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# Investigating Viral Proteins and Intact Viruses with Mass Spectrometry

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Mass spectrometry has become a fundamental technology for virology and an important tool in probing the structure and function of viruses. It has been used to identify viral capsid proteins, detect viral mutants, characterize post-translational modifications, and measure intact viruses. Mass-based analysis techniques including time resolved proteolysis, tandem mass spectrometry, and computer-assisted database searching are also providing new insights into viral structure and the dynamic changes in the capsid domains at the onset of infection. This capsid mobility is allowing researchers to develop and evaluate new drugs for viral inactivation. More recently, new mass spectrometry approaches have been used to detect intact viruses. Overall, its broad application to local and global viral structure is providing unique insight into the solution and gas-phase properties of viruses.

**Keywords.** Virus, Viral, Viral structure, Capsid, Dynamic, Capsid mobility, Whole virus, Intact virus, MALDI, Electrospray, Proteins, Inactivation

## 1 Introduction

Mass spectrometry is an emerging technology and now a core resource in all areas of viral research [1, 2]. It is becoming a fundamental tool for work in areas such as protein characterization [3–6], structural virology [7–12], drug discovery [12, 13], and clinical chemistry [14]. Most of the increased interest in this technique has been due to the development of matrix-assisted laser desorption/ionization [15] (MALDI) and electrospray ionization (ESI) [16], which are highly sensitive and "soft" ionization techniques.

Electrospray ionization involves the introduction of a liquid solution directly into the atmospheric pressure source through an emitter. The liquid forms a droplet at the end of the emitter, where it is exposed to a high electrical field (Fig. 1). This results in a buildup of multiple charges on the surface of the droplet. The coulombic forces from these charges ultimately result in the droplet's expulsion from the surface. The ions produced in the ion source are then extracted into the mass analyzer. ESI is now widely used for identifying small molecules, proteins, studying large non-covalent complexes, structural analysis, and as a detector for separation methods such as HPLC and capillary

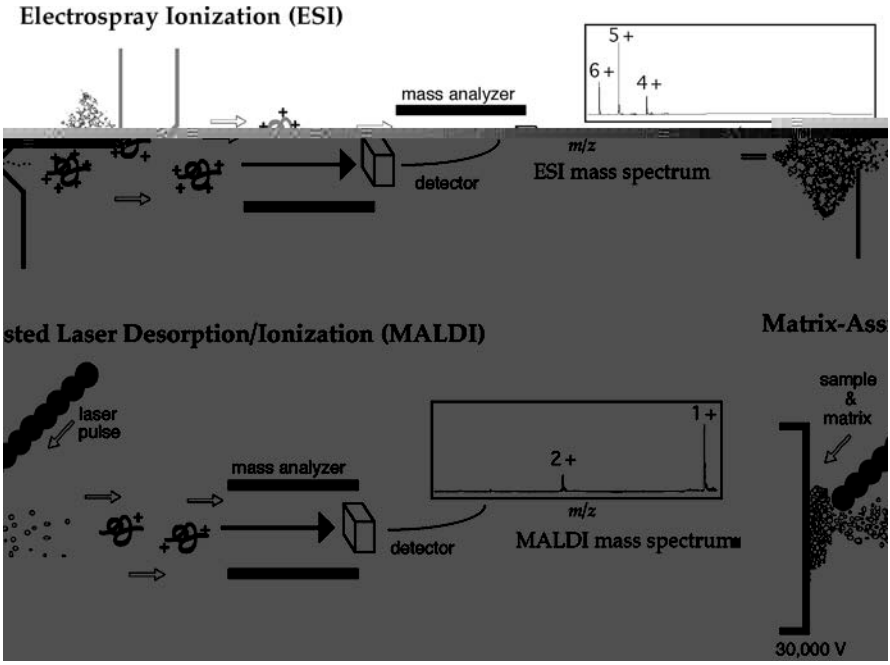


**Fig. 1.** MALDI and electrospray processes are represented schematically. Electrospray ionization involves the formation of charged droplets, while MALDI occurs through the laser evaporation from a crystallized sample/matrix mixture. In both techniques, the sample ions are extracted and transmitted through electrostatic lenses to a mass analyzer

zone electrophoresis (CZE). The low tolerance of ESI to salt (<10 mmol/l) has been overcome by coupling liquid chromatography to ESI-MS. Nanoliter/min low flow nanoESI-MS/MS has extended the application of ESI to highly sensitive means of protein identification.

MALDI MS has also emerged as an extremely useful method in biological analysis. A pulsed laser is used to irradiate a sample that is co-crystallized on a surface along with a matrix (e.g., 2,5-dihydroxybenzoic acid). The matrix absorbs the excess laser energy and facilitates ionization of the sample as the sample/matrix mixture is vaporized (Fig. 1). The matrix acts as a receptacle for the laser energy, therefore minimizing the decomposition of the analyte ion that would normally occur from direct laser desorption. The ions are then directed into the mass analyzer using a combination of electrostatic lens elements. While MALDI is not amenable to direct LC/MS applications, MALDI is more tolerant to salts and also better suited for direct complex mixture analysis than ESI. For this reason MALDI is very useful in the direct analysis of proteolytic digests with reported sensitivities in the low femtomole to attomole level.

