

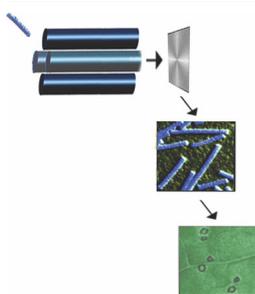
Staying Alive: Measuring Intact Viable Microbes with Electrospray Ionization Mass Spectrometry

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Abstract. Mass spectrometry has traditionally been the technology of choice for small molecule analysis, making significant inroads into metabolism, clinical diagnostics, and pharmacodynamics since the 1960s. In the mid-1980s, with the discovery of electrospray ionization (ESI) for biomolecule analysis, a new door opened for applications beyond small molecules. Initially, proteins were widely examined, followed by oligonucleotides and other nonvolatile molecules. Then in 1991, three intriguing studies reported using mass spectrometry to examine noncovalent protein complexes, results that have been expanded on for the last 25 years. Those experiments also raised the questions: How soft is ESI, and can it be used to examine even more complex interactions? Our lab addressed these questions with the analyses of

viruses, which were initially tested for viability following electrospray ionization and their passage through a quadrupole mass analyzer by placing them on an active medium that would allow them to propagate. This observation has been replicated on multiple different systems, including experiments on an even bigger microbe, a spore. The question of analysis was also addressed in the early 2000s with charge detection mass spectrometry. This unique technology could simultaneously measure mass-to-charge and charge, allowing for the direct determination of the mass of a virus. More recent experiments on spores and enveloped viruses have given us insight into the range of mass spectrometry's capabilities (reaching 100 trillion Da), beginning to answer fundamental questions regarding the complexity of these organisms beyond proteins and genes, and how small molecules are integral to these supramolecular living structures.

Keywords: Mass spectrometry, Intact, Virus, Bacteria, Viability, Charge detection, Electrophoretic mobility, Noncovalent interactions

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Introduction

The origins of mass spectrometry (MS) being applied to biological noncovalent interactions can largely be traced to 1991 when three seminal papers reported the observation of known protein complexes in the gas phase using electrospray ionization (ESI). These included the immunosuppressant ligand-binding to human immunophilin FKBP (Figure 1a) [1], the heme-myoglobin complex [4], and the ternary complex of dimeric HIV-1 protease binding to an inhibitor [5]. This work was quickly

followed by numerous other reports on noncovalent structures, such as the duplex DNA–drug complex [6], calcium mediated cell-surface carbohydrate association [3] (Figure 1b), and catalytic antibody-hapten/substrate interactions [7, 8]. As these applications further developed, it became increasingly clear that the noncovalent analysis capabilities that ESI-MS could be used for a variety of purposes. For example, monitoring binding site kinetics by titrating increasing concentrations of ligand [1], and even binding site position by using collision induced dissociation (CID) to disrupt both covalent bonds [9] and noncovalent interactions [5, 10]. These results motivated new instrumentation designs of large complexes, such as DNA (Figure 1c) [2], which were otherwise difficult or impossible to analyze.

