

Mass Spectrometry Advances Translational Medicine Trends in Proteomic and Small-Molecule Applications

Enabling targeted quantitative proteomics applications and hypothesis-driven inquiries will help researchers to understand how proteins function in living systems. The discovery and validation of small molecule and protein-based biomarkers, and the eventual translation of these discoveries from the research lab to the clinic, involves robust mass spectrometry (MS) systems and software that make it easier for technicians to perform routine sample analyses on liquid chromatography (LC)–MS–MS systems, which continue to be used in an increasing number of both protein and small molecule analysis applications.

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Proteomic applications of mass spectrometry (MS) have evolved from the early identification of proteins extracted from two-dimensional gels (2D) to the use of a combination of liquid chromatography (LC) and MS for the cataloging of lists of proteins in living systems. The quantification of proteins for the purpose of understanding complex mechanisms and pathways within living systems is increasingly becoming important to scientists. Moreover, the significance of quantitative proteomics discoveries continues to indicate potential importance in the clinical field as potential applications of MS technologies help to advance translational medicine.

Discovery and Early Validation of Candidate Biomarkers

The field of quantitative proteomics has sparked efforts by scientists to discover potential candidate protein biomarkers and then qualify and validate these biomarkers for the purposes of drug efficacy, off-target effects, patient stratification, disease monitoring, and toxicology studies, and to help scientists decide whether to proceed with drug development activities. MS is increasingly being used for protein

biomarker-based clinical research and pharmaceutical manufacturing applications, particularly research in the areas of oncology, cardiovascular disease, metabolic pathway analysis, and inflammatory disease.

For example, based on the relative quantification of differentially expressed proteins in cancerous versus normal tissue samples, scientists discover candidate protein biomarkers for disease. Once validated, a biomarker might eventually impact how a scientist or clinician makes decisions that involve diagnosis of disease, or, in the pharmaceutical industry, the development of drugs.

However, rather than discovering individual protein biomarkers, scientists believe that identifying groups or panels of relevant biomarkers might prove to be both more selective and sensitive for identifying a particular biological condition. To assist with the discovery of panels of biomarkers, researchers can use protein-labeling reagents that employ isobaric tagging for protein quantification (iTRAQ reagents, Applied Biosystems). These reagents enable scientists to multiplex as many as four samples in a single experiment. In the future, this multiplexing capability will be extended to enable the analysis of eight samples simultaneously.

Recently, researchers from the University of Washington, Applied Biosystems, and three other research facilities used a matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) analyzer in combination with the isobaric-tagging protein-labeling reagents and an LC MALDI workflow to discover a panel of candidate biomarkers that could potentially help researchers develop more effective diagnostic tests and treatments for Alzheimer's disease, Parkinson's disease, and Dementia with Lewy Bodies — three age-related diseases that affect more than seven million people in the United States today (1).

During the discovery phase of biomarker development, experiments generally focus on a few samples and the analysis of sometimes thousands of analytes. However, as candidate protein biomarkers emerge from a discovery investigation, researchers must verify and validate the candidate biomarkers. Typically, validation work involves use of western blots and enzyme-linked immunosorbent assays (ELISA), which use antibodies developed for the specific candidate proteins in the analysis. Following a discovery investigation, researchers decide which of their candidates qualify for further development into antibody-based assays. For example, in a biomarker discovery experiment, researchers might identify 100 candidate protein markers. It would not be practical to then develop antibody-based assays for all 100 markers, creating a need for a high-throughput assay to verify which candidate biomarkers are worth developing into full validation assays.

One approach to breaking this bottleneck involves the use of multiple reaction monitoring (MRM) MS assays, widely used in the pharmaceutical industry to detect and quantify drugs and drug metabolites based upon prior knowledge of the mass and structure of a drug. This technique couples LC with a specific scan function on a triple-quadrupole mass spectrometer and is increasingly being used to target and quantify specific candidate biomarkers in serum and plasma.

A recent publication underscored the value of being able to use MRM-initiated detection and sequencing software

(MIDAS software, Applied Biosystems) in conjunction with a hybrid triple-quadrupole linear ion-trap MS system (Applied Biosystems/MDS SCIEX 4000 Q TRAP) to conduct assays of a number of cardiovascular proteins in plasma (2). The software uses the capability of this MS system for both the selective and sensitive quantification, as well as the sequencing of targeted peptides.

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In this finding, the researchers described the evaluation of proteins previously indicated to be involved in cardiovascular disease, and the development of assays that detect 47 of these proteins in human plasma samples, a particularly difficult kind of sample to analyze because of the extreme dynamic range (>10 orders of magnitude) and complexity of the proteins in this kind of sample. The authors noted that this study exemplified the development of targeted specific assays needed to enable statistical validation of candidate biomarkers in human plasma.