ESI and MALDI-MS are routinely used for both accurate mass information on intact proteins and their proteolytic digests. As a result these methods have already helped detect viral mutants, identify capsid proteins, and post-translational modifications. Recent work has also included the detection of the first intact viral particles as well as a viral protein capsid. Other mass-based approaches like time-resolved proteolysis is giving new insight into the dynamics of viral capsid proteins in solution. This information, when combined with complementary information from X-ray crystallography studies is leading to a better understanding of viral structure and function.

## 2 Identification of Viruses and Viral Proteins

Mass spectrometry has been highly successful for identifying all classes of proteins including those originating from viral capsids and membrane proteins from enveloped viruses using existing genomic databases. Protein identification generally involves purification (commonly performing using gel electrophoresis), proteolytic digestion, and mass analysis. Trypsin is most often used to generate fragments since it cleaves at Lys and Arg, two amino acids which are in high abundance and usually situated in accessible regions in most proteins, therefore resulting in a significant number of peptides. Computer-aided data analysis conducted by comparing observed mass of proteolytic fragments against the predicted mass can be used to generate a list of potential matches. The computer-based analysis also provides confidence levels for each match that helps in distinguishing between statistically random and significant hits. This approach relies on the availability of the genomic sequences within the database. However, because of the small size of viral genomes, their genomes are readily available.

As a demonstration of the power of mass mapping for viral identification MALDI-MS and LC/MS/MS experiments were performed on Hong Kong 97 (HK97) icosohedral virus capsid [17]. HK97 presents an interesting system for these analyses because it has a highly stable interlinked "chain-mail" capsid that

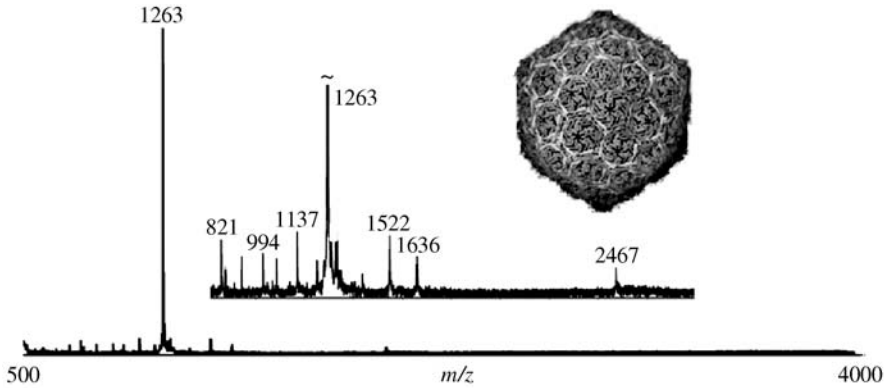


**Fig. 2.** Thermolysin rapidly cleaves the highly interlinked capsid from Hong Kong 97 virus at 65°C. Cleavage reaction was performed for 2 h in 100 mmol/l Tris-HCl, 10 mmol/l $CaCl_2$. Trypsin cleavage showed no significant fragmentation within this time. *Inset*: an image of HK97

is relatively impervious to enzyme digestion. Attempts at trypsin digestion of this capsid generated minimal peptide cleavage, even after 24 h. However, high temperature digestion with thermolysin generated significant cleavage within 2 h (Fig. 2). The 1263 (1263.66) mass peptide fragment appears most rapidly during the digestion process. This peptide corresponds to residues 172–181 or 173–182 in the capsid sequence. These peptides are situated at a beta sheet interface between capsid proteins and demonstrate this to be the first region of the virus coat to be susceptible to denaturation.

To establish whether thermolysin was a viable enzyme for identification using mass mapping, BSA and HK97 virus were digested and further analyzed. The molecular masses of the fragments were obtained and searches were undertaken using Profound. Using only F, L, I, and V as amino acid cleavage sites, we obtained an unequivocal identification of both BSA and HK97 virus capsid proteins. These identifications were made using the molecular weight range of 0–100 kDa and the database searches were constrained to mammals for BSA and viruses for HK97. Expanding the searches to include *all taxa* in the mass range of 0–500 kDa did not alter the identification significantly. This data indicates that thermolysin allows for identification of proteins that would be difficult or impossible to identify otherwise.

## 3
## Identification of Mutations and Post-Translational Modifications

DNA sequencing methods have been traditionally used to identify viral mutants yet peptide mass mapping has recently been successfully used to localize mutations. By comparing proteolytic fragments from wild-type viruses and genetic mutants their characterization has been successful. The mass mapping strategy schematically, represented in Fig. 3, was first shown for identifying the mutants of the human rhinovirus (HRV) and the tobacco mosaic virus (TMV) [10].

