Virology, as a branch of the life sciences, discovered mass spectrometry (MS) to be the pivotal tool around two decades ago. The technique unveiled the complex network of interactions between the living world of pro- and eukaryotes and viruses, which delivered “a piece of bad news wrapped in protein” as defined by Peter Medawar, Nobel Prize Laureate, in 1960. However, MS is constantly evolving, and novel approaches allow for a better understanding of interactions in this micro- and nanoworld. Currently, we can investigate the interplay between the virus and the cell by analyzing proteomes, interactomes, virus-cell interactions, and search for the compounds that build viral structures. In addition, by using MS, it is possible to look at the cell from the broader perspective and determine the role of viral infection on the scale of the organism, for example, monitoring the cross-talk between infected tissues and the immune system. In such a way, MS became one of the major tools for the modern virology, allowing us to see the infection in the context of the whole cell or the organism. © 2019 John Wiley & Sons Ltd. Mass Spec Rev

Keywords: viral infections; capsids; GEMMA-MS; charge-detection mass spectrometry

I. INTRODUCTION

Mass spectrometry (MS), since its invention by J. J. Thompson in 1911, has become increasingly more popular. The technique is also constantly evolving due to the development of ion sources and the construction of analyzers, improvements in the fragmentation process, and progress in software capabilities. Consequently, the application area is broadening. Starting as a tool to solve particular problems in physics and chemistry, from the mid-1980s, MS has become one of the essential tools for a broad range of sciences and industries. The dynamic development of “biological” MS was initiated by the discovery of soft ionization techniques: electrospray by Yamashita and Fenn (1984) and matrix-assisted laser desorption ionization (MALDI) introduced by Tanaka et al. (1988). Both inventors were rewarded with a Nobel Prize in Chemistry in 2002. Since the introduction of both ion sources into laboratory routine, we have observed milestone discoveries in life sciences, medicine, nanotechnologies, environmental protection, and chemistry, which would not be possible without mass spectrometers. Moreover, some fields were formed because this tool was created, and this includes proteomics, metabolomics, and tissue MS imaging.

Virology is a branch of the life sciences, which focuses on the viral infection of the cells. To begin with, this field was mainly clinical, focusing on epidemiology and therapy; however, it has since become obvious that it is a fascinating field for basic and applicatory sciences. The virus, perceived as the information transferred between cells and organisms, is a ready-to-use tool to modify the cellular environment of the host by hijacking this pathogenic process. Of particular note, oncolytic viruses, redesigned to cure cancer, have already been approved by the Food and Drug Administration (FDA), the European Medicines Agency, and others.

However, to develop novel therapies, to design a vaccine or harness a wild-type virus to serve our cause, we need to thoroughly understand its biology and interactions with living cells. The interaction between the virus and the host which has developed over time is complex and sometimes unexpected. Thus, virology requires increasingly more sophisticated tools to progress. MS is one of the unique tools allowing us to look into the fate of the information delivered to the cell by a virus, and visualize the changing microenvironment of the infected cell. Some of the applications of MS encompass the physicochemical characterization of virions and viral proteins during the infection, evaluation of the interactions between viral proteins and between viral and cellular proteins, analysis of proteome alterations in the infected cell, and the design and testing of the efficacy and cytotoxicity of antivirals. A separate branch includes high molecular weight (MW) measurements of the whole viruses and identification of their assembly dynamics during capsid formation in a time-dependent manner. Using this technique, it is possible to observe the formation of capsids and then filling with nucleic acids, providing insight into the fascinating process of viral replication. In such cases, MS achieves new frontiers in the mass measurement range, almost reaching mass values of the microscopic objects.

MS has also become more popular in the rapid, unambiguous, and cost-effective identification of pathogens in clinical samples which may be difficult to process (tissues, body fluids, food, cell extracts, natural products, and others). In some
cases, especially in medical applications, the time required for the identification of the pathogen is a critical factor for the application of appropriate therapies. To date, MS has been routinely used for the identification of infectious microorganisms, but current MS-based approaches could also be effective toward the identification of viruses infecting humans. As some viral infections develop relatively rapidly and, due to their high infectivity, pose a threat to the human or animal population, fast methods of detection of the etiological factor are required. However, such analyses seem to be difficult due to viral biology, as only scarce amounts of viral proteins are present in body fluids during the early stages of the infection. One may speculate that the infection may be sensed earlier, by the analysis of antigens presented in the context of major histocompatibility complex type II (MHC II) by infected cells; however, analytical approaches identifying such presentations are missing thus far. A similar analysis has also been carried out for the development of effective vaccines, which is another field in which virology and MS mingle. MS is used to design vaccines, but it is also used to verify the content and quality of this versatile medicinal product. Thus, the importance of MS in virology seems to be obvious, as combining those two fields offers new research perspectives. Although there are some reviews on this subject (Cobo, 2013; Greco et al., 2014), the area is developing rapidly, and any attempt to summarize the latest achievements may be helpful. Moreover, the broader presence of this subject to the scientists involved in MS may result in new solutions to the obstacles in this field.

II. VIRAL WORLD

The classification of viruses remains a challenge. These complex structures of macromolecules may not be classified as living organisms, as they are inert beyond the cell. However, once they enter the host, they hijack the cellular machinery to serve their purpose, changing the cell into a virus factory that becomes invisible to defense systems. A virus particle carries information in the form of RNA or DNA, which, in complex with the capsid proteins, forms the viral core. This nucleocapsid is protected by the shell built of proteins (naked viruses) or a lipid bilayer (enveloped viruses), which protects the viral genetic material in the hostile extracellular environment. Regardless of the virus type, the shell is always decorated with the proteins responsible for attachment and entry to the host cell, which initiates the replication cycle.

The virus may utilize virtually any molecule on the surface of the cell as a receptor, and these may include proteins, sugars, or lipids constituting the natural landscape of the cell surface. Each viral species is fitted to use a limited number of receptors, which is one of the most important determinants of tissue and species specificity. The binding initiates the complex process of virus entry, which ends up in the delivery of the viral genetic material to the replication site: the cytoplasm or the nucleus, where the replication starts. Viral proteins are expressed and initiate remodeling of the cell by the formation of intracellular structures serving as viral factories and protecting viral components from detection by the natural defense mechanisms. Eventually, the viral replication machinery initiates production of progeny nucleic acids, which interact with the capsid protein(s) forming nucleocapsids. These macromolecules are further matured, and the shell is formed. The release may occur simply due to the disruption of the host cell, but cellular transport routes are frequently utilized, and virions are slowly released to the extracellular space, which finalizes the viral cycle.

While the replication cycle of viruses is common for all species, viruses are an incredibly diverse group of microorganisms. In most cases, it is difficult to compare them. While picornaviruses carry a small, few-thousand nucleotides-long single-stranded RNA as genetic material, Mimiviridae have double-stranded DNA genomes which exceed 1 million base pairs, and carry more information than the DNA of some bacteria. The molecular mechanisms of the infection, virus structure, and virus biology are simply incomparable. For that reason, the International Committee on Taxonomy of Viruses regularly makes an effort to provide the most current classification of different viral types, with virology divided into sections specializing in certain virus types.

Considering the variability of viruses, the complex and very close interaction between the virus and the host, and the size of these pathogens, studies on viruses are difficult and often require sophisticated techniques. MS is one of the most important tools that allow us to analyze the infection process at the single molecule level and observe the interplay between the cell and the virus. In this review, the possibilities offered by the tool are discussed in detail.

III. PUT THE VIRUS ON THE SCALE. WHAT IS INSIDE?

With the aid of MS, it is possible to determine the molecular masses of substances with very high accuracy. However, with an increase in mass, there is a decrease in resolution due to the physical limitations of the method and the properties of the samples (e.g., natural heterogeneity of the molecules, oligomerization in solution, incomplete desolvation, and low MW salt molecules attached (Fuerstenau & Benner, 1995; Lössl et al., 2014)). Along with the molecular mass increase, even in cases of highly homogenous material, average mass dissipation due to isotopic patterns of atom building molecules, becomes notable. Furthermore, viral units of the same species should have the same MW, but such sample homogeneity is rarely achieved. Until recently, other analytical techniques like dynamic light scattering, electron microscopy (mainly in transmission mode, transmission electron microscopy (TEM)) or laser diffraction spectroscopy have seemed to be more adequate for large particles (weighing hundreds of megadaltons (MDa) or even gigadaltons (GDa)). Currently, MS proposes some solutions for the analyses of monomolecular or multimolecular particles in a given range of MW. Viruses, bacteriophages, and other small infectious agents are at the edge between MS mass measurement capabilities and other, previously listed, techniques. However, the increasing precision of MS encourages its use in analyses in the viral world. The most commonly used assays in virology focus on measurements of the proteins building capsids, their interactions with the cell membrane, assembly dynamics, envelope composition, and the role of chaperone proteins in the construction of fully functional capsids. Such approaches lead to the development of new techniques in the analysis of high MW using MS and broaden the frontiers of this type of analyses. The most commonly used analytical techniques, involving MS, applied in the detection of
high MW molecules or molecular aggregates (like viruses) are: charge-detection mass spectrometry (CDMS) and ion mobility spectrometry (IMS) or differential mobility sectrometry (DMS)-based analyzers like gas-phase electrophoretic mobility molecular analysis (GEMMA). There are also newer modifications of electrospray ionization (ESI)- or MALDI-based systems, such as detection by nanomechanical resonators, which could provide additional insights in the viral microenvironment.