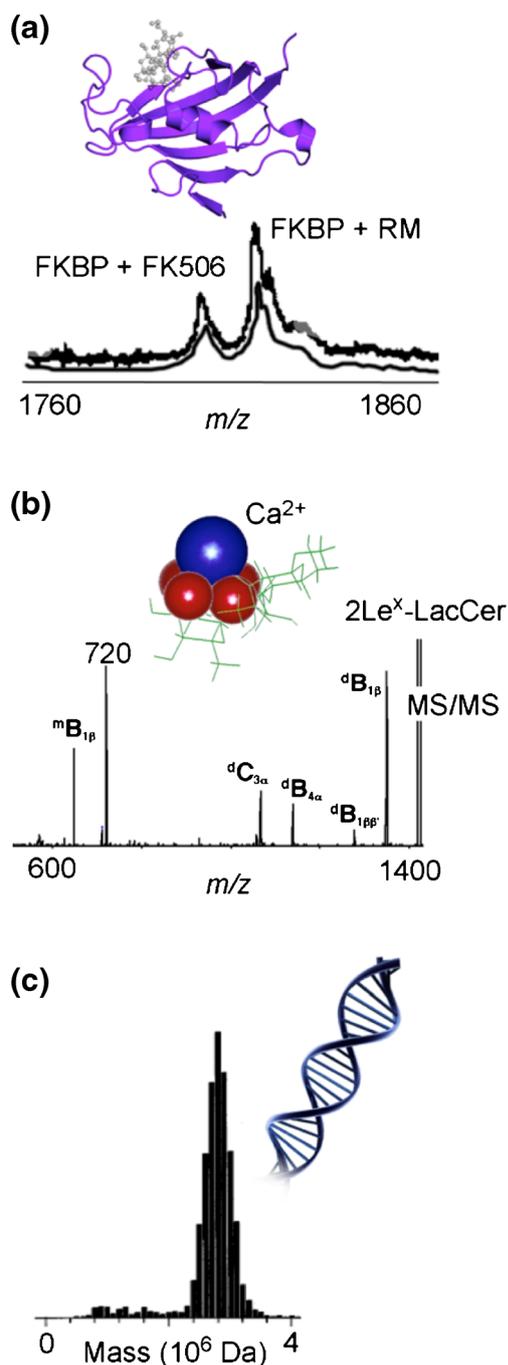


Figure 1. Mass spectrometric analysis of biomolecular complexes **(a)** the binding of immunosuppressants FK506 and rapamycin (RM) to human immunophilin FKBP [1], **(b)** Ca^{2+} mediated dimerization of the glycosphingolipid Lex–LacCer with MS/MS providing site-specific information of Ca^{2+} binding, and **(c)** the analysis of DNA using charge detection mass spectrometry [2]. Images reprinted with permission from [1] and [3], copyright 1991, 1993 American Chemical Society, and from [2], copyright 1998 Springer Publishing

These breakthroughs were compelling for both chemists and biologists, and for our lab they begged a bigger question: Can whole organisms be examined with mass spectrometry? By virtue of their homogeneity and relatively small size

(megadaltons), the logical organism to test to answer this question was a virus. An intact virus, or virion, consists of genetic material in the form of RNA or DNA surrounded by a protein shell known as a capsid and may be helical or icosahedral in nature. Some viruses are further encased by a lipid membrane called a viral envelope, which helps in evading the immune system. While advances in viral research have been largely associated with the development of physical techniques such as X-ray crystallography [11, 12] and electron microscopy [13, 14], the development of mass spectrometry to mass measure viral ions was recognized as having the potential to further facilitate their characterization. Another motivation for analyzing viruses was that while convincing evidence existed regarding the observation of noncovalent interactions with mass spectrometry [1–8], a common question was (and still is) whether native conformations are preserved throughout the vaporization, ionization, and mass analysis within the vacuum of the mass spectrometer [15]. A third question, reminiscent of the Manhattan Project where Calutron mass spectrometers were used to separate uranium isotopes [16], is whether this technology can be used as a viable separation and collection device for biomolecules. Our work on viruses attempted to address these issues with the analysis of the 40 MDa tobacco mosaic virus.

Instrumentation for Viral Analysis

The first breakthrough in accurately measuring the mass of a complete virus particle required the development of a novel detector capable of simultaneously measuring charge, m/z , and velocity of an ion. Dr. Henry Benner created this technology by modifying an ESI-TOF mass spectrometer with an additional metal flight tube attached to a charge-sensitive preamplifier [2, 17, 18]. The charge-sensitive preamplifier creates an image current for single ions as they pass through this device and have the added benefit of a signal with a magnitude proportional to the number of charges they are carrying. To demonstrate the capability of this charge detector design, an entire intact genome was initially analyzed [19]. This represented, at that time, the single largest molecule to be successfully mass analyzed. Charge detection mass analysis circumvents the problem of detecting large ions by making a simultaneous measurement of charge (z) and m/z ratio for individual ions; therefore it enables the direct measurement of mass (as opposed to m/z). Strictly speaking, one could say that this was the first true “mass spectrometer.”