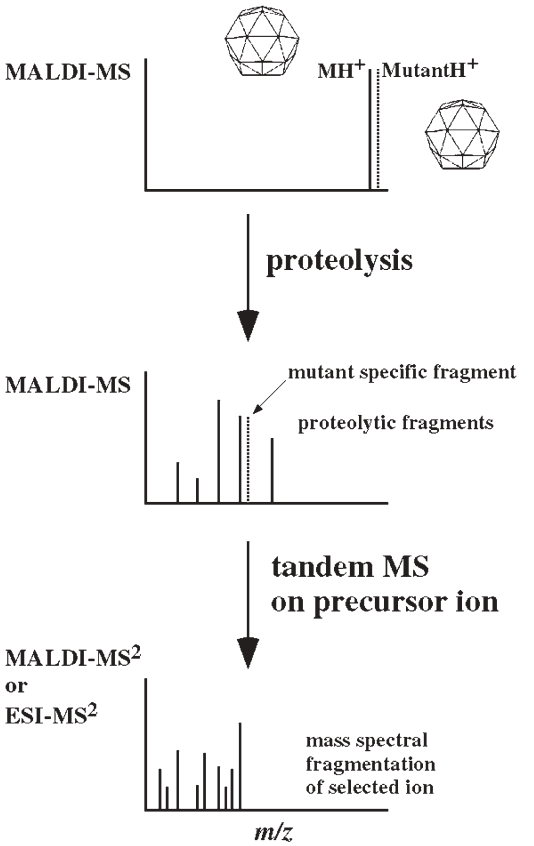


**Fig. 3.** The strategy for detecting viral mutations is represented. Differences between two strains of a virus are characterized by comparing mass spectra of proteolytic fragments. The specific peptide ion modification can then be localized by tandem mass spectrometry in combination with MALDI or ESI

Trypsin digests of both wild type HRV virus and the mutant were analyzed using MALDI-TOF and MALDI Fourier transform mass spectrometry (FTMS). For HRV, the mass spectra for both wild-type and mutant were identical except for one peptide occurring at *m/z* 4700. This corresponds to residues 187–227 in the wild type sequence. The corresponding peak in the mutant mass spectrum occurs at 4783.5 (Fig. 4, inset). This mass difference of 83 Da corresponds exactly to a mutation of a Cys to Trp residue and there are no other possible mutations that would be separated by 83 Da. Since there is only one Cys in the peptide 187–227 at position 199, the mutant can be localized as HRV14-Cys199Trp, which contains a Trp at position 199 instead of Cys in the wild type.

Another mutant for the tobacco mosaic virus (TMV Asp77Arg) (Fig. 4), was identified using the same approach. MALDI mass spectra of the digests of the capsid protein were identical except for the fragment occurring at 2051.4 Da in the wild type and 2091.8 Da in the mutant. The mass of the peptide for the wild

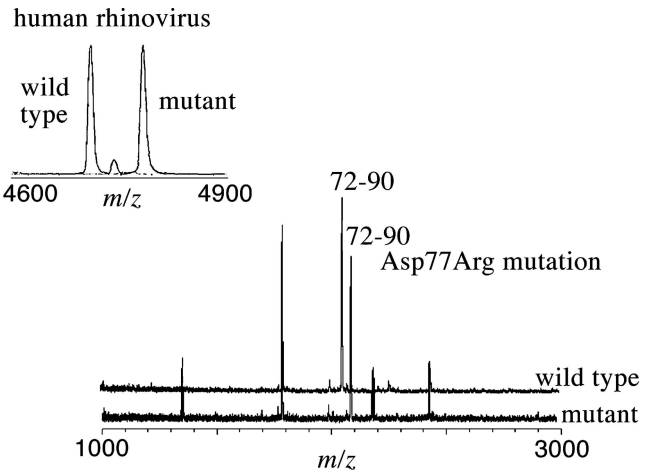


**Fig. 4.** The detection of mutations is shown for two viruses, HRV 14 and tobacco mosaic virus. The 83 Da mass difference for the HRV14 mutant helps identify the mutation as Cys199Tyr as the only possible mutation. For the tobacco mosaic virus, a mutation at Asp77Arg is identified similarly by comparison of mass difference with the known sequence for the tryptic fragment

type corresponds to the amino acids 72–90 in the sequence. Of the three possible mutations that cause an increase in the mass of the mutant by 40 Da, only one was possible in residues 72–90. The mutation at residue 77 replaces the Asp with Arg. Other mutations that differed by as little as 1 Da could be identified by a combination of high resolution/accurate mass measurement, tandem mass spectrometry, and genomic sequencing. In cases where genomic sequencing was required, the region to be sequenced was greatly reduced by using mass spectrometry.

Another wide application of mass spectrometry is the detection and characterization of post-translational modifications such as myristoylation, phosphorylation, disulfide bridging, etc. The detection and localization of post-translational modifications has been a rapidly developing area of mass spectrometry due to the functional importance of these modifications in biological systems. An example of this was recently shown for the case of the human rhinovirus HRV14 [10]. Electron density maps from crystallography data indicated a myristoylation of VP4. Mass analysis of VP4 also indicated a mass difference of 212 Da (consistent with myristoylation of VP4). Additional experiments with proteolytic digestion and tandem mass spectrometry were able to localize the modification to the N-terminus of VP4.

MALDI quadrupole ion trap mass spectrometry has also been used to localize and identify the post-translational modifications on the Sindai virus [18]. The polymerase associated protein (P protein) from this virus is reported in the literature to be highly phosphorylated. In vitro studies have detected phosphorylation in different regions of the protein, while a single phosphorylation site was found in the in vivo studies. Mass spectral data, along with computer-aided analysis, enabled the identification and localization of two phosphorylation sites.

