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Review Article

Getting to the core of protein pharmaceuticals – Comprehensive structure analysis by mass spectrometry

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ABSTRACT

Protein pharmaceuticals are the fastest growing class of novel therapeutic agents, and have been a major research and development focus in the (bio)pharmaceutical industry. Due to their large size and structural diversity, biopharmaceuticals represent a formidable challenge regarding analysis and characterization compared to traditional small molecule drugs. Any changes to the primary, secondary, tertiary or quaternary structure of a protein can potentially impact its function, efficacy and safety. The analysis and characterization of (structural) protein heterogeneity is therefore of utmost importance. Mass spectrometry has evolved as a powerful tool for the characterization of both primary and higher order structures of protein pharmaceuticals. Furthermore, the chemical and physical stability of protein drugs, as well as their pharmacokinetics are nowadays routinely determined by mass spectrometry.

Here we review current techniques in primary, secondary and tertiary structure analysis of proteins by mass spectrometry. An overview of established top-down and bottom-up protein analyses will be given, and in particular the use of advanced technologies such as hydrogen/deuterium exchange mass spectrometry (HDX-MS) for higher-order structure analysis will be discussed. Modification and degradation pathways of protein drugs and their detection by mass spectrometry will be described, as well as the growing use of mass spectrometry to assist protein design and biopharmaceutical development.

1. Introduction

The worldwide annual revenue of biopharmaceuticals is more than $165 billion. With 588 biosimilars and 434 biobetters in development, this number is likely to increase over the coming years [1]. Protein pharmaceuticals are large molecules containing hundreds of amino acids and having molecular masses from tens to hundreds of kDa. Protein pharmaceuticals thus represent a different class of drug compounds to be analyzed and characterized at the molecular level compared to small molecule drugs [2]. Any modification of either the primary, secondary, tertiary or quaternary structure of a protein pharmaceutical can in principle impact its function, efficacy and safety profile [3,4]. It is therefore important to be able to detect and characterize these modifications. Since the development of soft ionization techniques in the 1990s [5–9], mass spectrometry (MS) has developed into a powerful technique to characterize both primary and higher order structures of protein pharmaceuticals [10,11]. These developments of MS have recently been the subject of excellent specialized reviews (ion mobility spectroscopy [12], HDX-MS [13–17], higher-order structure analysis by MS [18,19]). However, as the analysis of chemical and physical stability and comparability of protein drugs, both *in vitro* and *in vivo*, increasingly relies on an expanding number of MS based workflows, a broader and more comprehensive understanding of the application of MS in biopharmaceutical development is needed by the pharmaceutical scientist. In this review, we seek to provide a broad introduction to the use of both established MS workflows and emerging MS technologies for in-depth qualitative analysis of proteins from the perspective of pharmaceutical research.

1.1. Mass spectrometry of peptides and proteins

With the advent of soft ionization, the analysis of macromolecules such as peptides and proteins by mass spectrometry became feasible. Soft ionization maintains a low internal energy in the analyte throughout the ionization process, ensuring that the backbone polypeptide structure is left intact. The most commonly used soft ionization techniques for protein mass spectrometry are matrix-assisted laser desorption/ionization (MALDI) [7] and electrospray ionization (ESI) [8]; their principle mechanisms are depicted in Fig. 1. In MALDI, the analyte is co-crystallized with a matrix that allows absorption of a certain wavelength. Upon

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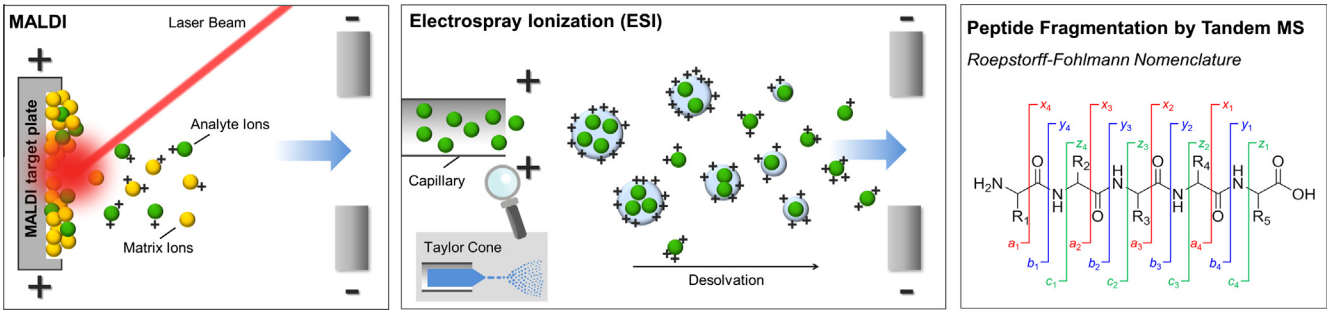

Fig. 1. Principles of MALDI and ESI ionization, as well as peptide fragmentation by tandem mass spectrometry leading to the formation of a-, b-, c-, x-, y- and z-ions, as defined by Roepstorff-Fohlmann [22].

irradiation of the sample with a laser under high vacuum, the matrix material absorbs the energy and ablates. Proton transfer reactions in the hot plume are thought to lead to ionization of sample molecules but the exact mechanism of ionization in MALDI remains to be elucidated [20]. In ESI, ions are produced by dispersing the sample solution at the tip of a capillary into a fine aerosol, due to a large potential difference placed between the positive capillary and a negative anode inside the ionization chamber. Desolvation of the microdroplets in the aerosol is achieved by a flow of counter-current heating gas, resulting in a series of coulombic fissions into droplets of decreasing size and the eventual emergence of desolvated analyte ions. However, as is the case for MALDI, the exact mechanism of ESI remains to be elucidated [21].