A. CDMS

CDMS is the method of choice for the analysis of high MW molecules. The methodology is relatively old: it was proposed by Shelton et al. (1960) during simulations of micrometeoroid behavior. CDMS focuses on the simultaneous measurement of the charge \( z \) and \( M/z \) ratio of the single ion rather than on the calculation of the MW of the molecules, based on the mass spectrum (Fuerstenau & Benner, 1995). Results are shown as a plot of MW (usually presented in MDa) as a function of the density of ions charges detected by the instrument (see Fig. 1). This methodology was adopted in the area of biological applications to molecular mass estimation of DNA (Schultz et al., 1998) and viruses (rice yellow mottle virus and tobacco mosaic virus; Fuerstenau et al., 2001). Here, typical MS was also unable to directly measure MWs of such large objects. Instead, a smart modification of the mass spectrometer was applied: after ESI, highly charged, viral capsids were guided through an amplifier, able to estimate their total charge, which was proportional to the current measured. As an additional verification, the time of flight of the molecules measured in the time-of-flight (TOF) analyzer was evaluated. \( M/z \) ratios were calculated based on both measurements, allowing for the acceptable precision of the measurement.

CDMS was used in a wide range of experiments in the field of life sciences, including investigations of the interaction between feline/canine parvoviruses (FPV/CPV) and antibodies synthesized during the infection. The authors measured quantity and time curve of binding between various types of antibodies and viral capsids, which resulted in deeper insight than mathematical models usually used to predict such type of interaction. Theoretical models focus on interactions between single types of molecules and their ligands (e.g., the antibody and the viral capsid), but in an experimental model, CDMS enabled the detection of interactions between four types of antibodies and a CPV, yielding more accurate values (Dunbar et al., 2018).

CDMS is seen as a technique of choice during analysis of high MW molecules as its range of measurement is far higher than, for example, MALDI-TOF capabilities. On the other hand, accuracy and speed of analysis is limited due to measurement methodology, as well as physical limitations. CDMS measures single ion parameters with a speed reaching 1,000 ions/min, which means that the time of analysis is long in comparison to other MS-based techniques. Additionally, the resolution of CDMS is also limited, but in a measured range of MDa or even GDa, precise MW calculations has minor importance. Continuous improvement of this method is also observed, like in case of increase in sensitivity of CDMS using so called pulsed mode (Todd & Jarrold, 2019).

B. GEMMA (Also Called Electrospray-Differential Mobility Analysis (ESI-DMA), DMS, or Scanning Mobility Particle Spectrometry)

GEMMA is a method of molecule/particle diameter measurement, when applied to high MW objects like viruses. This works as a connection of electrospray or nanospray ion sources and the viral capsid), but in an experimental model, CDMS enabled the detection of interactions between four types of antibodies and a CPV, yielding more accurate values (Dunbar et al., 2018).

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FIGURE 1. CDMS histogram of bacteriophage P22 with a bin size of 168 kDa (left panel). In the spectrum, there is a procapsid shown at ca. 24 MDa, with a smaller peak at ca. 20 MDa attributed to empty capsids. The histogram was calculated based on charge-to-mass plot (right panel) where points in the charge range 300–350 represent mature capsids, while points in the charge range 400–450 represent procapsids with scaffold proteins, detached during capsid maturation (after Keifer et al., 2014). © 2014 John Wiley and Sons. [Color figure can be viewed at wileyonlinelibrary.com]
same trajectory in typical IMS, differing by the time of travel. It makes DMA analyzers similar to sector instruments allowing for work in the mass filter mode.

GEMMA is widely used in investigations related to viral interactions with eukaryotic cells. Specifically, it has been used to estimate the binding ratio between antibodies and viral capsids when conventional MS-based methods failed. Bereszczak et al. (2014) used ESI-Q-TOF and ion mobility MS to measure MWs of antibody-hepatitis virus complexes up to ca. 8 MDa with the additional validation of results with the aid of GEMMA and cryoelectron microscopy (cryo-EM). As it would be difficult to directly measure molecular mass, based on Q-TOF system in the given MW range, the authors successfully used estimations based on predicted charge load on the ionized molecules and verified the results with other methods (Bereszczak et al., 2014). Far earlier, the ESI-GEMMA technique was developed as a convenient method for the analysis of high MW molecular complexes (Loo et al., 2005; Allmaier et al., 2008). Kaddis et al. (2007) have proven the usability of this methodology for the analysis of large molecules as well as high MW noncovalent complexes up to more than 9 MDa (Kaddis et al., 2007). Nowadays, GEMMA-based systems seem to be reliable alternatives to other analytical methods, typically used in the investigations of interactions between big molecules. An excellent discussion of the advances of this technique is provided in the work of Havlik et al. where interactions between tick-borne encephalitis virus and the vaccine formulations were investigated by various techniques. The authors compared results from GEMMA, atomic force microscopy (AFM) and TEM. They found that the size distribution pattern of viral capsids at various stages of vaccine preparation defines the behavior of capsids assembly dynamics significantly better than microscopic methods, which mainly comes from sample preparation methodology for each of the techniques. It seems that sample preparation for GEMMA is the least invasive and does not significantly affect the final result (Havlik et al., 2015). Compared with other methods used for molecular mass estimation, this technique is cheaper, less time consuming and simpler in application than concurrent techniques, like ESI-TOF-MS, CDMS (more time consuming), or microscopic techniques like TEM, AFM, cryo-EM (higher costs, sophisticated sample preparation, result dependent on sample prep; Weiss et al., 2019).

Viral protein-antibody interactions are not the only area of interest for MS-based investigations of the viral world. It is generally an agreement in the literature that it is possible to measure total viral capsids as well as their fragment parameters, like dimensions or MW, with sufficient accuracy using methods already described in this chapter. Some interesting data in the measurement range reaching even GDa have been shown by Hogan et al. (2006): during the electrospaying of bacteriophage MS2 with further analysis by DMA, the dimension of their capsids was estimated to be 24.13 ± 0.06 nm. Measurements did not influence the viability of the phages, which was confirmed in bacterial cells, since phages were still able to infect Escherichia coli strains after electrospaying. The limit of measurement was rather related to physical frontiers of the ESI than to dimensions of the particles, that is, bigger phages, such as T2 or T4, were not fully covered by the solvent droplets during electrospray formation. This resulted in fragmentation and the complete loss of phase function. Furthermore, the authors were able to separately detect capsids and tails of phage Lambda. This observation verified the possibility of in-source fragmentation of the bigger phages during ionization process, which was somehow related to dimensions of spraying droplets. Unfortunately, the exact fragmentation mechanism was not proposed and remains unknown.

MS-based analysis of viral capsid assembly and stability is a good example of another interesting application. Pease et al., using ESI-DMA technique, have shown, in addition to previously described experiments (Bacher et al., 2001; Hogan et al., 2006), that it is possible to measure the geometry of the viral particles, their structure and integrity (Pease et al., 2011). As a model object, phage PR772 was used, which belongs to the Tectiviridae family. Using the ESI-DMA system, the authors were able to determine the time-dependent formation of the icosahedral capsids. With this approach, it was possible to observe the intermediates (i.e., incompletely assembled capsids) formed by viral proteins. Other experiments showed capsid degradation kinetics in the natural environment and during heating of the sample. The ability to determine the symmetry of capsids, the number of major protein copies per capsid in the walls and vertexes and other geometry-based data using ESI-DMA is an excellent, fast and cost-effective supplement for the most expensive and time-consuming techniques, i.e., cryo-EM, small-angle neutron scattering, or X-ray crystallography.
FIGURE 3. nES-GEMMA acquired spectra of EM diameters of: (a) cowpea mosaic virus, (b) West Chester norovirus, (c) phage P22, and (d) phage T5. Spectra were acquired based on three different concentrations of viral structures (colors on plots) (after Weiss et al., 2019). EM, electrical mobility. © 2019 The Authors, under the terms of the Creative Commons. [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 4. Scheme of instruments using nanomechanical resonators for single particle mass sensing. SAWN or nanoESI sources generate a nebulized analyte, directing aerosol into a set of chambers where molecules are separated. Instead of charge, aerodynamic force is used for separation. Finally, molecules interact with nanomechanical resonators able to detect (resonate) only a defined MW range. The dimensions of resonators are width, 300 nm; thickness, 160 nm; and length, 7–10 μm. SAWN, surface acoustic wave nebulization. © from Domínguez-Medina et al., 2018. Reprinted with permission from AAAS. [Color figure can be viewed at wileyonlinelibrary.com]
C. Nanomechanical Resonators in Detection of Viral Capsids

One of the newest approaches to measuring objects at the MW range (from ca. 25–100 MDa) is the application of so-called nanomechanical resonators in MS (for mass spectrometer set-up see Fig. 4), which are mounted in a form of the silicon chip in place of a typical detector (Dominguez-Medina et al., 2018). Moreover, in such types of mass spectrometer, ionization of the measured objects does not necessarily increase the quality of analysis. The system uses an electrospray ion source, or a surface acoustic wave nebulization source, but focusing and guiding of the particles measured is carried out based on molecule inertia rather than the charge. The authors of this experimental system obtained satisfactory results in the measurements range from ca. 20 to 110 MDa, analyzing empty capsids and those filled with plague DNA of the bacteriophage T5. It is also worth mentioning that polystyrene nanoparticles at a diameter of ca. 45 nm (validated by scanning electron microscopy) were used as a reference material. This seems to be an interesting calibration technique, where the dimensions of objects are used for calibration purposes, as it is rather difficult to obtain a reliable reference material with a precisely defined molecular mass in the analyzed mass range.

In summary, the dynamic progress in the field allows the assumption that MS analysis will take over the field traditionally reserved for advanced microscopic techniques in the near future, becoming a tool allowing the more quantitative analysis of virus structure. Constant improvements in the available equipment break subsequent barriers in measurements of high MW and dynamics in big molecules interactions what makes MS method of choice for virological investigations.

IV. INVESTIGATIONS OF CAPSID COMPOSITION

In recent years, MS has become a core technique for the identification of viral particle content (Lewis et al., 1998). As viral proteins have various roles during infection, knowledge of the protein composition of the infectious particle is necessary for functional studies. This is increasingly important, as tools for oncolytic viruses and virus-like particle-based vaccines become common. The development of the highly sensitive MS techniques, usually used for protein identification (MALDI-TOF, nanoESI-MS/MS, nanoliquid chromatography (nanoLC)-nanoESI-MS/MS, etc.) strongly improved the quality of data.