Mass spectrometry was also used to localize disulfide bridging in the human respiratory syncitial virus (RSV) [19]. The attachment protein was digested with trypsin and separated using reversed phase high-performance liquid chromatography (HPLC). One tryptic peptide in the digest was detected using MALDI time-of-flight mass spectrometry that corresponded to residues 152–187 in the sequence, with four Cys residues in a disulfide linkage. Further selective digestion with thermolysin and pepsin was able to identify the linkages between Cys 173 and Cys 186 and between Cys 176 and Cys 182.

## 4 Peptide Mass Mapping for Protein Structure

The same proteolytic digestion methods used to analyze primary protein structure can be applied to the study of higher order structure of macromolecular assemblies. This is because the degree of proteolytic degradation for a protein complex will depend on the exposure of accessible regions on its surface. Enzymes such as trypsin when used under native conditions will be limited to surface accessible regions of the protein [4, 20–22]. The choice of proteases selected to probe the structure of the complex depends on the distribution of amino acids within the protein. Trypsin and V8 are frequently employed since these have a cleavage specificity for the hydrophilic amino acids, generally found on the surface of native proteins (R, K and D, E). The basic strategy for mass mapping for protein complexes involves the contrasting of associated and unassociated states after proteolytic digestion. The adduction of a macromolecule on the surface of the protein protects the surface in such a way as to reduce access of the protease to potential cleavage sites. This effect results in a dramatic reduction in proteolysis in the region of complexation. The basic experiment is shown in Fig. 5. Arrows mark susceptible cleavage sites on the surface and flexible loop regions. Only some of the possible tryptic fragments are generated due to presence of inaccessible regions on the protein surface.

Mass mapping for protein structure has been utilized for studying protein-protein [4, 15] and protein-DNA complexes [23]. An example of this is shown in Fig. 6 for cell cycle regulatory proteins Cdk2 and p21. A comparison of the proteolysis products of p21B for the associated and unassociated states was used to identify a 24 amino acid region on p21B as the binding site. The relative intensity of the proteolytic fragments was reduced for the p21/Cdk2 complex. Due to limited mass accuracy of the available equipment at the time and the general complexity of the mass spectra, $^{15}N$ labeling was used to identify unambiguously peaks originating from the p21B subunit. With the availability of more accurate MALDI-TOF mass spectrometers that facilitate ppm mass accuracy, the interpretation of mass spectra obtained from the digestion of complex assemblies is becoming easier without the need for additional steps taken in this experiment. However, it is important to note that information on the binding site of these two proteins was obtained in a matter of minutes in a mass spectrum without the need for additional separation and purification steps such as SDS/PAGE and reverse phase chromatography. This work was later corroborated by Pavletich and co-workers [24]. The structures of more complex viral protein assemblies have

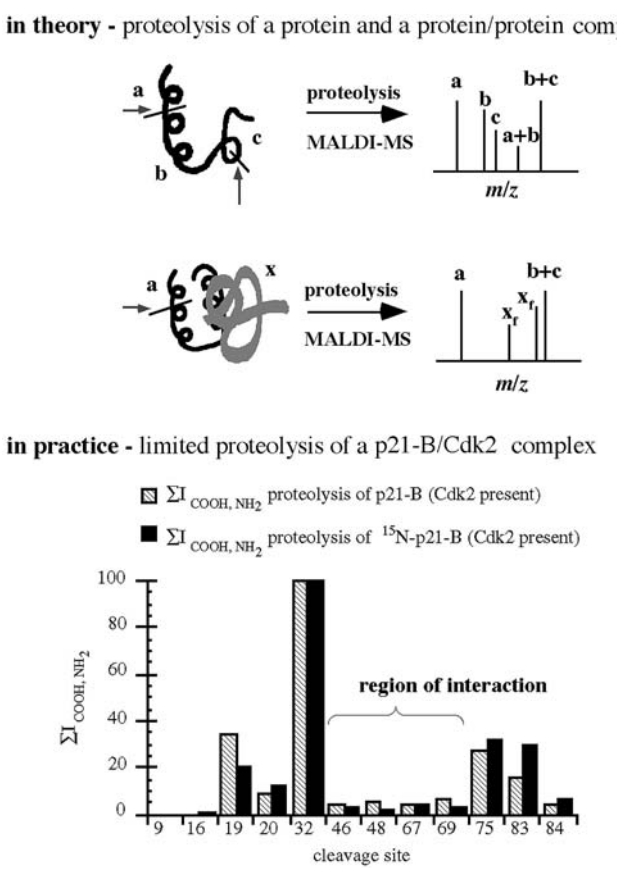


**Fig. 5.** The approach to probing higher order protein structure by mass spectrometry is shown. The cleavage of a hypothetical protein generates fragments (a, b, c, a+b, and b+c) that can be monitored by MALDI-MS. Here, *arrows* denote possible cleavage sites for proteolysis. The adduction of this protein with another to form a complex leads to a suppression of proteolysis in the region of association as well as fragments from the other protein ($x_f$). An example is shown for the limited proteolysis of p21-B/Cdk2 complex. The suppression of proteolysis in a 24 residue region of the sequence helps identify this area of association

since been explored using protein mass mapping and building on the success of the earlier work described above [8, 9, 11, 25].

## 5 Probing Viral Capsid Dynamics

A natural extension of protein mass mapping is getting a greater understanding of the local and global viral capsid structure. The viral capsid consists of a protein coat that can be mapped using limited and selective proteolytic digestion, since the proteins lying exterior to the viral surface are expected to be more susceptible to cleavage. Because proteolytic digestion is performed in solution, this

technique is capable of detecting multiple conformers of the viral capsid and thereby extending our understanding of the dynamic domains within the virus structure.

