Letter to the Editor

Top-down proteomics—a near-future technique for clinical diagnosis?

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We thank Dr. Laszlo Prokai, Dr. Jae-Seok Kim, and Dr. Hyun Sik Kim for their insightful editorial commentaries on our recent publication "*Diagnosis of hemoglobinopathy and β-thalassemia by 21 Tesla Fourier transform ion cyclotron resonance mass spectrometry and tandem mass spectrometry of hemoglobin from blood*" (1-3). As described by the editorial authors, 21 Tesla Fourier transform ion cyclotron resonance mass spectrometry (21 T FT-ICR MS) and top-down MS/MS set a new benchmark for precision diagnosis of hemoglobinopathies and thalassemia with fast sample preparation (dilute and infuse) and data acquisition (3 min).

Top-down mass spectrometry analyzes proteins in their intact state without prior enzymatic digestion. As a result, individual proteins with corresponding proteoforms (4) are completely characterized with fast sample preparation and minimal artifacts. Compared to bottom-up MS/MS (with enzymatic digestion), which yields more limited peptide sequence and post-translational modifications (PTMs), top-down MS/MS provides a "birds' eye" view of a complete proteoform to reveal the correct combinatorial PTMs pattern, even when more than one PTM is present (5).

Recently, top-down mass spectrometry has been successfully applied to plasma cell disorders diagnosis, in which clonal plasma cells secrete identical immunoglobulins (M-proteins) in excess, enabling patient monitoring by mass spectrometry (6). Traditional M-proteins detection methods (serum protein electrophoresis and immunofixation) have limited resolution and cannot accurately monitor therapeutic response for patients with plasma cell disorders whose M-protein co-migrates with therapeutic monoclonal antibodies (mAbs). In contrast, the top-down MS approach clearly differentiates endogenous M-proteins from therapeutic mAbs for accurate diagnosis (7). In addition, the higher resolution of mass spectrometry compared to electrophoresis-based methods potentially allows the detection of minimal residual disease at lower M-protein levels.

We further employed top-down MS/MS for *de novo* sequencing of monoclonal immunoglobulin light chains for classification of plasma cell disorders, and demonstrated 100% sequence characterization accuracy by comparison to gene sequencing (8-10). The fast and noninvasive MS/MS *de novo* sequencing method also yields PTMs (e.g., glycosylation, terminal truncation) as potential biomarkers not accessible by gene sequencing.

Identification of hemoglobin (Hb) disorders is

another excellent target for top-down MS/MS because a large number of sequence variants at different sites of hemoglobin (>1,500 hemoglobin variants discovered so far) can be directly characterized by this approach, and the concentration of hemoglobin in blood is so high as to allow minimal sample preparation to be employed. In addition, the limited resolving power of current screening methods (electrophoresis and high-performance liquid chromatography) can yield ambiguous results, and so a labor-intensive and confirmatory genetic test is frequently performed (11). High resolving power mass spectrometers [Orbitrap and time-of-flight (TOF)] have been used for top-down characterization of common Hb variants. However, only Hb variants that are known in the Hb database were analyzed, and the more difficult Hb heterozygote cases were not studied (12-14). A color-code strategy for fast localization of a Hb beta chain mutation was reported, but it relied only on MS/MS and did not attempt to further *de novo* sequence the mutant (15).

Therefore, we developed a top-down MS/MS approach for precision diagnosis of hemoglobin disorders. The advantages of fast turnaround (3 min data acquisition after blood sample dilution and infusion to the MS), ultrahigh mass accuracy, and abundant MS/MS product ions contributed to correct identification of all Hb variants in blind analyses of eighteen samples. Even a difficult Hb heterozygote case with β chains of Hb A (normal Hb) and Hb D (E to Q mutation, Δm =–0.98402 Da), differing by 0.0194 Da in isotopologue spacing was resolved by MS1 and MS2. We also showed for the first time that beta-thalassemia (both minor and major types) can be unambiguously screened by the ratio between the abundance of intact δ and β subunits (δ/β). In addition, we established human Hb alpha chain and beta chain standard curves for absolute quantitation of hemoglobin (e.g., for simultaneous diagnosis of anemia) with spiked bovine hemoglobin and no additional instrument data acquisition time (unpublished data).

The editorial authors raised the question of whether or not the 21 T FT-ICR MS result provides a realistic perspective for mass spectrometry-based clinical diagnosis in the near future. The 21 T FT-ICR MS results with the extreme MS capabilities for protein analysis in the cited publication provide baseline information to guide the implementation of methods with lower resolving power mass spectrometers (e.g., lower-field FT-ICR MS and Orbitrap MS). Moreover, the top-down MS/MS methods in the cited publication represent a potentially efficient protocol for clinical laboratory testing with improved data quality and faster turnaround time.

In order to implement top-down MS/MS as routine clinical tests, several technique improvements are still needed. First, for methods that rely on database searching, the current database search method is not applicable to proteins not in the database (e.g., endogenous monoclonal immunoglobulins and undiscovered hemoglobin variants), and an incomplete protein sequence in the database leads to low identification rate and high false discovery rate (16). In addition, the presence of unknown or unexpected mutations and PTMs leads to fragment mass shifts which further complicate database matching. Therefore, top-down *de novo* sequencing (database-independent) software needs to be further developed to yield confident protein sequences characterization. The orbitrap could potentially resolve the ~0.02 mass spacing for MS/MS product ions of some heterozygous Hb variants. However, the number of trapped ions would need to be reduced from ~1,000,000 to ~50,000 in order to prevent peak coalescence (17), so it would be necessary to sum ~400 transients to match the same signal-to-noise ratio, and the experiment would take correspondingly longer to perform. In summary, with the rapid growth of top-down proteomics, it is reasonable to expect that top-down MS/MS based clinical diagnosis is not far away.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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