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Review Article

Clinical protein mass spectrometry

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ABSTRACT

Quantitative protein analysis is routinely performed in clinical chemistry laboratories for diagnosis, therapeutic monitoring, and prognosis. Today, protein assays are mostly performed either with non-specific detection methods or immunoassays. Mass spectrometry (MS) is a very specific analytical method potentially very well suited for clinical laboratories. Its unique advantage relies in the high specificity of the detection. Any protein sequence variant, the presence of a post-translational modification or degradation will differ in mass and structure, and these differences will appear in the mass spectrum of the protein. On the other hand, protein MS is a relatively young technique, demanding specialized personnel and expensive instrumentation. Many scientists and opinion leaders predict MS to replace immunoassays for routine protein analysis, but there are only few protein MS applications routinely used in clinical chemistry laboratories today. The present review consists of a didactical introduction summarizing the pros and cons of MS assays compared to immunoassays, the different instrumentations, and various MS protein assays that have been proposed and/or are used in clinical laboratories. An important distinction is made between full length protein analysis (top-down method) and peptide analysis after enzymatic digestion of the proteins (bottom-up method) and its implication for the protein assay. The document ends with an outlook on what type of analyses could be used in the future, and for what type of applications MS has a clear advantage compared to immunoassays.

1. Introduction

Proteins and peptides are essential biomolecules, composed of individual amino acid residues linked together via peptidic bonds. Their biological functions are multiple: structural and mechanical roles, responses to stimuli, gene replication, chemical transporters, catalyzing metabolic reactions, and many others. Proteins are also used as drugs for the treatment of diseases. Typical examples are insulin, growth hormones, and therapeutic antibodies. Protein analysis is thus an important activity in clinical laboratories. Nonspecific detection methods such as chromatographic or electrophoretic separation followed by UV detection or staining are nowadays replaced by specific detection methods. These methods include indirect antibody-based immunoassays such as enzyme-linked immunosorbent assay (ELISA) and, more recently, mass spectrometry (MS). For many years, specific quantitative protein assays were performed almost exclusively using immunoassays. But rapid progress of protein MS has taken this method into the spotlight. There is much discussion in the field about the advantage of MS compared to immunoassays, including reagent cost, specificity, throughput, and the possibility to multiplex assays [1–7]. The following report will first discuss the principles, advantages and inconveniences of mass spectrometric assays compared to immunoassays from the point of view of a clinical laboratory. In opposition to other recently published reviews [7–10], an important distinction is made between top-down and bottom-up MS assays, where respectively the full length protein or only a short specific signature peptide after it's enzymatic digestion is analyzed. Second, the few most used or most promising clinical MS assays for peptides and proteins will be reviewed. The primary focus of this review is to highlight their limits and advantages compared to immunoassays, not to provide an exhaustive list of what has be proposed by the community. The two last sections will focus on the challenges to be overcome to implement more MS assays in clinical laboratories, and an outlook on what type of assays might be used in the future.

2. Immunoassays

Immunoassays use the specificity of an antibody to detect an antigen, for example a peptide or a protein. The concentration of

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antigens is then deduced from the number of antibodies bound to antigens, via a signal amplification technique such as a radiolabel, a fluorescent label, or an enzymatic conversion of a reagent to a detectable product. A typical immunoassay assay is summarized in Fig. 1.

Immunoassays are today clearly the most common quantitative protein assays in clinical laboratories. They are very sensitive, and protein concentrations in the ng/ml range can be detected with good specificity and linearity, if an antibody with the desired performances is available. The assays are performed in solution, making automation easy with the help of liquid handling robots. At the department of genetics and laboratory medicine of our institution, a university hospital serving a population base of one million habitants, about 320,000 protein immunoassays are performed each years, to measure 72 different parameters. However, it should be noted that the most common protein assays are enzymatic tests, with 470,000 analyses/year and 60 different parameters. Far behind are colorimetric assays, with 77,000 tests on 13 different parameters. No protein mass spectrometry assay is currently used on a routine basis at our department. Although very common, immunoassays are not perfect, and some of them suffer from limitations. Antibodies recognize a polypeptide epitope of typically eight to seventeen amino acids in length. Thus, an antibody might recognize a common portion of a protein present in different proteoforms[1] [11] without being able to differentiate between them. In some cases, a distinction of different proteoforms is impossible or simply not known. Antibody specificity for a particular isoform is suited for clinical data interpretation, but is not always possible. Such interferences are for example described for antibodies against the glycated hemoglobin form HBA1c, interacting also with the carbamylated form of hemoglobin [12]. Autoantibodies, *i.e.* antibodies expressed by a subject against the analyte can also result in false reporting. When such autoantibodies are present, the analyte of interest is captured by them and does not bind to the assay's antibodies. The measured analyte concentration is thus below the real physiological concentration. The presence of autoantibodies is known against thyroglobulin, a protein routinely measured for the therapeutic follow-up of thyroid cancer [13]. Other examples where autoantibodies interact with proteins are described, and includes mucin-1, PSA, TSH, CRP, troponin I and insulin [4]. Nonspecific interactions between antibodies and other compounds, typically other antibodies present in the patient's serum, could also be a source of errors. In addition, saturation and limited dynamic range is also a limiting factor for immunoassay. The titer of antibodies linked on the solid surface for the test has to be in the same order of magnitude as the number of analyte molecules binding to it. If the concentration of analyte molecules is higher, the excess will be washed away. This will, in turn, result with an incorrect concentration value. This saturation effect is also called the "hook" effect due to its characteristic shape when the detection signal is plotted against the analyte concentration. The different causes resulting in incorrect test results for immunoassays are summarized in Fig. 2.

