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ABOUT MASS SPECTROMETRY: FROM ELEMENTAL ANALYSIS TO DIAGNOSTIC IMAGING

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Summary

Almost hundred years after the first demonstration of exact mass determination of chemical elements by J. J. Thomson's in 1912, the method discovered by him – mass spectrometry – reached a high level of development in different fields of science and applications to life sciences, technology, medicine and public life. New aspects of ionization of molecules from all aggregate states contributed largely to develop new ion sources for almost universal usage. By development of distinct ion optics the secondary ion beams can be manipulated in mass spectrometers, which may contain cells for ion concentration or fragmentation of entire ion clouds or their parts. Modern mass spectrometry is characterized by unique properties for research and analytics like specificity, sensitivity, speed, the possibility of automatization and of application of algorithms for interpretation of experimental data. These properties are already integrated in commercial instruments and used for "omics"-type of projects, like genomics, proteomics, glycomics, lipidomics, and so on.

In this article, own investigations and method developments are presented, in which high resolution mass spectrometry, hyphenated techniques to MS and automated data interpretation are playing a crucial role. These technical solutions were applied to projects focussed to discovery of potential biomarker of human diseases. In the present vision of the future MS in medicine, MS imaging will play an important role. Using laser desorption for ionization, the images similar to those obtained by microscopy can be obtained, where they contain additionally also the information on the molecular structure as an extra value.

Keywords: Instrumentation for mass spectrometry, MALDI- and ESI-ionization, proteomics, glycoproteomics, glycourinomics

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Abbreviations

EI	electron impact
ESI	electrospray ionization
MALDI	matrix-assisted laser desorption/ionization
FAB	fast atom bombardment
CID	collision induced dissociation
FD	field desorption
PD	plasma desorption
HPLC	high-performance liquid chromatography
Q-TOF	quadrupole/time-of-flight
TOF–TOF	time-of-flight/time-of-flight
m/z	mass-to-charge
MS	mass spectrometry
MS/MS	tandem mass spectrometry
IMS	ion mobility mass spectrometry
MS^n	multiple stage mass spectrometry
PMF	peptide mass fingerprinting
ppm	parts per milion
PTM	post-translational modification

Mass spectrometry: historical remarks

The importance of mass spectrometry arose from its remarkable utility for studies of molecular structure and reaction mechanisms in a wide variety of contexts. In both its theoretical and experimental aspects, mass spectrometry does provide an integrative platform for discovery in numerous specialized areas of natural and life sciences. Thus, the significance of mass spectrometry to the research community encompasses presently wide area of physics, chemistry, biology, informatics and medicine, reflecting its unique interdisciplinary character. Since the discovery of separation of ions in the gas phase on the basis of mass to charge ratio in 1911 by Joseph John Thomson (1856-1940) as the "determination of atomic composition by mass" and followed by his student Francis William Aston (1877-1945) mass spectrometry has developed into a general field of great scope and power [1,2].

Thomson and Aston determined the isotopic composition of the element neon in 1913 by „mass spectrograph". Aston's new mass spectrograph built in 1919 made use of a collimated beam deflected by

Fig. 1. Joseph John Thomson (1856-1940)

a parallel-plate electric field, passed through a slit, and deflected by a magnetic field to be recorded on a photographic plate. Masses were measured by interpolation between lines of known mass with an experimental error of 0.1%. By 1921 Aston published his findings on the isotopic composition of several other elements, including Ar, Kr, Hg, and, of Cl. By 1925 the isotopic composition of over 50 elements had been determined, by 1933 the number had increased to 66, and by 1948 to 83 [3] However, development of laboratory instruments for mass spectrometry until mid-1940's was still in infancy, where only four university laboratories in USA were doing mass spectrometric work, Minnesota, Princeton, Harvard, and Chicago.