Limited proteolysis was used on the human rhinovirus 14 (HRV14) and the Flock House virus (FHV) [9]. Four proteins, VP1, VP2, VP3, and VP4 comprise the protein coat of the HRV. According to crystal structure, VP4 lies inside the virus at the RNA/capsid interface. FHV has a protein coat which is made up of a single protein that autocatalytically dissociates to $\beta$-protein and $\gamma$-peptide during maturation. These, according to the crystal structure, have regions which lie internally and externally on the surface (Fig. 6). Time resolved proteolysis, in combination with MALDI MS analysis, was used to map the surface accessible proteins in the virus capsid. The experiment involved sequential digestion by trypsin (endoprotease), followed by carboxypeptidase Y (exoprotease). In both HRV and FHV, the analysis was able to identify surface proteins. However, fragments resulting from the digestion of internal regions, as determined by crystallography data, were also observed very early on in the time course of the proteolysis experiments. Although these results were initially perplexing, additional studies along with crystallography data suggest that the internal proteins within the capsid are highly mobile. The domains lying internally can be transiently exposed on the surface of the virus in solution. For FHV, the regions of the $\beta$-protein and $\gamma$-peptide that were transiently exposed correlate with the regions implicated in the RNA release and delivery. More recently, mass spectrometry, along with proteolytic digestion, were used to distinguish between FHV and virus-like particles (VLP) [26]. These virus-like particles are cystallographically identical to the wild-type virus except that they are generated in a special baculovirus expression system to contain the cellular RNA instead of the viral RNA. The degree of proteolysis was found to be much higher for the VLP, suggesting that the viral capsid mobility and stability in solution is significantly affected by the specific interaction at the RNA-capsid interface.

Another way of examining viral dynamics is through chemical modification. Proteins within the capsid can be exposed to modifying agents. The analysis of

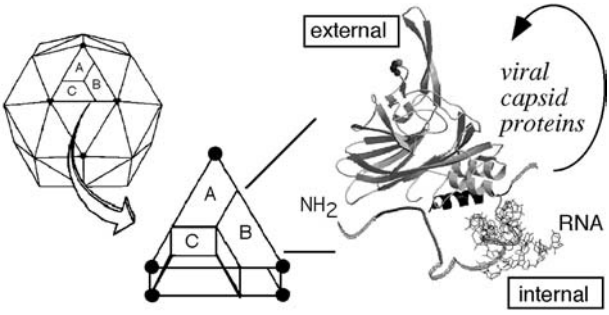


**Fig. 6.** The Flock House virus has an icosahedral symmetry with the $\gamma$-peptide and the C and N termini of the $\beta$-protein lying internally and away from the surface. However, time resolved proteolysis data indicates that the viral capsid is highly mobile and that internal domains are transiently exposed on the surface

the products helps identify regions of the proteins that are transiently exposed on the surface. In addition, hydrogen-deuterium (H/D) exchange experiments can yield information on different conformer populations present for a protein. For example, denatured proteins will be more susceptible to H/D exchange than proteins in a more folded state. These experiments can give helpful information on both the degree of folding and the number of conformations present. Two examples of structural characterization of viruses using chemical modification and H/D exchange are shown in Fig. 7. Here, acetylation is using to help localize the surface exposed region of the γ-peptide. An H/D exchange experiment is also shown in which the number of exchangeable hydrogen atoms reveals information about the structure of the capsid protein. Recently, H/D exchange experiments have been used to probe stages of viral capsid assembly for the dsDNA bacteriophage [27]. Here, limited proteolysis and the degree of back exchange of deuterated capsid protein were used to characterize the surface exposed regions of the virus during its dynamic transformation to the mature capsid form. In another study, pH-induced structural changes in the viral capsid that cause destabilization were characterized using mass spectrometry [28]. Therefore, time-

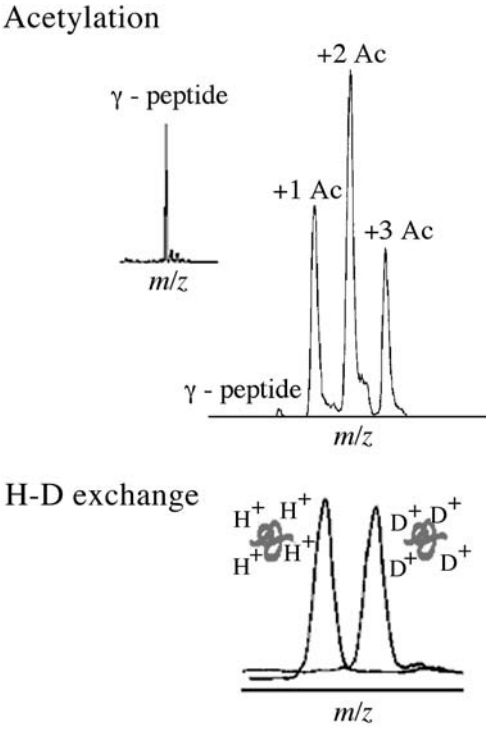


**Fig. 7.** *Top*: mass spectra of FHV γ-peptide after acetylation. The degree and site of acetylations, determined by tandem mass spectrometry, can be used to characterize surface accessible regions of the peptide. *Bottom*: a viral capsid protein ion generated by ESI undergoes hydrogen-deuterium exchange. The multiple populations of ions generated can help distinguish between multiple conformers present for a capsid protein

course proteolysis and chemical modification methods, when combined with mass spectrometry, provide complimentary information to X-ray crystallography. These, when taken together, can help broaden our understanding of the dynamic domains of the viral capsid.