3. Mass spectrometry

Mass spectrometry (MS) is an analytical method that detects analyte ions in the gas phase. The detection is specific to the mass over charge ratio (m/z) of the ions. In an MS experiment, the analyte is first ionized, *i.e.* converted into gas-phase ions. Many different ionization methods have been described, including electron impact, fast atom bombardment, chemical ionization, photoionization, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The latter two are particularly soft ionization techniques, *i.e.* fragmentation of the analyte during the ionization process is very limited. They are thus particularly useful to convert large biomolecules such as DNA and proteins into molecular ions. The inventors of these soft ionization techniques were recognized by the 2002 Nobel Prize [http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2002/]. With MALDI, the analyte is co-crystalized in a matrix, usually an organic acid, and introduced in solid form into the mass spectrometer. A laser pulse is then used to desorb and ionize the sample. The energy of the laser is absorbed by the matrix, resulting in a rapid gas-phase expansion. During this expansion, charges are transferred from the matrix to the analyte molecules, resulting with gas-phase ions (Fig. 3A). With ESI, the liquid analyte solution is directly transformed from the liquid phase into the gas phase. For this, a high potential difference is applied between the tip from which the analyte solution elutes and the mass spectrometer inlet. ESI can be hyphenated to a liquid chromatograph in a process called LC–MS. ESI is widely used in routine and research laboratories to analyze various analytes in complex matrixes, such as biofluids or complex mixtures (Fig. 3B). The exact mechanisms of MALDI and ESI ionization is still under debate, and the subject is extensively reviewed [14,15].

Once into the gas phase, the ions are directed toward a mass selective analyzer and detector, and their m/z value is precisely determined. A mass spectrum represents the ion abundance as function of the m/z value. The relative molecular mass (m) is expressed in unified atomic mass units [u] or Daltons [Da], defined as 1/12th of a ^{12}C atom. The charge (z) is the number of elementary charges. Sometimes, the Thompson [Th] is used as mass to charge ratio unit [16]. If the number of charges of an ion is known, for example by measuring the m/z distance between different isotopes in isotopic clusters, the m/z value can be directly converted into mass units.

Different types of mass analyzers can be used. Quadrupoles and ion traps are mostly used today in clinical laboratories. Also popular are time-of-flight, ion cyclotron and orbitrap analyzers, but these high-end instruments are mostly used for research applications. Quadrupoles (Q) consist of four parallel rods (electrodes), diagonally electrically connected. A radiofrequency (RF) is applied between them, and a direct current is superimposed on it (RF + DC). The electric field generated focuses the ions into the axial center of the quadrupoles. For each electric potential and radiofrequency amplitude pair, only ions at unique m/z ratio have a stable trajectory and pass across the quadrupole. Ions at other m/z values hit the electrodes [17]. The quadrupole can thus be used as mass filter to selectively transmit ions at a particular m/z value, or be used as scanning device to acquire a mass spectrum, *i.e.* a plot of m/z values and their relative abundances. Three-dimensional ion trapping devices (3D-IT) are operating on the same principle, but consist of a ring electrode and two endcap electrodes [17]. They can be used as ion storage and ion manipulating devices, and a mass spectrum is acquired when they are ejected out of the ion trap according to their m/z value [18]. Quadrupoles can also operate as ion trapping devices if an electrode at each end is used to create a potential well in the quadrupole. Such devices are called linear ion traps [19,20]. Time-of-flight (TOF) analyzers are field-free tubes that ions travel through after an initial acceleration using an electrical field pulse. All ions are accelerated with the same kinetic energy, and their flight time toward the detector is measured and converted into an m/z value. Ions with low m/z value will travel at higher velocity and reach the detector first [21,22]. Ion cyclotrons use a high magnetic field. The ions are accelerated orthogonally to the field, which induces a cyclotronic motion. The image current of this cyclotronic motion is recorded on

[1] Different proteoforms could be for example different cleavage products, different splice variants, different isoforms or the presence or absence of post-translational modification(s) [11].