A. Analyses of Nonenveloped Viruses Capsids

Nonenveloped viruses are generally small, and the amount of viral proteins in the capsid is limited. Further, these viruses are formed in a highly organized manner, adopting certain symmetry with a well-defined number of protein mers forming the shell. This makes the analysis rather straightforward and, to date, several reports on nonenveloped virus composition have been published. Adenoviruses are among the best-studied. In a work by Chelius et al. (2002), the human adenovirus (hAV) type 5 was used as a model virus and its capsid composition was analyzed using LC-MS/MS. As all of the major proteins were identified using one- and two-dimensional chromatographic separations, it was concluded that LC-MS/MS was an appropriate tool for such analysis. More recently, Benevento et al. demonstrated a more comprehensive method to evaluate hAV capsid protein composition. The researchers employed LC-MS/MS and selected reaction monitoring (SRM), which allows the abundance of small molecules or group of proteins after their enzymatic digestion to be quantified based on the unique peptides released. Next, they monitored the protein content changes in virions upon heat-induced disassembly, which was aimed to mimic the entry process. As a result, some proteins were identified as important players during virus release from the endosome to the cytosol (Benevento et al., 2014). Interestingly, MS studies on nonenveloped viruses revealed the usefulness of these methods in evaluation of viral protein activity. Salganik and colleagues studied the adeno-associated viruses (AAV) to identify proteolytic active sites which catalyze autolytic cleavage of viral capsids. The researchers employed LC-MS/MS analysis to identify the cleavage sites within capsid proteins and to show that AAV particles contain an intrinsic protease activity that is activated in the acidic environment of cellular endosomal compartments. Using both mass spectrometric analysis and N-terminal Edman sequencing, they identified unique autocleavage sites in AAV type 1 (AAV1) and AAV9. Results showed that AAV protease may have an important role in virus infection (Salganik et al., 2012). Furthermore, a direct LC-MS/MS analysis proven to be effective in characterizing AAV capsid proteins (VPs) in variety of AAV serotypes (Jin et al., 2017). Soon after, Zhang et al. described a novel method to characterize AAV capsid proteins. A microfluidic ZipChip capillary electrophoresis linked to mass spectrometer (CE/MS) was employed for the identification of three AAV2 proteins: VP1, VP2, and VP3. Researchers described the ZipChip CE/MS as a powerful method to confirm AAV serotype identity, which could support recombinant AAV gene therapy development (Zhang et al., 2018). Recently, MS techniques were also utilized in the analysis of the AAV capsid glycosylation profile. Aloor et al. employed high resolution MALDI-TOF and identified N-glycosylation of AAV8 capsid protein. These results showed that, contrary to previous data, AAV capsids are prone to glycosylation, which may influence AAV tropism (Aloor et al., 2018). In fact, MS-based methods enable the robust characterization of N- and O-linked glycans in complex virus samples, especially in glycoprofiling viral surface proteins (see also in posttranslational modification (PTM) analyses, subchapter IV.C and useability of glycan analysis in vaccine development, subchapter VIII.B).

LC-MS/MS was also employed to better describe quasienvolved human hepatitis A virus (eHAV) capsid composition. HAV, an important cause of hepatitis transmitted by the fecal-oral route, egress from infected cells in a nonlytic manner as eHAV virions, which are similar in size and density to exosomes (Feng et al., 2013). Despite eHAV virions being as infectious as the nonenveloped HAV virions, the biology and origin of their quiasmembrane are not well understood. McKnight et al. employed a quantitative LC-MS/MS analysis to show that eHAV contain proteins of endolysosomal compartments and lack markers of autophagy. Obtained data showed that eHAV capsids are selected for export in a specific process, involving endosomal budding, rather than autophagosome-mediated release (McKnight et al., 2017).

B. Analyses of Enveloped Viruses Capsids

Determining the capsid composition of enveloped virions is more challenging for a few reasons. First, the content of
enveloped viruses is less defined, as they adopt different structures of different sizes, very different from crystal-like shaped nonenveloped virions. Second, aside from viral proteins, enveloped virions usually carry also host proteins submerged in their membrane. However, giving the variety of enveloped viruses and their impact on human health, the effort was taken to analyze several viral strains using MS.

LC-MS/MS was used to identify viral and host proteins in human immunodeficiency virus type 1 (HIV-1) virions. In studies by Saphire et al. and Chertova et al. HIV-1 virions produced in different cell lines or monocyte-derived macrophages were analyzed. As a result, numerous host proteins were identified, including histones or proteins of the endosomal pathway (Chertova et al., 2006; Saphire et al., 2006). Further studies also described other host proteins that are incorporated into HIV-1 particles and interact specifically with viral proteins and these included Tsg101, cyclophilin A, and Apobec3G. All of these appeared to affect HIV-1 infection: Tsg101 plays a role in virus assembly, cyclophilin A regulates infectivity, whereas Apobec3G promotes hypermutation of HIV-1 genome, which causes lethal mutational loads that terminate progeny virus production and propagation (Franke et al., 1994; Braaten & Luban, 2001; Garrus et al., 2001; Demirov et al., 2002; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003). Furthermore, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2D SDS-PAGE, followed by MALDI-TOF or nanoLC-MS/MS, were used in several studies leading to the identification of biomarkers in body fluids (cerebrospinal fluid, serum, cellular extracts) of HIV-infected patients (Ciborowski et al., 2007; Rozek et al., 2007; Wiederin et al., 2009). In addition, recent work by Schlatter et al. shows detection of latent HIV-1 reservoirs. Researchers employed SRM to unambiguously identify latent reactivated virus reservoirs using Gag protein detection (Schlatzer et al., 2017).

Similarly, the severe acute respiratory syndrome coronavirus (SARS-CoV) virions were analyzed by 2D-LC-MS/MS and SDS-PAGE followed by ESI-MS. The combination of these two methods enabled the identification of all SARS-CoV structural proteins. Also, MALDI-TOF, followed by N-glycosidase digestion, enabled the identification of several glycosylation sites in SARS-CoV spike protein (SARS-CoV S) (Krokkin et al., 2003). The authors claimed that further analysis may demonstrate the importance of SARS-CoV S N-glycosylation in receptor recognition and virus entry to the cell, but a report by Chakraborti et al. showed that glycosylation of SARS-CoV S did not influence virus binding to its entry receptor (Chakraborti et al., 2005). On the other hand, a few years later, Zhou et al. demonstrated that a single N-glycosylation site in SARS-CoV S is important for the interaction with the mannose-binding lectin (MBL)—a serum protein involved in activating the complement system. The identified N-linked glycosylation site N330 appeared to be critical for binding of S protein by MBL and further inhibition of virus infection. Interestingly, the interaction between virus fusion protein and MBL had a various effect on S-mediated binding to host cell receptors—it blocked SARS-CoV binding to DC-SIGN, but did not affect interaction with ACE2 (Zhou et al., 2010). MS was also used for determination of the infectious bronchitis virus (IBV) capsid composition. Kong et al. studied the purified IBV particles and analyzed them with two-dimensional gel electrophoresis fractionation, tryptic digestion, and MALDI-TOF. Apart from IBV structural proteins, 60 host proteins involved in the function of cytoskeleton, ribosomes, chromosomes, as well as in intracellular trafficking, signal transport were identified (Kong et al., 2010).

Another example may be provided by the hepatitis B virus (HBV), for which capsid assembly was evaluated using MS. Lutomski et al. employed CDMS to track HBV capsid assembly process in real time. The study showed that contrary to other icosahedral virus capsids, HBV capsid completion is the slow step in assembly (Lutomski et al., 2017). It is worth to note that they obtained similar results to Pease et al., who used ESI-DMA analysis and worked on icosaedral PR772 phage (Pease et al., 2011). Obtained data allowed for a better understanding of the virus replication cycle, which can be further used in drug discovery or nanomaterial development. MALDI-TOF was also used to determine HBV capsid phosphorylation sites and the role of these modifications in infection (Heger-Stevic et al., 2018). Based on the examples shown it seems, that from the analytical point of view, every methodology applied (CDMS, ESI-DMA, and MALDI-TOF) works very well in the field of capsid composition analyses. Although each scientific group used another technology, every team was able to receive satisfactory results.

MS analysis of herpesviruses is difficult, due to the complex structure containing viral and host proteins (Fields et al., 2013). Despite that several herpesviruses were analyzed using MS-based approaches including human cytomegalovirus (CMV), Epstein-Barr virus (EBV), Kaposi’s sarcoma-associated herpesvirus (KSHV), and several animal herpesviruses (Baldick & Shenk, 1996; Bortz et al., 2003; Johannsen et al., 2004; Kattenhorn et al., 2004; Varum et al., 2004; Zhu et al., 2005). In 2008, Loret et al. performed a comprehensive analysis of proteins found in virions of human herpes simplex virus type 1 (HSV-1). Using multiple reaction monitoring (MRM), researchers analyzed highly purified extracellularly isolated viruses. The study identified 37 of 40 known HSV-1 components, along with 4 novel virion proteins (U₇, U₁₂, U₅₂, and U₅₅). Furthermore, the analysis showed that host proteins may also be incorporated to formed virions (Loret et al., 2008). Also, in a recent work by Snijder et al. a quantitative analysis of HSV-1 capsids showed that pUL17 and pUL25 proteins are stabilizing capsid shells. Identified proteins were shown to play a role in the virus stability, which gave new insight on HSV-1 structure (Snijder et al., 2017).