The charge detector instrumentation was successfully used to measure the mass of two intact viruses: rice yellow mottle virus (RYMV) and tobacco mosaic virus (TMV) [18]. The signal from thousands of individual ions was averaged over 30 min (Figure 2a) with both RYMV and TMV possessing a charge distribution between 300 and 1000 positive charges resulting in measured masses between 6×10^6 and 7×10^6 Da for RYMV and between 39×10^6 and 42×10^6 Da for TMV. These masses agreed well with the calculated masses of 6.5×10^6 Da and 40×10^6 Da for RYMV and TMV,

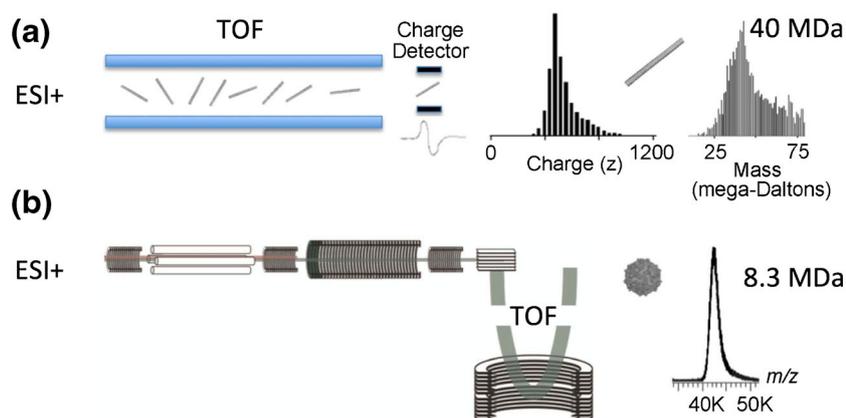


Figure 2. Intact non-enveloped tobacco mosaic and Norwalk viruses were electrosprayed into a time-of-flight (TOF) mass spectrometer with Benner's charge detection MS **(a)** and, more recently **(b)** a modified commercial TOF instrument

respectively. The error associated with these signals could be attributed to the charge detection signal amplification process and the system having a relatively short linear flight path, whereas the large tailing in the mass distribution is most likely the result of adduct formation and incomplete desolvation [10, 20, 21]. Previous studies have shown that desolvation becomes less efficient with increasing size of the biomolecules and a similar distribution was observed in Benner's original experiments on DNA [2]. More recent commercial instrumentation with traditional electron multiplier detectors have been especially successful at performing analyses of viruses and virus capsids. The addition of an inert countercurrent gas to time-of-flight (TOF) mass spectrometers, such as nitrogen [18] or xenon [22], to assist with droplet desolvation of large biomolecules, can produce charge-resolved spectra of masses up to approximately 5 MDa [22] (Figure 2b).

Although the initial observations of a single virus ion may seem trivial given today's mass spectrometry technology, back in the 1990s in the Lawrence Livermore lab, monitoring the data being generated by the instrument as each single ion passed through the detector was thrilling. At the time, this was a completely unique mass spectrometer at the forefront of sensitivity and mass measurement capability. These instrument alterations were highly influential in studying supramolecular living structures by mass spectrometry. Since the 1990s, even larger biomolecules have been analyzed using charge detection devices with improvements made by the Jarrold lab on the additional flight tube with optimized dimensions, alignment, and programming parameters. In this system, peaks can be rejected if they have baseline fluctuations, signals from more than one droplet, or when significant differences between the entrance and exit peaks are observed [23]. This improved setup results in a tighter distribution and detectable mass limits reaching 10^{14} Da (100 trillion daltons).