## 6 Screening for Antiviral Drugs

There are many human diseases caused by viruses, but not many anti-viral drugs that directly attack the virus. Screening of new and existing compounds for anti-viral activity is a necessary step towards the development of new drugs. Inhibition of viral enzymatic reactions can be monitored with mass spectrometry. These approaches have already been developed for non-viral enzymatic reactions such as glycosylation. In the mass based approach to screening activity, the substrate, inhibitor, product, and internal standard mixture are introduced into a mass spectrometer. The product formation for a reaction can be monitored by mass analysis. This methodology was illustrated for a galactosyltransferase catalyzed reaction (Fig. 8) [13]. The inhibition of this reaction was analyzed using a library of 20 small molecules. The experiment involved monitoring product formation for 22 parallel reactions which included two controls without any inhibitor (Fig. 9). These reactions were quenched by the addition of methanol before their introduction into an ESI mass spectrometer. Three new inhibitors were identified through the screening procedure. Although this example involves a non-viral enzyme system, this technique is a fast and efficient technique that can be used to screen for antiviral drugs that target viral enzymes.

The traditional method for determining anti-viral drug activity involves conducting a plaque assay. A preliminary mass based screening can be helpful in reducing the number of drug candidates that need to be screened by more

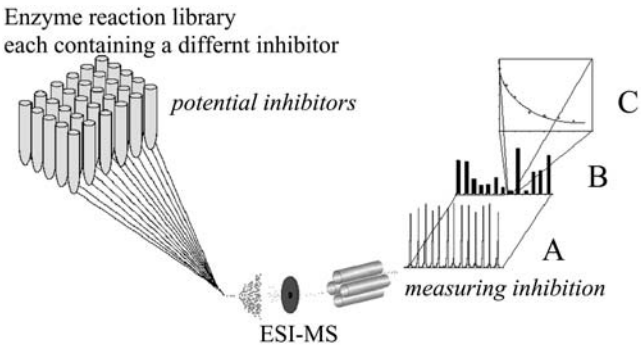


**Fig. 8.** A quantitative enzyme assay of enzyme inhibition is shown. The ESI/MS analysis can be automated. Samples introduced include enzyme, substrate, product, potential inhibitor and internal standard. (A) Total ion current is measured for each sample. (B) Product ion formation is plotted with respect to internal standard. (C) If an inhibitor is found, the degree of inhibition is derived using ESI-MS

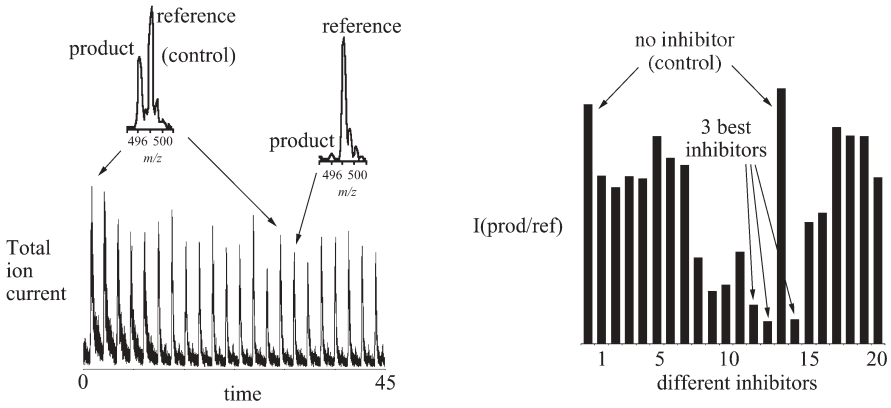


**Fig. 9.** **A** Total current observed for all 22 reactions; 20 of these had each a different inhibitor. *Insets* show data for control (*left*) and an effective inhibitor (*right*). **B** A bar graph was plotted of product ion/reference ion intensity ratio to help identify potential inhibitors

