Mass Spectrometry in Biomarker Applications: From Untargeted Discovery to Targeted Verification, and Implications for Platform Convergence and Clinical Application

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It has really been only in the last 10 years that mass spectrometry (MS) has had a truly important (but still small) impact on biomedical research. Much of this impact can be attributed to proteomics and its more basic applications. Early biomedical applications have included a number of efforts aimed at developing new biomarkers; however, the success of these endeavors to date have been quite modest—essentially having been confined to preclinical applications—and have often suffered from combinations of immature technology and hubris. Now that MS-based proteomics is reaching adolescence, it is appropriate to ask if and when biomarker-related applications will extend to the clinical realm and what developments will be essential for this transition.

Biomarker development can be described as a multistage process consisting of discovery, qualification, verification, research assay optimization, validation, and commercialization (1). From a MS perspective, it is possible to “bin” measurements into 1 of 2 categories—those aimed at discovering potential protein biomarkers and those seeking to verify and validate biomarkers. Approaches in both categories generally involve digesting proteins (e.g., with trypsin) as a first step to yield peptides that can be effectively detected and identified with MS. Discovery-based approaches use broad “unbiased” or “undirected” measurements that attempt to cover as many proteins as possible in the hope of revealing promising biomarker candidates. A key challenge with this approach stems from the extremely large dynamic range (i.e., relative stoichiometry) of proteins of potential interest in biofluids such as plasma and the expectation that biomarker proteins of the greatest clinical value for many diseases may very well be present at low relative abundances (2). Protein concentrations in plasma extend from approximately 10^10 pg/mL for albumin to approximately 10 pg/mL and below for interleukins and other cytokines. Proteins secreted or leaking into the blood from specific early-stage tumors could be present at even lower concentrations. Currently, most protein biomarkers that have been cleared by the US Food and Drug Administration fall in the range of 10^2 pg/mL to 10^3 pg/mL, a challenge for the broad discovery-oriented proteomics measurements that are still largely confined to proteins at the upper end of this range.

Because of the constrained dynamic range of present mass spectrometers [approximately 10^3 for a single spectrum from Orbitrap MS (Thermo Scientific) platforms currently popular for discovery efforts], broad coverage of lower-abundance proteins typically requires larger starting amounts of sample and extensive fractionation and/or separations that limit measurement throughput. An online high-resolution liquid chromatography (LC) separation requires approximately an hour, and the resulting approximately 10^4 spectra typically provide information on hundreds of proteins. A proteomic “deep dive” via the use of extensive fractionation (e.g., approximately 100 fractions with strong cation-exchange chromatography prior to LC-MS) to expand coverage to thousands of proteins further exasperates throughput, with such efforts requiring days or weeks of measurement time. The latter approach is highly attractive for detecting potential biomarkers, but the inherently low throughput largely precludes studies of populations that could effectively account for both human and disease diversity. Although the minimum useful study size for biomarker discovery remains an open question, it is expected to be much larger than is generally practical at present, likely in the thousands.

For these reasons, MS-based development efforts for blood protein biomarkers have increasingly been focused on verification and validation. These applications typically make use of targeted measurements that provide greater sensitivity, greater throughput, and more accurate quantification than broad discovery-
based measurements—without the need for extensive prefraccionation—but only for a limited number of "targeted" peptides/proteins. In particular, widely used triple-quadrupole selected reaction monitoring or multiple reaction monitoring measurement platforms afford targeted multiplexed tandem MS detection of up to hundreds of peptides during an LC separation, providing limits of detection and quantification several orders of magnitude lower than presently feasible with discovery-based platforms. The larger signals (peptide ion currents) associated with such measurements and the general application of stable isotope-labeled internal standards are primary reasons for the improvements in sensitivity and data quality and for the lower CVs. Moreover, recent platform advances, such as improved ion sources and interfaces that incorporate dual-stage ion funnels (3) and the use of immunoaffinity major-protein depletion or targeted peptide-enrichment methodologies (4), extend the practical limit-of-quantification values to nanogram-per-milliliter plasma concentrations. Low picogram-per-milliliter concentrations have recently been reported (4).