Another interesting offshoot of ESI-MS has been the coupling of ESI to ion mobility spectrometry (IMS) and differential mobility analysis, better known as gas-phase electrophoretic molecular mobility analyzers (GEMMA) [24]. These devices provide confident measure of

nanometer-sized biomolecules and noncovalent complexes with sub-nanometer resolution, and additional validation that these complexes survive the analysis process. IMS allows for the analysis of the cross-sectional area of an ion to be measured and has been widely applied to intact protein complexes [21, 25–29]. This technique has been used to detect intact bacteriophage MS2 at 24 ± 2 nm [30], and human rhinovirus with a diameter of 29.8 ± 0.3 nm [28]. Interestingly, since the measurements are based on the electrophoretic mobility (EM) measurements that are independent of known virus features, these results provide strong evidence that no large-scale disruption of tertiary or quaternary structure of the capsid occurred during ESI desolvation and ionization [31].

Although the ability to detect megadalton-sized particles is at the heart of investigating microbe behavior, it is not only size that matters. In native microbe analysis, the resolving power, sensitivity, and sampling rate are crucial for deciphering relevant and specific information about the intact particles and how they interact with modulators of function. Consequently, these aspects have been continually improved in both CDMS and GEMMA over the years. Today, even conventional mass spectrometers can be harnessed to obtain high-resolution mass spectra of megadalton-sized complexes. QTOFs are a particularly good choice for simple modifications to aid in the transmission of large ions. For example, analysis of an 18 MDa intact capsid from bacteriophage HK97 was performed on a QTOF with xenon collision gas at higher pressures and voltages. The instrument is capable of resolving masses up to 40 GDa, but the practical limitation lies within the ability to desolvate the ions, thus reducing the limit to ~ 20 MDa [32]. An Orbitrap mass analyzer has also been configured to accommodate the analysis of megadalton-sized capsids. With an additional gas flow to the HCD cell, optimized radio frequency (rf) voltages, and the removal of analogue filters, an upper mass limit of ~ 5 MDa was achieved, but restricted by the low pressures required in the C-trap [33]. The benefit of using a modified QTOF

or Orbitrap mass analyzer to measure megadalton particles is the capability of generating high-resolution spectra without the need for a completely different system.

Virus and Spore Viability

Beyond mass analysis, viruses represent an interesting analytical challenge and would make a good model system for testing the limits of ESI and its ability to examine noncovalent complexes. The primary goal of these first attempts to analyze an intact virus was to determine if viruses could be transferred into the gas phase intact, and at the same time perform mass selection [26]. The upper mass limit of our instrument was 2400 m/z , a limit that was overcome by operating the quadrupole mass analyzer in rf mode where only ions of high m/z were allowed to pass. The next step in these experiments was to determine whether the viral ions were intact and, finally, whether they were still alive and capable of their native biological function (i.e., infection). To accomplish this, we collected the viral ions from a glycerol-coated brass plate placed in front of the detector (Figure 3a). The separation and collection of ions within a mass spectrometer for purification was inspired by early Calutron mass spectrometers used to separate uranium isotopes [16]. The isolated virus sample was then directly analyzed by negative-stain transmission electron microscopy (TEM). Both RYMV and TMV (Figure 3a) particles remained intact based on the electron microscopy images, indicating that the native structure had been preserved. Even with the success of these experiments, it was clear that damage to the protein capsid or the packaged RNA could go undetected in the TEM images. Therefore, the viability of the collected virus following ESI mass spectrometry was tested to provide definitive evidence of whether the native state was retained. This experiment was conducted by inoculating tobacco cultivar Xanthi plants with TMV collected in the mass spectrometer. The tobacco plants developed lesions characteristic of

infection (Figure 3a), demonstrating that the viruses were viable. A similar viability experiment with RYMV was not successful, indicating it was more labile in its symmetrical icosahedral structure than TMV during mass spectrometry.

There have since been other reports of testing viability after the electrospray ionization of a virus. Hogan et al. used EM to analyze bacteriophage MS2, λ , T2, and T4 with a highly monodisperse electrospray source operated in cone jet mode to produce a near steady state diffusion charge distribution [34]. An airborne sampler was teed off from the electrospray source to collect and test the viability of the aerosolized virus particles. Bacteriophage MS2, a small, single-unit virus, was found to be viable, whereas the λ bacteriophage had low amounts of viability and the large, multi-subunit viruses T2 and T4 bacteriophages had no viability. The length of bacteriophages T2 and T4 exceeded the mean diameter of the droplets (171 nm), yet both T2 and T4 had mean diameters of 87.03 and 88.32 nm, respectively. It was surmised that the T2 and T4 bacteriophages were not the whole viruses, but only the protein capsid heads that remained intact through the electrospray process. Viruses with sizes exceeding the droplet diameter would be exposed to more mechanical and electrical stress, leaving them more susceptible to noncovalent bond disruption.