Identifying and Quantifying Posttranslational Modifications

Applications of quantitative proteomics have also helped researchers to make significant progress in the analysis of post-translational modifications (PTMs) and splice variants as well as to elucidate the roles of proteins in many different cellular signaling pathways.

Because most modifications to pep-

tides are in flux within a cell, MS tools help researchers quantitatively determine which amino acid residues in a protein are modified at a given time or cellular state, contributing to their understanding of PTM events that occur in cellular pathways. For example, through the use of isobaric-tagging protein-labeling reagents, researchers can quantify how much phosphorylation occurs on a specific peptide, or how many sites on a peptide have been phosphorylated.

Scientists at Applied Biosystems have partnered with academic and pharmaceutical research groups to use the isobaric-tagging protein-labeling reagents for the study of phosphorylation in biological systems. For example, the reagents were recently used in combination with an LC-tandem mass spectrometry (MS-MS) system (QSTAR, Applied Biosystems) to investigate a time course study of tyrosine phosphorylation on the EGF receptor signaling network (3). In contrast, other researchers have examined a potential novel therapeutic that acts on the Kit tyrosine kinase signaling (4).

Researchers also identify and quantify phosphorylation sites within a protein by using the more targeted approaches enabled by the unique scan functions of a hybrid triple-quadrupole linear ion-trap system (4000 Q TRAP, Applied Biosystems) to first discover potential phosphorylated peptides. Then, by using isobaric-tagging protein-labeling reagents, they quantified the abundance of the phosphorylated and non-phosphorylated peptides (5). An alternative approach involves the use of a hypothesis-driven method that makes use of a researcher's prior knowledge of a sample to hunt for potentially modified peptides from a known protein. This research demonstrates how MRM-initiated detection and sequencing software is used for the identification and sequencing of posttranslationally modified peptides (6).

Pathway Analysis

Despite the importance of quantitative analysis of proteins for understanding their function, a full understanding of the proteomics of a cell requires pathway analysis. Considering that proteins are dynamic and may not be localized to

any one particular organelle or compartment of a cell, researchers want to find out what kind of impact a particular protein or a specific modification to a protein has had on a pathway of interest.

Using the MRM capability on a triple-quadrupole mass spectrometer, researchers can analyze a sample and search for either a single peptide of interest, a single modified peptide, or simultaneously search for over 100 different peptides. In the future, using a scheduled MRM approach, researchers will be able to search for more than 1000 peptides in a single LC separation. When peptides of interest are eluted from the column, an MRM signal triggers the instrument to sequence the peptide and verify whether the modified peptides of interest are present in a sample. By using prior knowledge of when the peptide is likely to appear in the mass spectrometer, researchers can schedule each MRM assay within a specific time window, thereby significantly increasing the number of peptides detected in a single analysis. Researchers can accomplish this kind of application through the coupling of the hybrid triple-quadrupole linear ion-trap system and the MRM-initiated detection and sequencing software workflow, which enables simultaneous quantitative identification and analysis of all the proteins in a single pathway.

Because most proteins exert their function by protein-protein interactions, through automation of pull-down experiments, MS tools are used to determine how one protein interacts with another protein.

In pull-down experiments, researchers begin with a protein known to have a role in a pathway, or disease mechanism. By cloning that protein into an expression system, researchers make a fusion protein in which one end of the protein is amenable to some kind of affinity capturing. They then express the fusion protein in a cell system. The protein construct, through its fusion to the original protein, pulls down or captures any other proteins present in the cell system that might bind to it. Researchers use pull-down experiments as a fishing tool to observe how properties of proteins, such as binding ability, change between

a healthy and diseased state.

When performing pull-down experiments, researchers face the challenge of discerning a true binding protein from a false positive, which is an artifact of the experiment. High-performance LC-MS-MS instruments are currently being used for high-throughput pull-down experiments.

Data compiled from high-throughput pull-down experiments will potentially provide a valuable resource about protein-protein interactions, essential information for pathway elucidation. Ultimately, these kinds of experiments will further progress toward the development of an interactome, a map of all of the protein-protein interactions that occur in a living system.

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Small Molecule Analysis Applications Turn to LC-MS-MS

Of the many different MS systems and techniques in use today, LC-MS-MS has perhaps the greatest potential for advancing translational medicine because it accomplishes routine high-throughput analysis applications, similar to those required by both researchers and clinicians. One of the keys to adopting LC-MS-MS techniques for clinical applications will be the development of software and hardware interfaces for systems that accommodate users who are

not experts in mass spectrometry.