Modern instrumentation for mass spectrometry

Electron-impact (EI) was a single established method for ionization for more than 30 years, where in a relatively harsh form of ionization process molecular ions as cation radicals $M^{+\cdot}$ and their fragments were generated. Upon thermal vaporization of the sample the vapor diffused into the electron beam and become ionized, depending on the electron energy which was controlled by the accelerating potential on the ion trap electrode and accelerated to the analyser. Practical solutions to the quest for non-thermal evaporation of analytes from the solid or condensed phase to the gas phase appeared about 40 years ago, when the first cold ionization techniques, field desorption (FD) and plasma desorption (PD), followed by fast atom bombardment (FAB), were introduced. From this phase of development on, the ions were generated mostly in the external ion source and the refocused ion beam introduced to different types of mass analyzers. For FD ionization a high-potential electric field is applied to an carbon tip emitter, which was dipped to the solution of the analyte and briefly introduced into the ion source, producing molecular radical cations $M^{+\cdot}$ or protonated molecular ion to be extracted into the mass analyzer [4]. In PD the ^{252}Cf ion beam who hit the protein deposited on the polymer foil could be ionized and detected mostly as a singly charged species using the time-of-flight (TOF) detector [5].In FAB, the analyte in solution was deposited into a drop of a less-volatile liquid matrix and submitted to bombardment by a high energy beam of inert gas atoms Ar or Xe at 4,000 to 10,000 eV [6]. The latter technique became largely popular, representing a relatively soft ionization technique and producing primarily intact cationized or anionized molecular ions, e.g. $[M+H]^+$ and $[M-H]^-$. The best resolution has been achieved at that time on sector instruments, consisting of magnetic and electrostatic analyzers in different geometries, and peptide and carbohydrates could be analyzed under such conditions [7,8]. Radical changes in the size of ion-

ized molecular species came up with new techniques in the last century at the end of eighties, when laser desorption and electrospray ionization were presented as revolutionary methods to ionize very large molecules softly as expressed by John Fenn under the motto "making elephants to fly" [9].

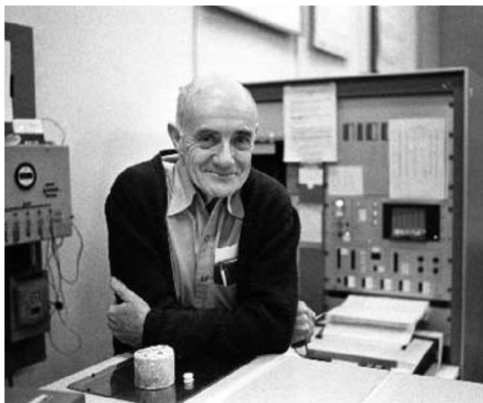

Fig. 2. John B. Fenn (*1917)

In the electrospray process of proteins, first published 1989 by his group at the Yale University, the ionization of intact macromolecules is taking place from solution in the electric field by formation of charged droplets on the capillary tip. These highly charged droplets shrink during the drying process and after entering the mass analyzer the distribution of their differently charged states can be documented in mass spectra [10]. Less soft Desorption Ionisation/Laser Desorption Ionization (LDI) of intact proteins from the solid state was first presented in 1988 by the industrial research group led by Koichi Tanaka in Japan [11], an achievement which was also honoured by the Nobel prize committee in 2002 [12]. In the same year Matrix-Assisted Laser Desorption Ionization (MALDI) for ionization of intact macromolecules larger than 100.000 Dalton was published by Franz Hillenkamp and Michael Karas from University of Münster in Germany, and became within short time a most popular method for protein and peptide analysis [13]. Using the Time-of-Flight (TOF) Analyzer, where the mass of the ionized species is measured by its time of flight, basically molecular species of any size could be detected, if well ionized. The present state-of-the-art in the size of molecules and assemblies was recorded to be 18 MDa virus protein coat, where the multiply charged molecular ions were detected by MALDI-TOF MS.

Concerning mass analyzers, presently one of the most popular devices is the Paul's ion trap. This radiofrequency quadrupole ion trap had its origins as far back as 1953, in the same patent as that in which Paul and Steinwedel at the University of Bonn first disclosed the operation of the quadrupole mass filter [14]. It has passed through several phases of development in which successive attempts have been made to exploit its potential for different analytical applications. At the same time there has been a distinct and parallel history of the evolution and

application of the trap in the field of atomic physics, culminating in being honored as one of the three co-recipients of the Nobel Prize for physics in 1989 [15].