Similarly, a comprehensive study involving MS analysis was performed to reveal the protein composition of influenza A virus capsids. In a work by Shaw et al., purified influenza A virus particles were analyzed using both LC-MS/MS and MudPIT columns (capillary columns filled with SCX/C18 beads allowing for 2D chromatography in a single capillary). As a result, 9 of 11 viral proteins were detected, together with 36 host-encoded proteins. These included both cytoplasmic, as well as membrane-associated proteins, some of which were present on the virus envelope. These data helped to define cellular requirements for virus replication, but also proposed novel targets for antiviral therapy (Shaw et al., 2008). Also, Hutchinson et al. used MS to detect phosphorylated proteins in influenza A and B virus capsids. The study involved LC-MS/MS analysis of phosphorylated viral proteins, followed by the Modification Localization Score (ModLS) tool, which enabled...
the mapping of phosphorylation sites. The analysis revealed 39 phosphorylation sites in influenza proteins, including the polymerase, glycoproteins, nucleoprotein, matrix protein or nuclear export protein. This further demonstrates the importance of protein phosphorylation for influenza viruses in regulating virus entry and egress from infected cells, nuclear localization, and replication (Hutcheon et al., 2012).

The surface-exposed influenza virus hemagglutinin (HA) and neuraminidase (NA) glycoproteins play a fundamental role in viral entry and egress. Further, they are also major antigens of influenza viruses, responsible for immune recognition of the virus (Johansson et al., 1987; Johansson et al., 1989; Sriwilaigorajon & Suzuki, 2012; Virk et al., 2016). For that reason, they undergo a constant antigenic drift (Medina et al., 2013; Hervé et al., 2015). Consequently, alterations in protein sequence result in an escape from immune clearance but may also mask epitopes, for example, changing the glycosylation pattern. Cruz et al. studied the glycosylation patterns of the HA protein. Researchers employed a high-energy collision dissociation (HCD) and collision-induced dissociation tandem MS (HCD/CID-MS/MS) for characterization of the site-specific profile of HA from A/New Caledonia strain (Cruz et al., 2018). The analysis experimentally confirmed previously identified glycosylation sites and determined a broad glycan heterogeneity of HA (Sun et al., 2011). Obtained data contributed to the understanding of influenza virus evolution. Better understanding the impact of glycosylation on influenza virus replication is highlighted by the fact that the alterations of glycosylation site locations on HA and NA proteins the H1N1 virus subtype was an important factor contributing to the pathogenicity of the 1918 pandemic strain (Sun et al., 2013).

Interestingly, a recent work by Reed et al. employed LC-MS/MS as a diagnostic tool to detect nucleoproteins of the rabies virus. A fast and reliable diagnostic method is essential, as infection can be prevented by post-exposure passive or active immunization. MS allowed to identify all rabies virus proteins in infected cell lysates, suggesting that future MS-based rabies diagnostics are of great promise (Reed et al., 2018).

A recent pandemic of Zika virus (ZIKV) in Brazil and the Americas increased the interest and research on Flaviviridae family (Valentine et al., 2016). These small (approximately 50 nm in diameter) enveloped viruses include over 40 pathogenic human species of flavivirus and hepacivirus genera. Viruses egress from infected cells through a secretory pathway. During this process, viral particles undergo diverse maturation steps, such as glycosylation or proteolysis (Voisset & Dubuisson, 2004; Fields et al., 2013). Additionally, a recent study by Rana et al. investigated the role of a capsid anchor (CA)—a hydrophobic peptide of a capsid protein, in the morphogenesis of ZIKV. Previously, CA was considered to direct the pre-membrane protein into the endoplasmic reticulum (ER). However, the study showed that CA has another function, as it controls E protein stability and availability. Thus, CA expression is believed to be necessary for the production of infectious ZIKV particles (Rana et al., 2018). Glycosylation and proteolysis also enables the incorporation of host proteins into the viral capsids, as shown for the hepatitis C virus (HCV) by Lussignol et al. Using different purification methods and LC-MS/MS, researchers identified 46 host proteins in HCV particles. Among several new host factors, a nuclear pore complex (NPC) protein was identified and further showed to participate in HCV infection (Lussignol et al., 2016). Thus, despite the protein composition of HCV still not being well described, it is believed that cellular proteins are important components of the virus particle (Pawlotsky, 2013). Future MS based studies on host proteins incorporated into virions should broaden current knowledge on other Flaviviridae members, such as ZIKV or West Nile virus (WNV).

The MS analysis for flaviviruses is not limited to the protein content analysis. Flavivirus replication induces invaginations in the ER membranes and forms vesicles in which RNA replication occurs. This results in a dramatic reorganization of membrane architecture and infected cells (Fields et al., 2013). A high-resolution LC-MS/MS analysis was employed by Perera et al. to evaluate global changes in lipid metabolism upon Dengue virus (DENV) infection (Perera et al., 2012). The study revealed great alterations in the global lipid profile in human and mosquito cells infected with DENV. Soon after, a study by Barletta et al. demonstrated that genes involved in lipid metabolism were regulated after DENV infection, suggesting that lipids have an important role not only in the flavivirus replication cycle but also in antiviral response (Barletta et al., 2016).

C. Posttranslational Modifications (PTMs) of Viral Proteins

PTMs of proteins are known to be important for the regulation of various cellular processes. Eukaryotic cells utilize PTMs to diversify their protein functions and dynamically coordinate their signaling networks. As viruses rely on the host protein synthesis machinery, many viral proteins are subjected to PTMs. The most common PTMs include proteolytic cleavage, acetylation, formylation, phosphorylation, ubiquitination, and glycosylation. In recent years, several PTMs of viral proteins have been identified using MS, including glycosylation, ubiquitination, and SUMOylation.

Glycosylation is one of the most common PTMs of viral surface proteins and it is believed to be important, for example, for virus maturation during egress and interaction with the receptor during entry to the susceptible cell. MALDI-TOF is one of the most commonly used tools for glycan analysis and its combination with lectin microarray is a powerful tool for complex analyses (Lei et al., 2015). For example, it has been successfully used for the identification of N-glycosylation of proteins of many virus species, including SARS-CoV, HIV-1, DENV, and Chikungunya virus (Krokhin et al., 2003; Raska et al., 2010; Lei et al., 2015; Lancaster et al., 2016). MALDI-TOF has recently been used for the analysis of O-glycosylation for Ebola virus, HBV, and HIV-1 (Schmitt et al., 1999; Go et al., 2013; Stansell et al., 2015; Collar et al., 2017). Moreover, this method has been used for the differential analysis of glycan profiles for recombinant virus proteins and proteins isolated from native viruses (Doore et al., 2010; Bonomelli et al., 2011).

Ubiquitination is another common PTM of viral proteins that has been analyzed with the aid of MS (Parker et al., 2019). The process mediated by ubiquitin plays a role in cell division, cell death, and signal transduction, and is exploited by representatives of many viral families. In fact, ubiquitination of certain viral proteins is necessary for replication of Poxviruses, Reoviruses, and Coronaviruses (Teale et al., 2009;
V. VIRUSES IDENTIFICATION BY MS

Rapid and proper diagnosis of a viral disease is essential for the application of adequate treatment. This is especially important for viruses, which may be treated, such as HIV-1, hepatitis, or influenza, but it applies to all viral pathogens in special groups of patients, such as pregnant women, young children, the elderly, and people with a weakened immune system. Moreover, the short time of the analysis is crucial, since the appropriate time of antiviral drugs application is important. In the case of influenza, the first dose of an antiviral should be taken within 48 hr after symptoms emerge, to reach the optimal efficacy (Chou et al., 2011).

There are a number of identification techniques available to date; serotyping (e.g., by identification of antibodies in the serum) and reverse transcription polymerase chain reaction (RT-PCR) are target-directed, which means that they could miss nonselected or emerging pathogenic viruses (Binnicker et al., 2013). Viral cultures, on the other hand, can identify all the cultivable viruses, but are time-consuming (from days up to 4 weeks, depending on the type of virus and method of cultivation) (Singhal et al., 2015), demand specialized skills and additional assays for virus identification (Calderaro et al., 2014). In this approach, collected samples are cultured in the special cell lines (e.g., LLC-MK2 or Hep2), which are then checked for the cytopathic effect (such as disruption of cells’ monolayer). If such an effect is visible, the identity of the virus is determined by specific antibodies in an enzyme immunoassay, immunofluorescence, electron microscopy, or by RT-PCR (Schepetiuk & Kok, 1993).

Although MS was not broadly used in standard diagnostics in the past, due to limited availability for routine analyses and difficulties in high throughput approach, the technique offers some superior parameters allowing for virus identification and becomes more common. In this section, we present strategies using the speed and ease of MALDI analysis of protein and peptide mass fingerprints of a particular virus, for their identification. Additionally, interesting hybrid techniques combining the specificity of PCR or antibody-based detection with the MS based analysis are discussed.

A. Analysis of the Whole Virus Proteins: Protein Mass Fingerprint

MALDI-TOF is an exceptional technique which drastically shortens the identification time and has a more cost-effective analysis than molecular or immunological detection methods, excluding the initial costs of MALDI instrument (Bizzini & Greub, 2010; Singhal et al., 2015). MALDI Biotype by Bruker Daltonics and VITEK system by bioMerieux Inc. are the first platforms approved by the FDA for in vitro diagnosis and identification of bacteria and yeast isolated from human specimens (Patel, 2015), and the routine typing of viral infections in human-derived material (biofluids, tissue samples, etc.) is a question of time. The high-throughput idea of MALDI analysis is based on the detection of the protein fingerprint characteristic for the certain pathogen. Spectra obtained for the clinical sample when compared with reference data allows for identification of such a pattern (Patel, 2015).

The major problem with MALDI-TOF identification of viruses is related to the low quantity of the viral proteins in...
comparison with human, endogenous proteins in the clinical sample. Additionally, the MW of viral proteins is relatively high (>20 kDa) in contrast with the ranges used for standard microbiology diagnoses (2–20 kDa in the case of MALDI Biotyper). Moreover, viruses have to be cultivated in vitro in the specific cell line to amplify their number, so there is a risk of sample contamination by proteins and cellular debris from the cell line. Additionally, the accurate fingerprint characteristic for the specific virus has to be already deposited in the library designed for clinical use, which may be problematic due to the rapid evolution of viral pathogens.