Another interesting study involving much larger bacterial cells was recently reported by Pratt and Austin [35]. Two different species, *E. coli* and *B. subtilis*, were electrosprayed, ensuring complete deagglomeration and desolvation, electrostatically deflected, and collected to assess whether individual cells in various charge states could survive. Bacterial particles with higher charge or lower mass were deflected more strongly as shown in Figure 3. This device, called a “Bug Trap,” consists of two parallel stainless steel plates with an applied electric field acting as a particle deflector, and a slotted collection plate to capture the charged particles. After electrospraying a bacteria sample, the collector channels were washed with

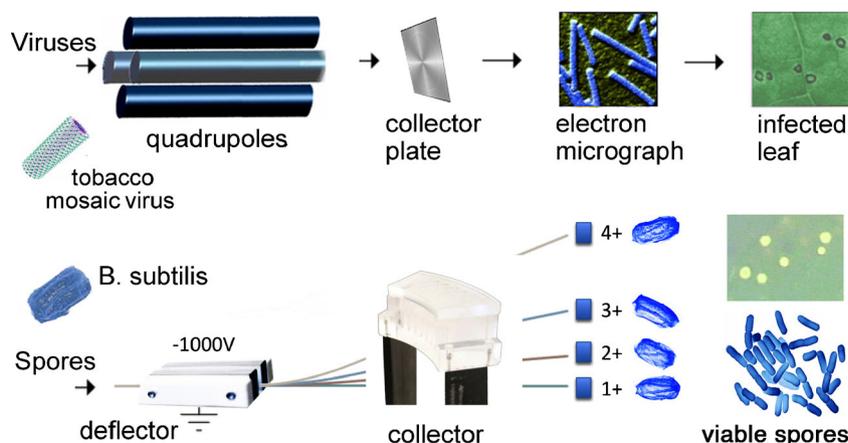


Figure 3. (Top) Intact viruses electrosprayed into the mass spectrometer, collected, detected with electron microscopy, and inducing an infection on a leaf. (Bottom) Intact spores are electrosprayed into a Bug Trap where they are electrostatically deflected and collected. Bug collector and *B. subtilis* images courtesy of Sarah Pratt

sterile water, transferred to petri dishes, and incubated overnight at 39 °C. The *E. coli* did not survive complete desolvation and charging under the applied electric field, whereas *B. subtilis* did survive. The major difference between these two strains is the ability for *B. subtilis* to form spores, whereas *E. coli* does not. This protective spore coat helped this bacterium survive the electrospray process in both atmospheric (~20% recovery) and vacuum (<0.1% recovery) conditions, albeit at low charge states. To keep microorganisms viable as they traverse the harsh conditions of electrospraying and acceleration through electric fields, the size of the microorganism with respect to the droplet diameter becomes important as does the strength of the noncovalent interactions maintaining the native structure. Reducing mechanical and electrical stress by controlling droplet size can be balanced with the ability to completely desolvate the particle to effectively separate it based on electrophoretic mobility and m/z , which is necessary to give accurate and well-resolved measurements. Taken together, these results show that the viability of the microorganism was strain-dependent, as determined by their size and structural stability.