Fourier Transform Ion Cyclotron Resonance (FTICR) as the high-performance nondestructive image-current detection device was introduced by Comisarow and Marshall in 1974 [16]. FTICR represents *de facto* a spectrometry as a tandem-in-time technique, meaning that all manipulations of the ions take place within the confines of a single space, but in temporally separate events. The ICR cell is located in the homogeneous region of a static magnetic field, where trapped ions undergo cyclotron motion in a plane perpendicular to the field at a frequency reflecting its mass-to-charge ratio. Confinement parallel to the magnetic-field lines is achieved by a static electric field generated by applying a small voltage onto two trapping plates that are oriented orthogonal to the magnetic field. The interactions of the ions with the magnetic and electric fields permit the ions to be trapped, manipulated, and ultimately detected. The frequencies of the image current can be measured to better than nine figures, corresponding to a mass measurement accuracy on the order of l part per billion (ppb), provided that a suitable mass calibration and multiple linear regression can be applied [17]. Gorshkov et al. determined by FTMS the mass of 20Ne to be 19.992440691(90)~with a precision that is a factor of 24 better than that in the 1983 Atomic Mass Evaluation; that latter measurement is based on nuclear reaction energy [18].

Fig. 3. Wolfgang Paul (1913-1993)

High mass resolution and the accurate determinations of ion mass and relative abundance are particularly important for the identification of the biological molecules, where the atomic composition information can be derived. For example, even with elemental composition restrictions that apply to a peptide containing some combination of the twenty common amino acids, 523 elemental compositions are proposed within 25 ppm of the molecular mass accuracy of the relatively small peptide, substance P (MW= 1346.7121). An improvement in the accuracy of the mass measurement to 10 ppm causes the number to fall to just 13.

A novel hybrid instrument in which the linear trap quadrupole is linked to the orbitrap is a compact and less costly analyzer of high resolution and mass accuracy developed by Alexander Makarov in 2000 [19] and shortly after brought

to the market. It is designed for protocols demanding for a short acquisition time and using a linear octopole collision cell, fragmentation analysis can be performed. Despite its relatively recent commercial introduction, the LTQ-Orbitrap has already proven to be a highly relevant analytical tool with a wide range of applications. Major applications are currently in proteomics, where its high resolving power (>150,000) and excellent mass accuracy of less than 1ppm under favourable conditions can contribute to significantly reduce false positive peptide identifications. A high number of publications appeared in this time window to report on identification of intact peptides and proteins of 3–150.000 kDa, on drug and metabolite analysis, lipidomics and on molecular structure characterization using hydrogen/deuterium exchange measurements [20].

Proteomics: changing the paradigma

Proteomics evolved as a field in which the total protein expression profile studies of a particular cell, tissue, or organism, at a given time can be systematically and in parallel studied. Proteomics and other complementary analysis methods are essential components of the emerging 'systems biology' approach that seeks to comprehensively describe biological systems through integration of diverse types of data and, in the future, to ultimately allow computational simulations of complex biological systems [21].

The analytical techniques involve primarily a work-flow which include the two-dimensional gel electrophoresis separation, followed by a proteolytic digestion of single spots, their extraction and peptide mass fingerprinting (PMF) by mass spectrometry, data analyses, and the use of databases [22]. In an alternative scenario, the total mixture is digested and submitted to the one- or two dimensional LC separation linked on-line to the mass spectrometer fitted with the ESI ion source or at-line to the analyzer fitted with a MALDI ion source. For the systematic study of the protein content under specific conditions, three main aspects must be efficiently solved by analytical methods: protein identification, characterization, and quantification. In clinical proteomics the study of disease-related molecular changes is focussed toward the discovery of diagnostic markers and new therapeutic targets. Mapping molecular ions of peptides in proteolytic mixtures by PMF is not a sufficient requirement for identification, even at high resolution/high mass accuracy conditions. Fragmentation experiments by tandem MS protocols (MS/MS) add sequence information required for protein identification. Due to the complexity of the proteome numerous pitfalls faced by software, or from the MS/MS data are still possible [23].

Recent efforts to integrate studies based on the targeted and the global approaches represent a promising strategy to be integrated to cell biology and biomedicine. Membrane proteomics is concerned with accurately and sensitively identifying molecules involved in cell compartmentalisation, including those controlling the interface between the cell and the outside world. Due to the high lipid content of the environment the protein material out of membranes must be isolated prior to HPLC-MS analysis.

Monitoring of red blood cells (RBC) as the solely oxygen transporter in the organism of particular value for clinical proteomics was recently reviewed [24]. From experimentally obtained RBC proteomics data major understanding toward interactions with plasma, endothelial cells, leukocytes, and platelets can be deduced, and matched to the deposits in open-access databases [25]. The contribution of large-scale, high accuracy mass spectrometric equipment, like QTOF and FTICR-MS, in combination with selected biochemical procedures for sample preparation, will contribute largely to the increase of the protein identification score. In the case of RBC identified proteins can be categorized in terms of subcellular localization, protein family, splice isoforms and functions. Encompassing 600 membrane and soluble proteins identified in a single study, this data set provides interesting tool for further targeted explorations.