Calderaro et al. (2014) used MALDI-TOF for the detection of human polioviruses serotype 1, 2, and 3. In this study, purified viruses were subjected to MALDI analysis, and careful statistical examination of the results revealed the peaks that can discriminate between the three different serotypes (in low and high mass range). Similarly, the same comparison was made for the LLC-MK2 cells infected with mentioned viruses and for uninfected cells. This allows viral biomarkers to be found—the peaks characteristic for each cultivated serotype—and to create an in-house viral protein database. The analysis of the cells infected with the poliovirus strains isolated from clinical samples and their proper identification showed the potential of this analytical technology based on MALDI analysis for virus identification (Calderaro et al., 2016). In the next study, the team continued the research and analyzed the most common viruses causing respiratory tract infections (influenza A and B viruses; adenovirus C; parainfluenza virus types 1, 2, and 3; respiratory syncytial virus [RSV]; echovirus; CMV; and human metapneumovirus). As previously reported, the cell culture step was included to amplify the signal, since the amount of analyte present in the sample is the major limitation of current MALDI protocols (Calderaro et al., 2016). For each virus, the spectra for uninfected and infected samples were compared and peaks that differentiated those two in the MW range from 2,000 to 1,1500 Da were indicated. Those peaks may represent the virus-specific proteins or might be the effect of the cell line response for the infection. Hence, the list of peaks was used to create the main spectrum profile (MSP) for each reference virus. The library that consists of all the MSP was then used to identify viruses from 58 clinical samples. The signal was initially amplified in the cell culture and further analyzed. The proposed system was able to discriminate between seven different viruses. In the case of influenza, it was possible to identify the sample as human influenza virus, but distinguishing between species A and B was not possible. The same problem occurred for parainfluenza types 1, 2, and 3. Mentioned studies showed that a MALDI-based approach can be used for virus analysis, despite some limitations, and could lead to the reduction of the time and costs of the analysis in comparison with classical methods.

B. Viral Peptide Mass Fingerprint

The more classical approach was utilized by Majchrzykiewicz-Koehorst et al. (2015). In her study, MALDI-TOF combined with LC, and in-house developed sample pretreatment protocol was used for the analysis. Serially diluted samples of different viruses (eight influenza A H1N1 strains, two influenza A H3N2 strains, human metapneumovirus [hMPV], and RSV) were prepared in CyMol medium. Obtained viral proteins were reduced, alkylated, and digested with the aid of Rapid Enzymatic Digestion System that allows for very fast sample preparation (usually not exceeding 70 min). After the whole procedure, obtained peptides were subjected to MALDI-TOF analysis or such analysis preceded by LC separation and then nanoESI-Q-TOF was used.

For direct MALDI analysis, the whole procedure lasted for 3 hr, and the equivalent of $1 \times 10^6$ viral genome copies in the total sample volume deposited on MALDI plate was necessary for correct virus identification. A relatively high quantity of the material was necessary for the analysis, authors tried to improve sensitivity of their assay. When LC separation of obtained peptides was introduced into workflow the analysis lasted for 6 hr, but was more sensitive. The identification limit was equivalent to ca. $7 \times 10^3$ viral genome copies in the sample volume subjected to LC-MS/MS analysis. Additionally, it was possible to identify influenza viruses with LC-MS/MS in mixed samples (containing also hMPV and RSV) and in reconstituted clinical samples (throat swabs spiked with the virus). The authors estimated that this method should be sensitive enough for clinical application.

Schwahn, Wong, and Downard proposed another strategy for virus identification called “proteotyping,” which is also based on viral peptide mass fingerprints (for workflow see Fig. 5). Here, high resolution and high mass accuracy (of 1–5 ppm) spectra are required (Schwahn et al., 2009). Initially, translated gene sequences for influenza HA, neuroamidase, nucleoprotein, and matrix protein M1 of all viral strains isolated from humans were used as an input (type A H1—931 sequences; type A H3—2,814; type B HA—1,057; B type Vic87-like—424; and B type Yam88-like—629 sequences). With the aid of bioinformatics tools, the masses of their theoretical tryptic peptides were obtained and matched. Then, the peptides characteristic for certain type and subtype of the virus (signature peptides) from each viral protein were chosen. The care was taken to choose the most conserved peptides for each type and subtype with the masses that differ by the largest value from theoretical tryptic peptides of all known influenza antigens whose sequences are not conserved. For example, the peptide with the mass 593.307520 (SSIMR) comes from HA and is characteristic for type A and subtype H3 of influenza virus. Such mass identified in the spectrum from the whole virus digest may correctly identify the strain and subtype but the data has to be acquired with the aid of a high-resolution mass spectrometer, for example, MALDI FT-ICR (Downard, 2013). This approach may be used for the analysis of mixed strain proteins in the vaccines, to distinguish seasonal from pandemic influenza strains and to determine currently circulating strains, which is essential for the production of effective vaccines.

C. Hybrid Techniques

As aforementioned, one of the major drawbacks of the MS based detection of viral antigens in the clinical samples has a sensitivity that is not high enough for direct detection of viral molecules. Typically, it is a result of small yield of viral proteins in the total protein content derived from the clinical sample. While some amplify the signal by cell culturing, such an approach has severe drawbacks, as majority of clinical wild-type viruses do not replicate or replicate poorly in vitro. Thus, well known methods taken directly from immunology or
molecular biology are additionally utilized for proper virus identification with the aid of MS techniques.

1. Immunodetection and MS

Chou et al. (2011) coupled MALDI-TOF with the highly specific immunodetection technique. Briefly, magnetic nanoparticles coupled with antibodies specific to HA were used to enrich the sample. Subsequently, the complex was mixed with the MALDI matrix and deposited directly on the MALDI plate. Low pH of sinapic acid was responsible for the removal of the protein from the antibody by breaking the weak bonds between both molecules. The purified proteins were further analyzed. Consequently, the major advantages of the two techniques were combined and the analysis employing nanoparticles coupled with H5N2-specific antibodies were used on H5N2 influenza sample and three recombinant H5N1 samples—and proved its usefulness.

2. PCR and MS

Another approach combines the selectivity and sensitivity of PCR with the downstream MS analysis to determine the molecular masses of PCR and in consequence, their base composition. Moreover in this case, PCR due to its nature, allows to increase the amount of the measured material (Metzgar et al., 2013). In the microbiological analysis, the primers are designed to bind to conserved regions present in about 95% of all the bacteria and amplify heterogeneous regions between the primer sites. Such an approach is more difficult for viruses, which are characterized by higher diversity and it is not possible to design broad-range primers for general diagnostics. However, the in silico analysis of the influenza virus sequences allowed the selection of primers in conserved regions of the core gene segments that allows for detection of all known influenza viruses and distinguish >90% of all species and types (Sampath et al., 2005). Following the RT-PCR amplification, the sample was desalted and analyzed with the electrospray. Sufficient mass accuracy of PCR amplicons allows for the unambiguous determination of the base composition (i.e., the number of As, Gs, Cs, and Ts) of each amplicon, which allows for the identification of the pathogen. The complementary nature of the DNA strains (in the case of DNA viruses) make such analysis possible at the level accuracy of around 20 ppm (see Table 1), but such analysis is also possible for RNA viruses (Sampath et al., 2007). Moreover, new pathogens may be assigned to the viral family based on the conserved primers. Such primers allow for the efficient PCR from all the members of the certain family, whereas the base composition is able to indicate the new member. In the case of SARS, it was shown that this approach was able to categorize it as a new member of the coronavirus family, based on the coronavirus primers designed and tested before its emergence (Sampath et al., 2005).

Pathogens identification based on PCR/ESI-MS assay is now commercially available as the Ibis Biosciences Plex-ID® Pathogen Detector (Omnica Corporation/Abbott Ibis Biosciences). This technology was first developed for the Department of Defense and known as TIGER (Triangulation
Identification for the Genetic Evaluation of Risks (Ecker et al., 2006) for pathogen surveillance. In its commercial form, it could be used for pathogen identification (Ecker et al., 2006).

Chen et al. (2011) used RT-PCR/ESI-MS platform (see Fig. 6) for identification of upper respiratory tract infections caused by RSV, influenza A and B, parainfluenza types 2 and 3, adenoviruses types A–F, coronaviruses (SARS), human bocavirus, and hMPV from nasopharyngeal aspirates in 280 retrospective samples. In the Ibis T5000 Respiratory Virus Surveillance II kit, used in this study, 16 primer pairs were distributed in a 96-well plate, in which each well contained one pair of primers, which allowed for the analysis of six patients per plate. Clinical samples collected and analyzed before, with classical clinical virological methods were used. This study proved the usefulness of the new technique, since it was characterized by 87.9% accuracy in comparison with conventional clinical virology assays, and was able to identify viruses in the samples previously unidentified. In particular, for RSV, 34 samples were positively identified with both RT-PCR/ESI-MS and clinical virology (rapid immunochromatographic tests), three samples unidentified by clinical virology were positively identified with RT-PCR/ESI-MS (the positive identification of two of them was further confirmed by another PCR-based platform) and two were misidentified with RT-PCR/ESI-MS, but their identification with clinical virology was correct. Additionally, the method was shown to be sensitive, rapid (time to detection, 8 hr) and was able to detect simultaneously multiple pathogens. Sampath et al. (2007) used the technique on 92 mammalian and avian isolates representing 30 different H and N types of the influenza virus. All samples were correctly identified. Additionally, 676 human clinical respiratory specimens were tested and both: sensitivity and specificity were satisfactory (97% and 98% approximately), showed the usefulness of this approach (Sampath et al., 2007). Moreover, using the technique it was possible to observe the evolution of the virus in time (in this case for 7 years), which allows for monitoring of the current epidemic situation. The RT-PCR/ESI-MS approach proved to be useful for detection of orthopoxviruses (Eshoo et al., 2009), coronaviruses (Sampath et al., 2005), and adenoviruses (Blyn et al., 2008). The commercially available device was also successfully tested by Tang et al. (2013) on the influenza virus.