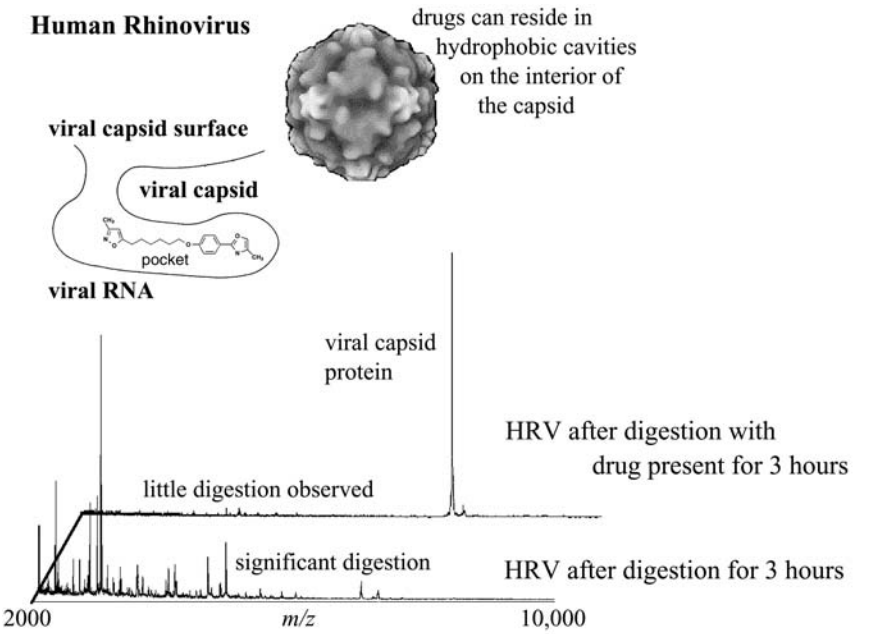


**Fig. 10.** Proteolysis is used to probe the antiviral activity of WIN52084, an experimental antiviral drug. The binding of WIN52084 to the viral capsid results in a suppression of proteolysis that can be monitored by mass spectrometry

time consuming plaque assays. Most antiviral drugs are targeted at the protein coat of a virus. The picornaviruses are a class of icosahedral viruses of which the human rhinovirus 14 (HRV14) is a member. These are known to have 25-Å canyons on each of the fivefold symmetry axes. The experimental drug WIN52084 is known to bind to a hydrophobic pocket within the canyon. This is of particular interest to researchers since the canyon is also believed to be the site of the cell receptor attachment before endocytosis. The binding of the antiviral drug WIN52084 has been observed to block cell attachment, inhibit uncoating, and make it easier to inactivate the virus thermally. A family of the WIN drugs were analyzed for suppression of capsid proteolysis [29]. This effect, which results from the drug binding to the protein coat, was observed for other drugs, but was most pronounced in WIN52084 (Fig. 10). Other viruses were also analyzed using this method and their capsid proteolysis was found not to be inhibited by WIN52084. This was further evidence of the specificity of the drug for HRV14, and that proteolysis suppression was not an effect resulting from the inhibition of the enzyme. The dramatic reduction of proteolysis for the virus in the presence of WIN52084 is believed to result from the interruption of virus capsid mobility which is essential for the virus to initiate receptor attachment and the ensuing endocytosis.

## 7 Differentiating Viral Genomes by Mass

The analysis of viral DNA and RNA directly using mass spectrometry has been challenging due to the low levels present in biological samples and the limited mass range offered by most commercial mass spectrometers. However, nested polymerase chain reaction (PCR), along with MALDI/TOF analysis, has been used to detect the presence of low levels of virus in hepatitis B patients [30]. The hepatitis B virus related products were purified, immobilized, denatured, and analyzed using MALDI/TOF mass spectrometry. Very massive single ions of DNA of several megadaltons molecular weight have been observed using ESI Fourier transform mass spectrometry (FTMS) [31] and charge detection mass spectrometry [32, 33]. Both techniques employ the amplification and measurement of a weak image current induced by the ion. In charge detection mass spectrometry, a very sensitive amplifier is used to measure directly the charge and velocity of an ion. The velocity is used to deduce the $m/z$ which can be used along with the measured charge to give molecular weights of very heavy ions. These techniques may be promising for measuring viral DNA directly as a means for identification and characterization of genetic variability. The first observation of an intact virus particle was made by charge detection mass spectrometry as discussed in the section below.

## 8 Analyzing Whole Viruses

There have been significant developments in the past few years in the area of macromolecular analysis of whole viruses and viral capsids. The first was the

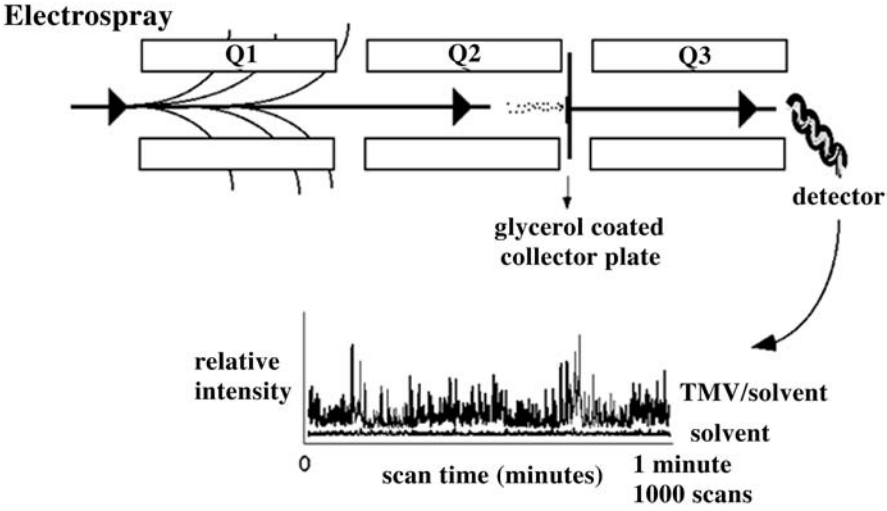


**Fig. 11.** The experiment of indirectly "detecting" an intact viral particle after its transmission through an electrospray mass spectrometer. The viral particles were observed under an electron microscope after collection since the massive particles exceeded the working range of the electron multiplier normally used with this mass spectrometer

examination of virus viability following transmission of the whole virus through a mass spectrometer [34]. A brass plate was placed between Q2 and Q3 in a triple quadrupole mass spectrometer where viral particles could be collected (Fig. 11). The plates, when examined using electron microscopy, were found to have intact viral particles. The mass analyzed and collected TMV viral particles were successfully used to infect tobacco plants. The surprising results of these studies demonstrated that intact viruses could be transmitted through the ESI mass spectrometer and that the ionization method was gentle enough for the viruses to retain their virulence. More recently, the measurement of an intact viral capsid was accomplished using ESI/TOF mass spectrometry by Robinson and coworkers [35].