Glycoproteomics: revealing the most common proteome modification

According to intensive research over the last 50 years glycosylation appeared as one of the most common and diverse posttranslational modification (PTM) of eukaryotic proteins. Presumably more than 50% of all proteins are glycosylated [26], while their carbohydrate content varies from less than 1% to more than 90%, by weight. A variety of biological functions have been proposed for the carbohydrate part of glycoproteins: the blood group antigens are composed of oligosaccharides bound to proteins or lipids, glycans are involved in the proper folding and the quality control of newly synthesized glycoproteins in the endoplasmic reticulum, and control the serum lifetime of glycoproteins, play a role in the polarized biosynthetic sorting of proteins, participate in the sperm-egg binding, just to mention a few. Glycosylation is a highly complex and dynamic process, which frequently results in the biosynthesis of multiple glycoforms of the same protein by either incomplete glycosylation or processing of the oligosaccarides.

There are two main types of protein glycosylation. For the *N*-glycosylation, the polypeptide sequence has to contain the consensus sequence asparagine-X-serine/threonine (Asn-X-Ser/Thr, where X may be any amino acid except proline, Pro) in which the oligosaccharide is attached to the Asn residue via an *N*-glycosidic bond. *O*-glycosylation

occurs at Ser or Thr residues, but no general consensus sequence for O-glycosylation is known so far. Therefore the structural analysis of glycoproteins to reveal the glycosylation sites and the corresponding glycoforms is still a challenge.

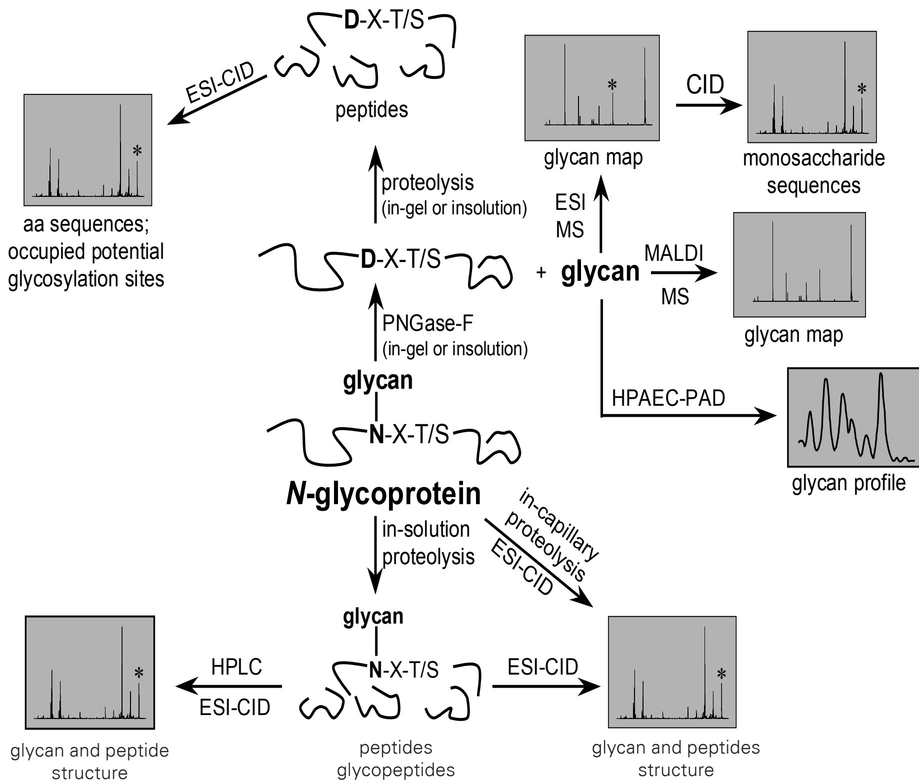

Fig. 4. Strategies for structure elucidation of *N*-glycosylated proteins [27].

