to bypass the issue of chromatography, several ambient ionization MS methods are available that are fast and require minimal or no sample pre-treatment steps. Although a number of ambient MS methods are available, Desorption Electrospray Ionization (DESI) and Direct Analysis in Real-Time (DART) are two well-established flagship ambient methods that enable the real-time MS analysis of complex mixtures[90]. These ambient ionization MS methods are very fast, robust with minimal sample preparation, and provide the direct MS analysis of biological specimens. However, these ambient MS methods offer a severe drawback that they can only provide qualitative information and suffer from unreliable and inaccurate quantification[91].

6. Conclusions

The development of the latest and emerging technological advances in NMR and mass spectrometry has expanded the application of metabolomics in various research fields, including medical research, endocrinology, reproductive biology, toxicology, nutrition, drug metabolism, food science, metabolic diseases, *in vivo* imaging, etc. MS and NMR have emerged as the most powerful analytical platforms in carrying out the global metabolic profiling in biological systems, and each has its strengths and weaknesses. NMR is quantitative, reproducible, and does not require extensive steps for sample preparation. The major drawback of this approach is its low sensitivity compared to MS. However, the sensitivity of NMR spectroscopy has improved considerably due to the technological advances in the NMR hardware, including the application of a higher magnetic field, cryoprobe, small volume probes, and hyperpolarization techniques.

On the other hand, the higher sensitivity and resolution of MS, and its compatibility with the chromatographic techniques (LC and GC), and its ability to cover a wide metabolome range make it an ideal tool for performing metabolomic studies in biological systems. In addition, various MS techniques, such as ambient ionization methods and mass analyzer technologies, are used to increase the metabolome coverage detection and to overcome the limitations of sample preparation in the MS-based metabolomics approach. We envisage that to obtain a holistic picture of the metabolome, NMR and MS approaches must be taken together in a complementary fashion for gaining deeper insights into the disease pathophysiology, effect of therapeutic interventions, and biomarker discovery. It will be particularly useful for complex endocrine disorders and metabolic syndromes.

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