VI. CHANGES IN THE PROTEOME OF THE CELL DURING VIRAL INFECTION

The viral infection may be compared with reprogramming of the cell, where the virus brings in the hostile information able to

| Mass uncertainty (ppm) | Number of consistent base compositions | For complementary pairs |
|------------------------|----------------------------------------|------------------------|
|                        | Strand I 37,374.266 Da                 | Strand II 37,231.153 Da |                     |
| 1                      | 101                                    | 130                    | 1                     |
| 5                      | 519                                    | 631                    | 1                     |
| 10                     | 933                                    | 934                    | 1                     |
| 20                     | 1,321                                  | 1,214                  | 1                     |
| 50                     | 3,703                                  | 3,524                  | 20                    |
| 100                    | 7,377                                  | 7,179                  | 81                    |

The measurements with lower accuracy are enough to assess the proper number of As, Gs, Cs, and Ts for certain m/z value, due to the complementary nature of DNA strains (Ecker et al., 2006).

FIGURE 6. The workflow of RT-PCR/ESI-MS analysis. [Color figure can be viewed at wileyonlinelibrary.com]
change the microenvironment of the cell into the virus-producing factory. While these changes may be driven directly by viral proteins, microRNA or noncoding RNA, all of them reflect themselves in the proteome of the cell, significantly changing their physiology. Understanding the process allows us to understand how the virus replicates and design therapeutic or preventive strategies. Screening of the changes in the proteome evoked by viral infection can be done only with the aid of MS. Indicated changes can be confirmed by other methods (like western blotting) but MS is the only method allowing for high throughput analyses in this field to date.

To identify cellular pathways that are modified during infection, virus- or mock-treated cells are subjected to a lysis procedure followed by proteomic analysis. The proteomic analysis using MS allows capturing the whole range of changes in the proteome of the infected cell, while other methods allow for tracing of isolated changes only. As an example, infection with the WNV leads to massive cell death. Investigation of this process with cell viability assays, DNA fragmentation assays, annexin V labeling, and flow cytometry analysis led to the conclusion that the infection induces bax-dependent apoptosis (Parquet et al., 2001). While the proteome analysis of the WNV infected cells led to a similar conclusion, a number of other proteins involved in this process were identified allowing for better understanding of the process. Using proteomic fractionation by 2D-DIGE analyzed by MALDI-TOF in primary rat neurons, proteins such as restin and β-amyloid were identified (Dhingra et al., 2005), and, in the case of vero-infected cells, the 2D-DIGE was followed by capillary LC-MS/MS and identified that programmed cell death 8 protein and apoptosis-inducing factor protein (Pastorino et al., 2009) were upregulated.

Observation of the proteome changes during the infection allows for even better understanding of the process. One of the studies describing the proteome during influenza A virus infection was carried out in human primary macrophages, and lysates from different time-points postinfection were analyzed with iTRAQ labeled samples by nanoLC-ESI-MS/MS. The most prominent changes in the nuclear fraction appeared 12 and 18 hr postinfection, after which there is a significant increase in histone proteins in the cytoplasmic fraction. Also, at these time points, the number of apoptotic cells increased drastically. Activation of the inflammasome has been shown to be dependent on the activity of cathepsins, which were present in the cytoplasm due to endolysosomal leakage (Lietzén et al., 2011). The employment of fractionization and the analysis of individual fractions enriched the information obtained about infection.

It is important to note that the model used in the analysis is of utmost importance. At present, most of the analyses are carried out on well-defined in vitro culture models. However, efforts have been made to use more natural models. Influenza A infection was studied by two-dimensional gel electrophoresis followed by MALDI-TOF using chicken brain tissue infected with H5N1 avian influenza. Novel, undescribed changes of the ubiquitin-proteasome pathway, neural signal transduction pathway and cytoskeletal proteins were observed (Zou et al., 2010). A similar study with a human virus, influenza A/California/07/2009 was carried out on normal human bronchial epithelial cells isolated from three different donors. Samples were analyzed by ultraperformance liquid chromatography (UPLC) and high-resolution MS. The results obtained revealed a great diversity of responses between patients and the obtained results allowed the authors to suggest proteins important for influenza A virus replication in the natural tissue (Mindaye et al., 2017).

However, the most clinically relevant data may be obtained only based on an in vivo models or naturally infected patients. Choi et al. made that effort and compared the plasma content from patients infected with influenza A H1N1 virus with the control plasma from healthy volunteers. Proteins were separated by 2-DE and differential analysis allowed to select for proteins that are differentially regulated during the illness. Protein identification was performed by UPLC-ESI-MS. Fourteen proteins were identified and some of these may be used as markers of the disease (Choi et al., 2014).

A perfect example of the importance of the model selection was provided by Sengupta and colleagues, who compared in vivo and in vitro models. Mouse brain tissue and neuroblastoma cells (Neuro2a) were infected with Japanese encephalitis virus and the proteome alteration was verified and compared. Forty-one proteins in brain tissue and 15 proteins in the cell line were differentially regulated. Proteins identified in both models were mainly involved in metabolism, protein folding or cytoskeleton forming, but only a few proteins were identified in both types of material (e.g., γ-actin, calreticulin precursor, heat shock protein-8) and consequently, conclusions drawn would be diametrically different for these two models. The proteomes were analyzed by 2-DE with spots identification by MALDI-TOF (Sengupta et al., 2014).

The proteomic analysis may also be used for the analysis of complex models. Proteomic profiles of human lung epithelial cells (A549) infected with paramyxoviruses pathogenic in humans (RSV, parainfluenza virus, human metapneumovirus, and measles virus) were obtained at different time points after infection. The cell lysates were separated with 2-D DIGE followed by nanoLC FTICR-MS. While obtained results showed some similarities between different viruses, some patterns were species-specific (van Diepen et al., 2010). Further, coinfection of mosquitoes with Chikungunya virus and DENV were studied. While these two viruses are very different, they share the vector and the host. Further, they cause a similar disease in humans and may occur simultaneously. The mosquitoes with mono-infection or coinfection were analyzed with isotope labeling by tandem mass tag and LC-MS was used. Infections with a single CHIKV or DENV virus have various effects on the mosquito’s body—modify the oxidative stress pathway and energy-related pathway, respectively. Coinfection causes an energy-related pathway disorder despite the fact that CHIKV showed a higher infectivity rate (Shrinet et al., 2018).

While the effect of the infection process on the proteome of the host may be studied, in some cases such analysis is not possible. Hepatitis D virus requires HBV coinfection of the cell, as it hijacks also viral replicative machinery (Fields et al., 2013). To identify changes of the proteome associated only with HDV infection, Huh-7 cells were transiently transfected with plasmids encoding small delta antigen (S-HDAG), large delta antigen (L-HDAG), genomic RNA or antigenomic RNA. After the transfection, cells were lysed and analyzed with 2-DE and the differentially expressed spots were submitted for MALDI-TOF identification. This approach allowed to recognize thirty two proteins important for virus replication and pathogenesis. Downregulation of three form identified proteins (hnRNP D,
HSP105, and triosephosphate isomerase) was further confirmed by real-time PCR, which showed a reduction in mRNA levels (Mota et al., 2008).

In summary, proteomics allows observation of the complete landscape of the infection in the cell. While we are still not able to understand some observed phenomena, the development in related fields of bioinformatics, cell biology, machine learning, and big data analysis allows us to hope that we will be able to see and understand the complete network of interactions between the virus and its host. In such a situation, MS being up to date the only effective methodology in proteomic approaches, is a game-changer that may alter our understanding of the infection and enable novel routes to develop antivirals and to fight the infection.

VII. INTERACTOME ANALYSIS

The concept of the interactome has been developed since 1999 when Bernard Jaqu and his group created the term interactome (Sanchez et al., 1999), defined as a branch of science combining bioinformatics and biology, which deals with the study of both the interactions and the consequences of these interactions between proteins and other molecules in the cell. Information about protein interactions is crucial for understanding cellular processes in physiological conditions and pathophysiology. Particularly, the net of molecular interactions between viral and infected cell’s proteomes are the interesting field of investigations to understand interaction of both objects. Interactome analysis can be used to build our knowledge about principles of viruses behavior in the cells, starting from the early infection phases, viral RNA or DNA processing, through cellular transcription, translation, and metabolism conquest, ending at new viral particles release and cellular death. The main goal of the interactome analysis is to show interactions between players (mainly proteins) in the examined, cellular or tissue model like: direct influence by activation or inhibition of one molecule by other, cleavage, modifications including remodeling, phosphorylation, glycosylation, and other PTMs, cooperation in the same metabolic pathway or in the same process. Results from interactome analysis are usually presented in the form of graphs of interacting proteins linked by the lines containing basic information about the nature of the interaction. It seems that interactome analysis could be one of the most powerful tools in understanding how viruses influence on the infected cells and how work the cellular defense strategies against viral conquest. Here it should be also noted that terms: interactome analysis and proteomic analysis are similar but definitely not the same. Proteomic analysis informs how the proteome of the investigated biological material (cell substructures, cells, tissues, whole organism) changes under interfering factors, while interactome analysis answers the question how individual players of the observed process influence on each other. Several methods have been introduced to identify and study protein-protein interactions (PPIs).