Different strategies are in use for glycan mapping. N-linked oligosaccharide moieties are enzymatically released as glycosylamines from the polypeptide chain by peptide-N-glycosidase F (PNGase F) for mammalian glycoproteins or A (PNGase A) for plant glycoproteins (Fig. 4). The former site of N-glycosylation Asn residue is converted to aspartic acid (Asp) inducing a mass shift in the remaining peptide of roughly 1 Da. The characterization of the overall glycan expression pattern and the determination of the previously occupied glycosylation sites occurs in separate MS experiments using either glycan mapping by MALDI or ESI-MS/MS, which together contribute to the glycosylation state overview as

demonstrated in the glycosylation pattern analysis of CDG patient plasma by in-gel N-deglycosylation followed by MALDI TOF MS for rapid diagnosis [28]. Alternatively, the proteolysis of the intact glycoproteins followed by identification using the on-line HPLC-MS and MS/MS should provide a direct information and overview of all glycan isoforms at the single site and encompass the glycosylation status at all glycosylation sites. However, there are still major ongoing efforts to obtain a high coverage of all native glycopeptides present in proteolytic mixtures obtained from purified or mixted preparations of glycoproteins [29].

Cell surface and extracellular proteins are O-glycosylated, where the most abundant type of O-glycosylation in proteins is the GalNAc attachment to serine (Ser) or threonine (Thr) in the protein chain by an ά-glycosidic linkage. Most eukaryotic nuclear and cytoplasmic proteins modified by ά-linked O-GlcNAc to Ser or Thr exhibit reciprocal O-GlcNAc glycosylation and phosphorylation during the cell cycle, cell stimulation, and/or cell growth. Less investigated types of O-glycosylation are O-fucosylation, O-mannosylation, and O-glucosylation, but they are functionally of high relevance for early stages of development and for vital physiological functions of proteins. Glycosaminoglycans are ά-linked to proteoglycans via a xylose-containing tetrasaccharide, represented by linear chains of repetitive disaccharides modified by carboxylates and O- or/and N-linked sulfates. Their functional role is described in crucial processes as coagulation, wound healing, cell adhesion, inflammation and interaction with fibroblast growth factors, but their detailed molecular structure is in many cases still unknown [30].

Analysis of O-glycosylation by mass spectrometry (MS) is a complex task due to the high structural diversity of glycan and protein factors. The parameters in structural analysis of O-glycans include determination of (i) O-glycosylation attachment sites in the protein sequence, (ii) the type of attached monosaccharide moiety, (iii) a core type in the case of GalNAc O-glycosylation, (iv) the type and size of the oligosaccharide portion, (v) carbohydrate branching patterns, (vi) the site of monosaccharide glycosidic linkages, (vii) the anomericity of glycosidic linkages, and (viii) covalent modifications of the sugar backbone chains by carbohydrate- and noncarbohydrate-type of substitutents [30].

Glycourinomics: a potential for biomarker discovery in human diseases

Enzymatic deficiencies of carbohydrate biosynthesis and metabolism are frequently regarded as glycoproteinoses [31] giving rise to massive accumulation of glycopeptides excreted in urine. α-NAGA deficiency known also as Schindler's

disease, is characterized by remarkable clinical heterogeneity, but patients without overt clinical symptoms have been also reported. The polar mixtures of middle molecular weight between 500 and 5000 Daltons obtained by work-up procedures of patients' urine were proposed to contain possible biomarkers for early diagnostic evaluation and possible treatment. However, at the time of discovery there were no analytical methods capable to encompass the existing carbohydrate complexity and to contribute to identification of single excreted intact and truncated complex carbohydrates. According to the progress in development of instrumentation technical options for structure elucidation of metabolically excreted glycans and glycopeptides by glycoscreening of complex mixtures by MS are becoming feasible [32].

The goal of metabolic screening is to identify all major and minor single components according to their m/z values as molecular ions in MS and to assign them *de-novo* by an automatic algorithm. The importance of high-end instrumentation, in particular in high resolution/high accuracy has been underestimated in the past. We could show, that for *de-novo* automatic compositional assignment of intact molecular ions high mass accuracy is highly relevant, in