Once ionized, the mass spectrometer records the *m*/*z* value of resulting peptide or protein ions (MS analysis). Tandem mass spectrometry, or MS/MS, can provide additional information about structure and composition of peptides and proteins. In MS/MS, initial MS analysis of the precursor ion is followed by gas-phase activation and subsequent MS analysis of the resulting dissociated "product" ions. Common types of ion activation for peptides and proteins include collision-induced dissociation (CID) [23], electron transfer dissociation (ETD) [24] or electron capture dissociation (ECD) [25]. CID is accomplished by colliding the gas-phase analyte ions with neutral gas atoms (e.g. He, N_2, Ar) [26]. A disadvantage of this approach is that labile posttranslational modifications such as phosphorylation and glycosylation are easily lost because the backbone amide bonds in proteins and peptides require higher collision energies to break, compared to e.g. glycosidic bonds [27]. However, use of either ETD or ECD can circumvent these problems. For ETD, an electron is transferred from a reagent radical anion (e.g. anthracene) to the analyte ion [24], while in the case of ECD a free low energy electron is captured directly by the analyte ion [25]. The prompt nature of radical-induced fragmentation *via* ECD or ETD prevents the loss of posttranslational modifications enabling localization of PTMs by mass analysis of product ions through a nonergodic process [28,29]. Depending on the type of fragmentation, different kinds of ions are generated; CID leads to b- and y-ions while ETD and ECD result in the formation of c- and z-ions (Fig. 1).

2. Characterization of protein pharmaceuticals – analysis of covalent (primary) structure

Primary structure analysis aims to determine protein purity, molecular mass, amino acid sequence and posttranslational modifications, often as a function of expression, purification, formulation, distribution, handling or storage. Fig. 2 provides an overview of general workflows for primary structure analysis of protein pharmaceuticals by mass spectrometry. The choice of an adequate MS workflow can vary depending on the nature of the

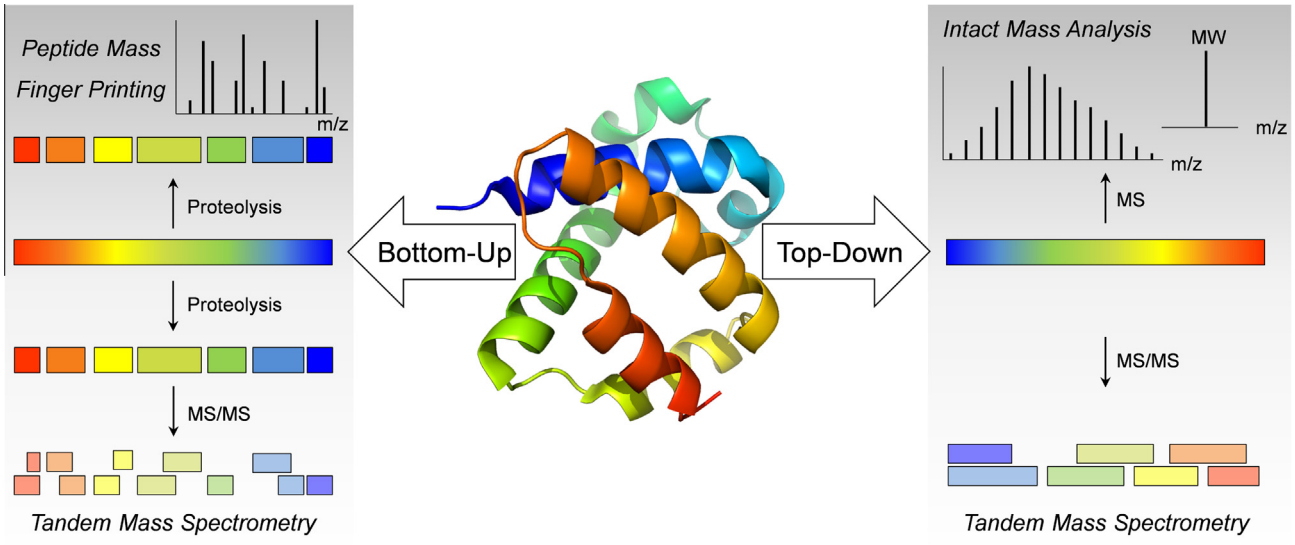

Fig. 2. Bottom-up- and top-down analysis for the structural characterization of peptides and protein pharmaceuticals by mass spectrometry. In bottom-up approaches, the protein is first digested into peptides, which are then analyzed by either MS (peptide mapping/peptide mass finger printing) or tandem MS (MS/MS). Top-down approaches include intact mass analysis (the inset shows the deconvoluted mass spectrum, displaying the molecular weight of the protein) and gas-phase fragmentation of the protein by tandem MS.