A. Affinity Purification Mass Spectrometry (AP-MS)

In this approach we can find cellular proteins interacting with viral ones, finding, for example, binding partners, cellular targets for viral enzymes or proteins recognizing viral DNA or RNA in the cytoplasm. In AP-MS assay bait protein is immobilized on a solid support such as magnetic beads oragarose, prey proteins are in the solution. In this case different types of baits such as specific antibodies, proteins with attached tag can be used (like: FLAG, HIS-tag, MYC, HA, GFP, streptavidin). After the affinity step proteins are digested with enzymes (proteases such as trypsin), the obtained peptides can be analyzed using the UPLC system connected with mass spectrometer. The AP-MS assay belongs to the group of library-independent methods, and is a universal technique allowing the usage of a wide group of molecules acting as baits. The application of a complex protocol involving cell lysis, purification, eliminates the possibility of detection spatial, temporal, weak, and transient PPIs. The development of PPI research strategies which are combined with tandem MS has become an important and powerful approach during interactome study. Various types of AP-MS assays have been introduced over time. Particular assays differ by the types of tags and the types of protein purification methods (Dunham et al., 2012). In native AP-MS, proteins are fused with antibody and linked to the magnetic beads or agarose. The antibody binds to the selected protein and forms an immunocomplex while unbound proteins are washed away. This assay requires high purity antibodies directed exactly against the prey protein (Gillen & Nita-Lazar, 2019). Taylor and Knipe (2004) used the native AP-MS method to identify 50 both cellular and viral proteins that interact with viral DNA-binding protein ICP8, important in the HSV-1 genome replication (Taylor & Knipe, 2004). Gillen et al. used the AP-MS technique to study the interaction of a protein involved in inhibiting the host’s immune response against the Kaposi’s KSHV. Applying the a group of selected monoclonal antibodies and MS for identification, the binding sites of the ORF45-binding protein were found (Gillen et al., 2015). As specific antibodies availability is limited in some cases, the introduction of GFP protein as a tag significantly simplified this problem. The main advantage of GFP is that the signal from this protein can confirm the expression of proteins of interest, being helpful in mapping the intracellular location of prey proteins. Munday et al. used GFP AP-MS method for investigation the cellular interactomes of the human respiratory syncytial virus L- and P-proteins. The GFP AP-MS approach showed that HSP90 and several other chaperones are involved in the replication of the viral genome by binding to polymerase and stabilizing L-protein (Munday et al., 2015). As shown by Crow et al., during HSV-1 infection, the IFIX protein responsible for the inhibition of expression of viral genes is bound by several E3-ubiquitin ligases which degrades the host’s immunity proteins. Magnetic beads connected with anti-GFP antibodies were used for affinity purifications and analyzed using MS. The identified IFIX interactions were verified by comparing biological replications of IFIX-GFP complex and pure GFP as a control using SAINT algorithms (Crow & Cristea, 2017).

Tandem affinity purification (TAP) is an efficient assay for fishing native protein complexes from cells and was used for the first time for protein complex identification in yeast and then for mammalian cells (Rigaut et al., 1999). It can be also used for finding a hybrid: viral/cellular protein complexes or analyzing of capsid assembly dynamics with types of protein involved in this process. The TAP method is based on a two-step purification procedure which increased the high-confidence interaction results, compared to standard AP methods. The use of a basic TAP tag with a high MW (21 kDa) has an adverse
Effect on the folding, activity and interaction of proteins; therefore, numerous modifications of the used tags have been introduced. Changing the tags to SF-TAP (streptavidin II and FLAG) resulted in a weight reduction to 4.6 kDa. Depending on the assay, different tag combinations are used (Rigaut et al., 1999; Puig et al., 2001). Applying the TAP-MS methodology (Eberle et al., 2014) examined the effects of viral HCV nonstructural protein (NS) 5A, responsible for interference with cellular pathways and the regulation of viral RNA replication. In total, 274 proteins were identified in this experiment; the CRAPome platform was used to verify protein interactions. As a final result, 24 high confidence proteins were obtained (see Fig. 7). Among them, fifteen were confirmed by an independent research group in another experiment (Germain et al., 2014). Germain et al. applied IP-MS/MS approach and ectopic expression of individually FLAGG-tagged HCV proteins such as Core, NS2, NS3/4A, NS4B, NS5A, and NS5B. All baits were tagged at the N-terminal, only NS4B was tagged at the C-terminal. Ninety-eight proteins were identified, including 74 new proteins, not previously described in the literature. The use of IP-western blotting confirmed eleven new virus-host interactions and gene silencing during HCV-associated proteins investigation, which allowed for the identification of eleven partners that are new modulators of HCV replication (Fig. 8).

The TAP-MS assay was also used to identify host-viral interactions of Nipah virus. Tags Strep_II and FLAG- at the N and C terminus of the selected virus bait proteins were used. The authors identified 88 new PPIs among 101 records. The PRP 19 complex turned out to be the most interesting. When interacting with the PRP 19 complex, the viral W protein may alter p53 protein control and gene expression (Martinez-Gil et al., 2017).
B. BioID-MS

The BioID-MS assay is based on biotin-streptavidin interaction. The bait protein is connected with biotin ligase molecule. In the cell, proteins interacting with bait protein are biotinylated. Using biotin-streptavidin affinity approach, those biotinylated proteins can be captured. Then, isolated ones are identified by MS. The use of appropriate bioinformatics tools allows a group of proteins that interact with selected bait proteins to be obtained. This method has similar advantages and disadvantages to the previously described AP-MS technique (Roux et al., 2012). Unfortunately, we can expect a number of nonspecific biotin-streptavidin interactions. Coyaud et al. used BioID-MS approach to create Zika interactome map. The experiment allowed the identification of 1,224 proteins interacting with the Zika virus that are involved in various processes such as translation, lipid metabolism, and vesicle trafficking (Coyaud et al., 2018).

C. Protein Reporter Interaction

The protein interaction reporter (PIR) assay, first published in 2005, is based on chemical cross-linking combined with MS (Tang et al., 2005). Chemical crosslinking of the covalently bound partners allows for the identification of interactions between protein complexes, as well as the identification of virus-host interactions in cells (Tang & Bruce, 2010; Sinz, 2014). The application of the PIR technique provided structural data on luteoviruses involved in plant-virus, as well as vector-virus interactions (Chavez et al., 2012; Alexander et al., 2017). DeBlasio et al. published the results of a host-pathogen-protein interaction network. The results also contains topological features for each identified cross-linked site. By applying the PIR ReACT (Real-time Analysis Cross-link Technology) technology (Weisbrod et al., 2013), researchers identified 375 unique cross-linked peptide pairs, including 19 between or within the two potato leafroll virus (PLRV) structural proteins, 285 were between hosts and 67 were between 33 hosts and 3 PLRV proteins. PIR technology is an interesting alternative for mapping protein interaction networks using chemical cross-linking.

D. Computational Methods

So far, no experimental method has been developed to detect interactions with 100% certainty, so a number of tools have been created to verify the obtained results, such as protein localization method, expression profile reliability (EPR index), paralogous verification method, and interaction generalities measures. Most of the experimental methods are time-consuming, expensive, require special equipment and have a high rate of false-positive results. During the past decade many computational methods have been designed to meet the challenge. Computational methods can be used to predict protein complexes based on PPI network analysis and network integration with various additional data, such as a gene function, PTMs modifications, coexpression or other introduced parameters. Various algorithms are available; however, all computational methods are based on several major assumptions such as assigning relevant scores to identified interactions, recognition of complexes by grouping available PPI networks or inclusion of additional data in the analysis and evaluation of the obtained complexes by comparison with the so-called golden standard datasets. However, so far, no existing method is able to predict efficiently the entire human, or other mammalian interactomes. Difficulties are provided by: instabilities in PPIs, the complexity of processes in which the selected protein is involved, dynamic changes that occur in cells (Rao et al., 2014). Experimental and predicted PPIs are collected in a number of different databases which are available on-line, such as database of interacting proteins accessed at: http://dip.doe-mbi.ucla.edu (Xenarios et al., 2000), FPCLASS (Interactions and Properties of Human Proteins) accessed at: http://phid.utoronto.ca/fplass/ (Kotlyar et al., 2015), HPDR (Human Protein Reference Database) accessed at: http://www.hprd.org, IntAct (Molecular Interaction Database) accessed at: http://www.ebi.ac.uk/interact (Hermjakob et al., 2004), iRefWeb a web interface to protein interaction data accessed at: http://wodaklab.org/iRefWeb (Turner et al., 2010), STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) accessed at: http://string-db.org (Szklarczyk et al., 2019), BioGrid (Biological General Repository for Interaction Datasets) accessed at: https://thebiogrid.org (Oughtred et al., 2019). Reference maps of interactions have been created to better understand cellular functions. Open reading frames (Wiemann et al., 2016) enabled progress in interaction mapping. So far, several maps have been published: BioPlex (Huttlin et al., 2015), Qubic (Hein et al., 2015), CoFrac-15 (Wan et al., 2015), HI_11_14 and novel generation HI-III-19 (Rolland et al., 2014) and HuRI (The Human Reference Protein Interactome Mapping Project). HuRI is the first human binary reference interactome map described by Luck et al. HuRI was generated by screening 90% of proteins coded in the human genome for binary PPIs by applying the Y2H assay. The integration of HuRI with different types of data such as the proteome, the transcriptome, and the genome allow the investigation of physiological and pathological cellular aspects.

VIII. MS IN VACCINE DEVELOPMENT

Vaccine technology shifted in the recent years from poorly characterized viral preparations to well-defined products, which were carefully crafted to induce proper and protective responses. Such a switch in methodology requires appropriate analytical methods. MS is one of the most important tools in modern vaccinology; in this chapter, the role of this method in vaccine design and preparation will be discussed.

A. Vaccine Design

The adaptive immune responses can be sorted into two groups—humoral (associated with B lymphocytes) and cell-mediated (associated with T lymphocytes) immunity. Classically, the vaccine is considered to induce humoral responses and provide humoral protection against viral pathogens. However, this effect is often not sufficient to control the infection and, in some cases, may result in antibody-mediated enhancement of the infection. Further, the rapid evolution of viral pathogens results in the emergence of escape mutants due to changes in highly variable viral surface proteins in terms of the primary sequence but also due to PTM (e.g., glycosylation). To develop a vaccine that can also induce cell-mediated responses it is essential to identify epitopes that are effectively presented in the context of MHC I and MHC II. Efforts to predict such epitopes in silico were undertaken, but due to the high variability of MHC molecules and unobvious rules of peptide presentation, the reliability of
these methods is limited. While these computational methods do not provide reliable data, they may provide valuable inputs for subsequent experimental studies.