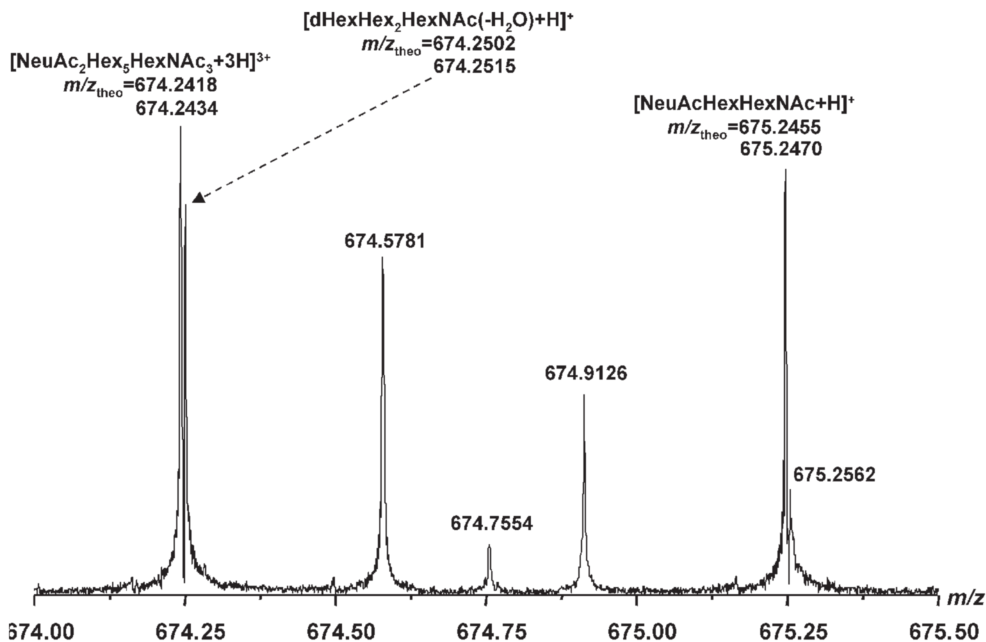

Fig. 5. Identification of glycoconjugates from human urine by high accuracy FT-ICR mass spectrometry [33].

which a number of previously unknown structures could be identified, which were overlapping in measurements on instruments with lower resolution.

Probing the analysis of urine samples by Fourier-transform ion cyclotron resonance mass spectrometry (FT ICR MS) under high resolving power and accuracy in mass determination it was demonstrated that this approach could be particularly suited for complex carbohydrate profiling in native mixtures. Multiple stage fragmentation (MS^n) on a FT ICR MS analyzer represents a particular advantage for structure determination of macromolecules, to provide high resolution and mass determination accuracy of the fragment ions at any stage of analysis. Particular attention has been consecrated to native sialylated glycans to prevent a possible loss of the NeuAc moiety due to the relative ease of its detachment by in-source fragmentation, requiring a careful optimization of the MS and tandem MS conditions. In experiments carried out by FT ICR SORI CID MS at 9.4 T glycoforms profile from urine of patients suffering from Schindler's disease were obtained, and optimized MS^n conditions provided a new option to analyze the degree of sialylation by the negative ion mode multistage analysis [32].

The FT ICR approach has been applied to screen urine of patients diagnosed with Congenital Disorders of Glycosylation (CDG) of unknown type. More than 150 molecular species were detected in a single glycoconjugate fraction obtained from urine of a CDG patient by use of accurate mass determination under high resolution, in which overlapping nearly isobaric ionic species could be resolved and identified in a single MS stage by computer-assisted calculations. In combination with monosaccharide building block analysis, the considerations of non-carbohydrate modifications, such as amino acids, phosphates and sulfates were taken into account. Taking advantage of this strategy the number of assigned peaks was significantly increased. Applying basic principles of *N*- and *O*-glycan assembling it was possible to indicate the type of the glycan involved according to the ratio between numbers of hexoses and *N*-acetylhexosamines. The $Hex_3HexNAc_2$ unit can be associated with the pentasaccharide $Man_3GlcNAc_2$ as a common core for all types of *N*-glycans, while a "high-mannose" type *N*-glycan can be estimated according to the Hex/HexNAc ratio of 2:1 or higher. For the hybrid type *N*-glycan this ratio decreases, whereas the complex type is characterized by the lowest ratio. In *O*-glycans the ratio is 1:1 or lower [33].

For *de-novo* sequence assignment of single components from fragmentation analysis by MS/MS the overlapping is a problem as well, which was investigated using the separation in the gas phase by ion mobility mass spectrometry [34]. Probing the IMS approach together with *de-novo* automatic assignment ac-

cording to fragmentation patterns high-efficiency procedure for glycourinomics could be designed [35,36].