Conclusion: Revolution to Evolution

The mass spectrometer has seen dramatic improvements in the past three decades and the revolution of ESI has generated an evolution in the entire mass spectrometer from front to back. ESI has clearly been the method of choice for examining noncovalent interactions in the gas phase, especially intact viruses, yet it is interesting that desorption/ionization techniques (e.g., MALDI) have not been used extensively, despite both of these methods also being relatively soft. This may be because analyzing macromolecular complexes by MALDI is confounded by the need for a denaturing sample preparation involving matrix application, laser optimization, and reduced detector sensitivity in the ultrahigh m/z range [24]. However, gentle ablation methods, such as DESI [36] or nanostructure-based desorption and ionization [37], coupled with CDMS or modified TOF-MS, may provide possible alternatives for soft desorption/ionization. For the ESI itself, nebulization techniques could also be improved to increase the viability of the microorganisms. As discussed above, previous studies observed that bacteria or viruses with a more spherical shape and smaller diameters were more likely to survive the journey through the MS. Further investigation on the size of nebulizer droplets, desolvation methods, and temperature/voltage during ion transfer would be useful in keeping larger microorganisms alive through the MS process.

Advances in analyzer technology have also greatly enhanced our ability to simultaneously and accurately analyze m/z and z of individual ions. With improved mass accuracy, limits of detection, and the ability to decipher charge states from heterogeneous mixtures, it has become possible to detect intermediates in the capsid assembly process (Figure 4) [39]

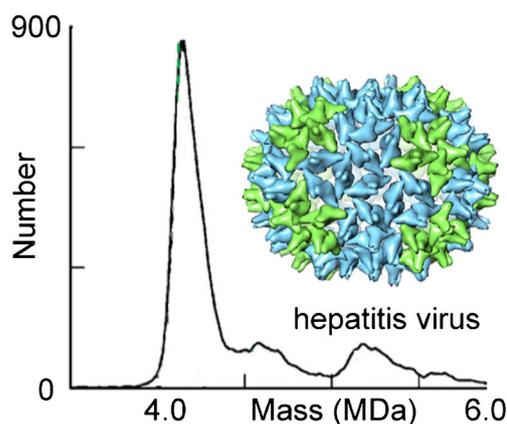
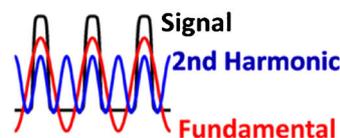
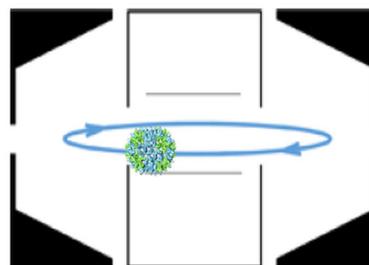


Figure 4. A “Dalek” Charge Detector designed to investigate heterogeneous organisms. Artist rendition of the Dalek Charge Detector courtesy of Martin Jarrold and reprinted with permission from [38] copyright 2015 American Chemical Society

and to examine how protein envelopes form on capsid surfaces [40]. Detector evolution has also played a significant role in the

ability to detect large megadalton species as limitations exist in both electron multiplier detectors and condensation particle counters (GEMMA). For example, the recently developed nanoelectromechanical mass sensors (NEMS) showed advantages over both of the currently developed detectors. This cantilever-based mass sensor operates at an ultra-high vibrational frequency, with the adsorption of analytes causing measurable alterations in the frequency that span a very large dynamic range [41]. In contrast to GEMMA, NEMS successfully avoids the drawback of charge neutralization, which makes most particles become uncharged and, therefore, not separated in the electric field, resulting in decreased sensitivity [34, 42]. Compared with CDMS, interpretation of NEMS spectra is simplified as there are no charge states associated with particles [39]. However, this technique suffers from relatively low mass resolution [41] compared with GEMMA and CDMS and requires experimental run times up to several hours [42]. Although this technique has great potential for analyzing supramolecular living structures, the NEMS-MS technology is still in its early stages of development.

Some would say that mass spectrometry is now a mature technology; however, a truly comprehensive understanding of a biological system requires the ability to examine all of its components: the genetic material, proteins, macromolecular structures, and small molecules. While there have been many advances in MS instrumentation for analyzing these systems, a complete solution remains tantalizingly out of reach. What are the next mass spectrometry technologies that will allow us to simultaneously observe genes, proteins, metabolites, and their interactions within the complexity that is a living biological entity? Perhaps we need to turn to our students for the next revolution.

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