For small viruses or single proteins, it is possible to synthesize a set of overlapping peptides covering the full sequence, which may be further incubated with recombinant or natural MHC proteins. The peptides that are effectively bound by the MHC may be further identified by the MS. Such an analysis was carried out for T-cell leukemia virus type 1 (HTLV-1), which is a broadly distributed pathogenic virus that is estimated to affect ~10 million people worldwide. In the majority of cases, the infection is asymptomatic, but in ~5% of patients, the disease manifests itself in a severe form, which may include adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis. Sundaram et al. (2003) synthesized HTLV-1 peptides and analyzed their processing using nanoLC-ESI-Q-TOF.

This approach is unfortunately not feasible for pathogens with larger proteomes. In such cases, analysis of the peptidome presented in the context of the MHC starts with the expression of viral proteins or infection with the virus (Lorente et al., 2019). Cells are then lysed, and MHC molecules are being pulled down using monoclonal antibodies allowing for the distinction of different MHC classes. Peptides associated with the MHC molecules are further released in acidic conditions and further analyzed by LC-MS/MS. Obtained data are searched against databases of viral proteomes, and peptides are mapped (Meiring et al., 2006; Weidanz et al., 2007). Importantly, this approach also allows for mapping of epitopes that are modified posttranslationally. Exemplary, (Mulherkar et al., 2018) isolated the MHC I associated peptidome using monoclonal antibody affinity purification, and then identified peptidome compounds by nanoLC-orbitrap-MS. The authors identified and confirmed six novel MHC-I restricted epitopes that were able to bind to HLA-A2 and HLA-A24 alleles and used in vitro and in vivo methods to generate CD8+ T cells specific for each of these peptides. Wu et al. (2019) used LC-MS/MS, also in the MRM mode, and identified 21 peptides derived from the influenza A virus proteins presented by immunocompetent cells with the further estimation of the response level of T cells. In similar experiments, 25 epitopes were found from human herpesvirus 6B (HHV-6B) presented in the MHC II context. They were able to activate T cells recruiting from CD3+ and CD4+ subpopulations. In natural conditions, observed T-cells reactivity toward presented antigens, identified during the investigation, allows controlling HHV-6B infection at a reasonable level (Becerma-Artiles et al., 2019). Ternette et al. (2016) immunoprecipitated peptides bound to HLA class I complexes from HIV-1 infected primary CD4+ T cells and C8166 cell lines, and further identified those by LC-MS/MS. A similar work was previously carried out, but the researchers here used primary T-cells infected with HIV-1 for the first time, which improves the reliability of the results (Hickman et al., 2003; Yaciuk et al., 2014). The analysis yielded a pool of epitopes, of which ~35% was never reported before.

Current understanding of the MHC-associated peptidome is also growing due to the large collections of data allowing the comparison of results from different studies, using databases such as the Immune Epitope Database (www.iedb.org). These data may be processed using existing and novel data mining tools and allow for recognition of peptidomes for different variants of the MHC molecules (Sette et al., 2005; Dhanda et al., 2019).

B. Vaccine Production

Considering that biological therapeutics are complex and challenging to standardize, it is important to utilize methods allowing for efficient and effective quality control. A number of techniques based on methods such as liquid chromatography, spectrophotometry, electrophoresis, immunoassays, or molecular biology tools are available, but only MS allows the exact content of the vaccine to be determined and characterized. Here, not only is protein content of importance, but also PTMs that may drastically change the immunostimulatory properties of the product. Exemplary review through an underlying methodology for vaccine analysis by MS-based and other methods was given by Hickey et al. (2016) and Sharma et al. (2018).

One of the basic applications of the MS includes the content analysis of the developed vaccines, as described for HAV (Hennessey et al., 1999). The more sophisticated analysis was described by Xie et al. (2011), who shows the benefits of LC-MS for characterization and monitoring of a recombinant influenza candidate vaccine formulated from purified rHA antigens. Application of this method allowed for the identification of impurities in the preparation, verification of rHA primary sequence, analysis of PTMs of the rHA and assessment of protein degradation. Similarly, Creskey et al. described an LC-MS method for the simultaneous quantification of HA and NA content in influenza vaccines (Creskey et al., 2012; McKnight et al., 2017). The developed technique has the potential to determine the precise HA content in vaccines and to increase the accuracy of reference antigen standards. It can also be used to monitor manufacturing consistency by assessment of other protein content, as NA or chicken egg proteins. This is especially important, as the HA/NA content is one of the major parameters describing the viability of the virus; these two proteins are major targets for neutralizing antibodies. The importance of such analyses was shown by Brgles et al. (2016) who compared the protein and lipid composition of mumps viruses, showing that the research model may severely affect the vaccine contents. Furthermore, MS allows for the analysis of vaccine contaminants, as described by Lei et al. (2018), who developed a sensitive analytical method, based on GC-MS to assess β-propriolactone content in the rabies vaccine. β-Propiolactone is used for virus inactivation. A previously described method employed gas chromatography but offered inferior parameters compared with the GC-MS method.

LC-MS allows for the characterization of the amino acid sequence of the protein but also for the assessment of PTMs. Among the common modifications which may alter vaccine effectiveness is glycosylation. Both, N- and O-glycosylation form a shield on the viral capsid proteins that is one of the most important modulators of immunogenicity, but which also affects protein folding and the consequent solubility (Varki et al., 2015). The glycosylation pattern differs significantly between different cell types and between organisms. Even more importantly, glycosylation patterns are important for the recognition (or lack of recognition) of viral epitopes by our immune system. This is mostly relevant for heterologous expression systems, where the viral protein is immunogenic but does not elicit protective immune responses. For that reason, vaccine antigens are often produced in mammalian cells, despite the high cost and low expression efficiency. Analysis of the glycan shield is a difficult task due to the high heterogeneity of the polymers and high
flexibility of sugar chains. Developments in MS allowed for the precise mapping of protein glycosylation and other PTMs, which could enhance vaccine effectiveness in the near future (Wyatt et al., 1998; Helle et al., 2010; Machiels et al., 2011; Burton & Hangartner, 2016; Cao et al., 2017; Lavie et al., 2018).

IX. CONCLUSIONS

MS is an exceptionally versatile analytical technique. Combined with sample separation methods, it allows for unprecedented development in various branches of life and medical sciences. Virology suffered for many years from the lack of support from powerful analytical techniques, suitable for answering the fundamental questions about interactions between viruses and cells. Since 1990, there has been increased interest among scientists working in virology regarding MS topics. This resulted in the immediate development of both areas of science: our overall knowledge about the viral world increased and, subsequently, the methods of analysis used in MS were better designed for the demands of this unique type of investigation.

The development of mass spectrometric applications in virology evolved in at least two directions. The first direction covered the design of special techniques or instrumental solutions, suitable for the measurement of viral physicochemical features (design of specific analyzers, nanoresonators, particle diameter measurements). The second direction adapts existing methods, mainly from proteomic and interactomic experiments, to solve the problems from the virology area of interest.

For investigations oriented directly on the measurements of viral MW, dimensions, assembly dynamics, etc., there are special types of mass spectrometers available. Initially, MALDI-TOF instruments were used for such purposes, but it was soon found that their capabilities are insufficient for the direct measurement of MWs of the whole viruses. Therefore, techniques measuring diameters were used instead of TOF analyzers. Currently, CDMS and GEMMA based on DMA seem to be the methods of choice. However, there are also attempts to design novel solutions, as in the case of nanomechanical resonators detecting virus particles depending on their masses.

Investigations of interaction between virus particles and host cells use methodologies that have been directly adopted from proteomic or interactomic studies, with additional, extensive sample preparation steps (like fishing, binding, DIGE, isobaric labeling, LC, 2D-LC, nanoLC or others). As such techniques are very widely optimized in a broad range of assays applied in life sciences, their adaptation for virology is not a difficult task. Therefore, this area uses all of the techniques derived from investigations of protein-protein or DNA/RNA-protein interactions. As in every other field of this kind of research, there is a constant pursuit to improve the quality of sample separations, resolution and sensitivity of the mass spectrometers as well as the design of novel and faster algorithms processing the big data buckets, but the principles of technology in general does not differ significantly.

Starting from the marriage of virology and MS, initial expectations were focused mainly on the estimation of the dimensions and composition of viruses. However, along with the evolution of these two fascinating areas of science, the expectations were rising. Currently, we can resolve how the virus enters into the cells, how it deceives the immunological system, and what processes serve to conquer cellular metabolism. We can detect crosstalk between viral and cellular proteins at the basal level, investigate viral strategies, leading to the omission of cellular defense systems. In some cases, we are able to detect viral particles, directly or indirectly, in the clinical material, supporting physicians in the identification of viral infections. We can also assess how the cellular proteome changes during infection, and what the defense strategies of cells are, as well as the immunocompetent cells. Finally, using MS, we can design and test mechanisms of vaccination and prepare effective vaccines, taking into consideration many more variables than to date.

Currently, it is difficult to predict directions in the development of MS-based analytical techniques toward finding answers for questions arising in virological sciences. However, the future will be exciting in this area of life sciences. Apart from other topics, it seems that the problem of the rapid identification of viral strains in human infections is insufficiently explored and needs significant improvement. Similarly, the design of novel vaccines and other anti-viral treatment strategies will be based mainly on the results from MS in the future. Also, there is a constant need to improve the sensitivity, resolution and speed of analysis, especially in the direct measurement of viral capsid dimensions, their assembly dynamics and potentially, the direct typing of strains. Methods used to date, like CDMS, GEMMA, or nanomechanical resonators, need significant improvements to serve as precise and efficient analytical techniques in MDa and GDa range of analysis. Fortunately, due to the rapid development and technical improvements in known technical set-ups, we hope that the numerous breakthroughs in analyses of viral nanoworld will be observed soon.

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