Conclusions

A major change in paradigma took place within the last two decades turning our way of designing and performing experiments in life sciences, beyond reductionism approach to the systems biology. Development of analytical tools and instruments reached a mature stage for high accuracy/high speed data acquisition. Recent efforts to integrate studies based on the targeted and the global "omics" approaches represent a promising strategy to be integrated to cell biology and biomedicine. Mass spectrometry appears presently as a global analytical method, which is aimed to be integrated in different type of strategies for biomedical projects to increase the efficiency of identification and speed of data acquisition. High perspectives can be forecasted for use of mass spectrometry in diagnostics of human diseases and for new concepts in studies toward discovery and validation of biomarker molecules and their profiles. Identification, validation and quantification are strictly required to increase the output of proteomics in systems biomedicine. The targeted proteomics can contribute more validated evidence then in the data-driven experiments [37]. Glycomics, still underdeveloped in comparison to proteomics, will be in focus for the next decade, in which robust integrated platforms will be necessary to validate high-throughput procedures. Novel MS systems for tissue microscopy and for the surface ablation will probably play an interesting integrative role for interdisciplinary projects between medical practitioners and basic scientists. Such molecular imaging of proteins in rat brain tissues by mass spectrometry were already shown, where the images are constructed at spatial localization with a lateral resolution of 10–100 μm [38].

On the other side the technical progress into miniaturization, automatization and nanodevices for sample delivery [39] will largely contribute to in-depth investigations, concerning in particular the identification of components barely visible in the mixtures of high dynamic range and open new horizons for development of in-care devices. Construction and curation of general extended data bases as well as those focussed for distinct applications will enable a rapid access to the accumulated knowledge for comparison and data mining. A concept of tight collaboration between biochemists and bioinformaticians on one side could give rise to higher efforts to establish robust software for automatic assignment of acquired MS data and their automatic deposition in data bases. On the other side a tight collaboration between medical practitioners and ba-

sic scientists will be necessary for new protocols for standardization of clinical samples' preparation and storage.

The platforms based on integration of the aspects described above will represent a beginning of more efficient, more rapid and less costly health care, based on systems biology, in which a close placement of MS to the patients' bedside may become possible. Many challenges are still to be overcome.

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Sažetak

O spektrometriji masa u molekularnoj medicini: od elementarne analize do dijagnostičkog slikanja

Gotovo sto godina nakon što je J. J. Thomson's 1912. prvi put pokazao da se masa kemijskih elemenata može eksperimentalno točno odrediti metoda koju je on otkrio – spektrometrija masa – dosegla je visok stupanj razvitka i raznolikosti na raznim poljima znanosti i svoje primjene u tehnologiji, medicini i javnom životu. Nove spoznaje o ioniziranju molekula iz svih agregatnih stanja omogućile su razvoj novih ionskih izvora za gotovo univerzalne primjene. Razvojem instrumentalne ionske optike omogućeno je manipuliranje ionskih zraka u spektrometrima masa, koji sadrže ćelije za ili sakupljanje ili fragmentiranje čitavih ionskih oblaka ili njihovih dijelova. Moderna spektrometrija masa raspolaže jedinstvenim svojstvima za istraživanje i analitiku kao što su specifičnost, osjetljivost, brzina, mogućnost automatizacije i primjene algoritama za interpretaciju eksperimentalnih podataka. Sve te instrumentalne metode danas su već u cijelosti integrirane u „omics" poljima istraživanja kao što su *genomics, proteomics, glycomics, lipidomics* itd.

Ovdje su prikazana pojedina autoričina istraživanja razvitka metoda za visokorazlučenu spektrometriju masa i za njezino direktno priključivanje na instrument za razdjeljivanje te razvitka algoritama za interpretaciju eksperimentalnih podataka. Ta tehnička rješenja bila su primijenjena na projekte usredočene na otkrivanje potencijalnih biomarkera u ljudskim bolestima. U momentalnoj viziji primjene spektrometrije masa u medicini se već ocrtava dijagnostičko slikanje, pri čemu se laserskom desorpcijom dobivaju slike slične onima dobivenima mikroskopijom, ali koje sadrže i dodatnu informaciju o molekularnim strukturama.

Ključne riječi: Instrumentarij za spektrometriju masa; MALDI- i ESI-ionizacija; proteomika; glikoproteomika; glikourinomika