Most exciting are platform developments and incipient trends pointing toward a convergence of the "untargeted/discovery" and "targeted/verification" dichotomy. One driver for this convergence is the dramatic increases in the effectiveness of MS ion sources. For example, it has been demonstrated that approximately 50% of peptide molecules in a sample can be ionized and effectively transported through the MS interface to the analyzer (5), a 10^- to 10^5-fold improvement compared with conventional sources. Moreover, >10^10 peptide ions per second can be formed and introduced into the mass analyzer, with an upper limit of approximately 10^15, owing to the disruptive effects of space charge (e.g., on ion focusing). These developments are requiring a considerable redesign of MS platforms to avoid the charging of surfaces by larger ion beams that then distorts electric fields, degrades ion focusing, and leads to lower sensitivity and fluctuating performance. The developments particularly benefit designs that are capable of using large ion currents (e.g., triple-quadrupole or TOF analyzers), as opposed to those that have limited charge capacities per scan—specifically linear ion traps (approximately 10^4 charges) and Orbitrap/Fourier transform MS (approximately 10^6 charges).

Another trend is the use of broader targeted analyses in which sets of ions are selected for simultaneous tandem MS analysis with "hybrid" quadrupole–Orbitrap or quadrupole–TOF MS platforms. In contrast to targeted multiple reaction monitoring measurements in which multiple species are actually sequentially monitored, this type of multiplexing involves the simultaneous selection and dissociation of multiple peptides by one of any number of first-stage selection events (including the use of limited m/z ranges) and the detection of a spectrum of their combined dissociation products. These measurements benefit from tandem-MS resolution and mass-measurement accuracy that enable effective deconvolution of the multiplexed peptide fragmentation spectra, as well as from other improvements. The ability to correctly discern contributions from low-level species in the presence of much more abundant species in these measurements requires sufficient signal intensity in addition to specificity and detector dynamic range.

Such developments provide the basis for appreciably more sensitive targeted measurements that are currently limited by some combination of ion signal intensity and analyzer specificity. For example, the limit of detection or quantification in targeted selected reaction monitoring measurements is limited either by the selected peptide ion current or by the presence of interfering ions from such sources as coeluting species or "chemical noise." The developments noted above are increasingly addressing the sensitivity limitation and suggest the likelihood of gains of approximately 10^3 over the best present performance, as well as the possibility of extending the detection of plasma proteins to picogram-per-milliliter concentrations without the use of multiple fractionation or immunoaffinity-enrichment stages. These gains can potentially be achieved by higher specificity, either from increased MS resolution or additional analyzer stages, such as MS^3, or to a more limited extent by broad detection of MS^2 fragment transitions, in which, again, MS resolution and detector performance are presently limiting.

A key trend to be discerned here is whether we can expect technological developments, such as faster separations, more-effective ion sources, higher MS resolution, and detectors with higher dynamic ranges, to increasingly allow broad untargeted measurements that retain the benefits of targeted measurements. An initial step in this direction exploits very fast gas-phase ion mobility separations (IMs), which take place on a time scale of tens of milliseconds and can provide peak widths of less than a millisecond in combination with a TOF mass spectrometer that can acquire approximately 10 spectra every millisecond. This capability allows placement of IMS between the LC and TOF-MS stages, whereas the use of ion funnels makes operation essentially lossless, thereby making 2-dimensional separations possible without the need for LC prefraccionation, as well as without any loss of sensitivity or throughput. As an example, an early LC-IMS-MS platform implemented in our laboratory (6) consistently reached detection concentrations of 1–10 ng/mL for 20 peptides spiked into mouse plasma, an order of mag-

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itude better than achieved with ion trapping–based Fourier transform MS platforms. LC-IMS-MS platforms also allow highly multiplexed peptide dissociation (e.g., between the IMS and MS stages) (7), which translates to more effective information on "all the ions, all the time."

Such developments are just a first step in the coming convergence of untargeted and targeted platforms, which will be accelerated by the emerging capabilities for faster and higher-resolution separations, improved MS resolution, and extended detector dynamic range. The potential for higher-throughput measurements with such platforms also presents an opportunity for considerably more effective discovery efforts and, ultimately, a "grand convergence" of discovery and verification measurements. In the shorter term, more sensitive and increasingly multiplexed selected reaction monitoring measurements will lead this advance.

Despite the common assertion that MS-based platforms are simply too complex, too expensive, and insufficiently robust for clinical application, these platforms are in fact being broadly used for such applications as blood-based drug and environmental measurements. With sufficient sensitivity, accuracy, and throughput, it is clearly feasible for MS-based proteomics platforms to move to clinical application if they can effectively overcome concerns associated with cross-reactivity and/or the high costs of developing affinity reagents. Expect this value to become increasing evident.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors' Disclosures or Potential Conflicts of Interest:** No authors declared any potential conflicts of interest.

**References**