Today, many scientists and technicians are turning to LC-MS-MS techniques to perform small molecule analysis applications that they might have performed previously using other analytical techniques. Because LC-MS-MS systems are now faster and require less sample preparation than do other techniques, they have become more cost-effective for high-throughput routine analysis applications. Consequently, industries such as environmental, food, drug screening, and forensic toxicology that have long invested in other techniques are now discovering that LC-MS-MS results in higher performance and lower cost per analysis than do traditional techniques. The capabilities of LC-MS-MS that make the technique ideal for these types of applications are the same capabilities needed to perform MS analysis of clinical samples.

One of the traditional techniques that has been used in clinical-forensic and food testing laboratories is gas chromatography (GC)-MS, in which a gas chromatograph is coupled to a single-stage electron ionization or chemical ionization mass spectrometer. GC-MS techniques provide good sensitivity for most species analyzed and have reached a level of cost and performance that is accessible to small and large laboratories alike. GC-MS, however, separates molecules in the gas phase, so it is not immediately amenable to many of the biomolecules of interest. GC-MS methods therefore frequently require complicated and time-consuming sample preparation, often involving the derivatization of larger molecules to more volatile species that can then be separated in the gas phase.

Food analysis is a good example of an application in which LC-MS-MS demonstrates the type of capabilities that would also be required of a mass spectrometry technique used for routine analyses of samples in the clinic. For instance, because LC-MS-MS combines the natural affinity of liquid separations for biological compounds in biological matrices, with the sensitivity and selectivity of tandem MS detection, it detects both the presence and the amount of banned compounds in food samples.

LC-MS-MS techniques cover a wide range of potential analytes, have a low potential for false positives, and deliver high-throughput analysis.

The development of MS systems that are easier to use and feature solution-oriented software will make MS more accessible to the average chemist or technician and enable workflows for routine analysis.

The LC portion of the system separates compounds based upon the chemical properties of the analytes of interest. In complicated biological mixtures, which might consist of thousands of different compounds of both endogenous and exogenous origins, the chromatographic column separates those compounds for a chromatogram, and the mass spectrometer gives users a way to look at those pieces independently and identify individual compounds in a complex sample. Electrospray LC-MS-MS analysis is especially well suited for analysis of biological fluids because a large proportion of the chemicals found are readily ionized.

LC-MS-MS in the Development and Screening of Candidate Compounds

LC-MS-MS has already become the standard technology for other kinds of routine analysis applications. For example, LC-MS-MS, in particular triple-quadrupole analyzers, have become the standard technology for routine quantitative studies in the pharmaceutical industry. Worldwide, in high-throughput laboratories, thousands of these instruments are employed to perform in vitro and in vivo experiments in the support of new drug development. Over the past 10 years, the pharmaceutical industry has embraced triple-quadrupole systems, driving vendors to develop faster, smaller, and more sensitive instrumentation.

Recently, LC-MS-MS has reached the sensitivity level required for the support of microdosing studies. Microdosing experiments challenge the performance of analytical systems by dosing patients with

extremely small, innocuous amounts of investigational compounds to investigate in vivo metabolism. Traditionally, these types of experiments require ultrasensi-

tive accelerator mass spectrometers. Now, such instruments have demonstrated sensitivity at levels that can support microdosing experiments (7).

LC-MS-MS has recently bridged the gap between qualitative and quantitative analysis by providing high performance for both applications on a single platform. In the past, these two types of analyses were carried out on two types of instruments. Now, with hybrid triple-quadrupole linear ion trap systems, the selectivity and sensitivity of a triple-quadrupole instrument is combined with the fast scanning and resolution of an ion-trap instrument. This versatility can become useful for applications beyond drug discovery and development, such as drug screening in athletes, which requires that a technician both positively identify a compound and quantify how much of the compound is present in a sample.

Software Enables Routine Analyses by LC-MS-MS

The application of LC-MS-MS techniques for a growing list of routine analyses means there is an increasing need to make MS systems available to a larger number of users. However, there is a growing gap between the number of mass spectrometers in the marketplace and the number of qualified mass spectrometrists available to use these systems. For example, some organizations have laboratories running dozens of MS systems 24 h/day. In these organizations, experts must teach a large number of people how to use the systems to perform routine analysis.

Once the instrument and the LC system are installed, over 90% of what a

person does with the system involves the use of the software. In routine mode, often the most interaction a scientist or technician might have with the hardware is to place samples in an autosampler tray. The development of MS systems that are easier to use and feature solution-oriented software will make MS more accessible to the average chemist or technician and enable workflows for routine analysis.

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