Because of the size of viruses the challenge of measuring them intact required the development of new technology. The direct measurement of the charge state of very massive ions has been shown using charge detection TOF/MS through image current measurement. In charge detection mass spectrometry, both the charge and the time-of-flight, yielding *m/z*, are measured one ion at a time. Intact viral particles of the rice yellow mottle virus (RYMV) and the tobacco mosaic virus (TMV) were studied using this methodology [36]. The RYMV is an icosahedral non-enveloped virus consisting of a single strand of RNA and multiple copies of a single protein. The RYMV and TMV have theoretical molecular weights of $6.5\times10^6$ Da and $40.5\times10^6$ Da, respectively. The mass spectra obtained for RYMV and TMV are shown in Fig. 12. The broad peaks obtained for each virus correlated with the calculated masses. This work demonstrates the use of mass spectrometry for identifying pathogenic viruses.

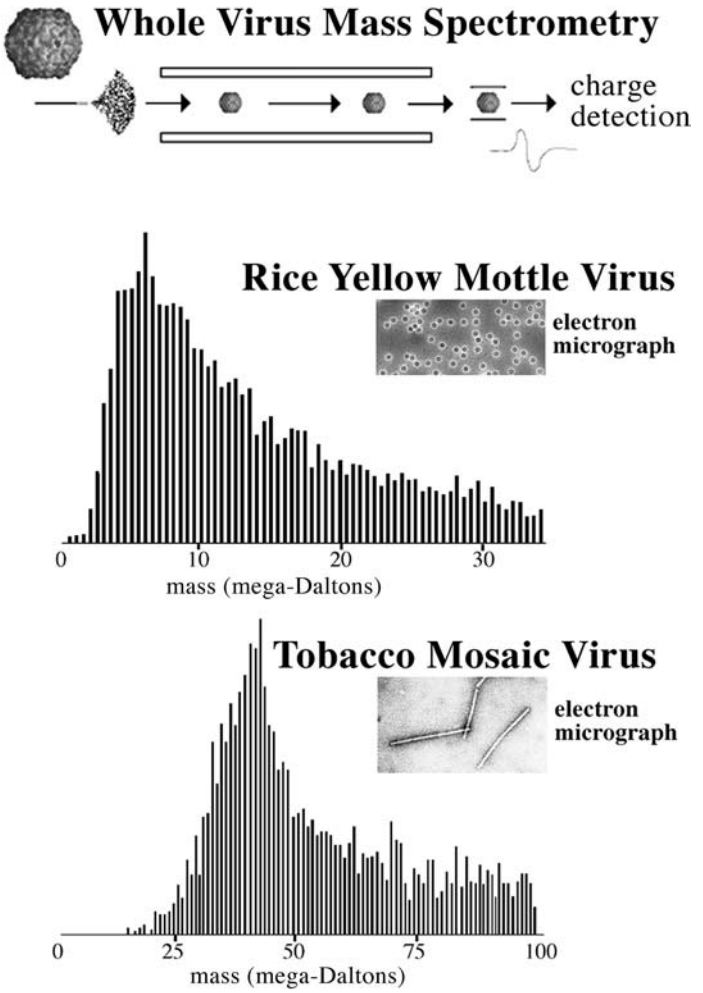


**Fig. 12.** Mass spectra of Rice Yellow Mottle Virus (RYMV) and Tobacco Mosaic Virus (TMV) particles analyzed with an electrospray ionization charge detection time-of-flight mass spectrometer. *Inset*: electron micrographs of the icosahedral RYMV (diameter 28.8 nm) and the cylindrical TMV (~300 nm long and 17 nm in diameter). The known molecular weight of RYMV and TMV are $6.5\times10^6$ and $40.5\times10^6$ Daltons, respectively

## 9
## Future Prospects and Conclusions

Mass spectrometry has opened up new opportunities for understanding viruses. ESI, MALDI, and emerging new technologies such as desorption ionization on silicon (DIOS) [37–39] are in a continual state of flux, with significant improvements accruing yearly. The ultra high accuracy provided by FTMS is being increasingly applied to solve biologically significant problems and promises to become an important tool for probing viruses. The use of proteolysis, with mass based approaches is helping researchers not only to probe the functional and genetic diversity within viral species, but also to understand the dynamic nature of the viral capsid. One of the most exciting developments has been the use of time-resolved proteolysis to study capsid dynamics, and as a means to characterize protein-protein and RNA-capsid interactions. Since proteolysis is performed in solution, it can yield complementary information to crystallography data. These techniques, when extended to even more complex enveloped viruses like HIV and influenza, will surely provide valuable information about these important pathogens, and may lead to the development of new and more effective anti-viral drugs.

## 10
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