Applications of Mass Spectrometry in Molecular Diagnostics

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***Abstract*— This non-exhaustive review is intended to introduce the reader to Mass Spectrometry (MS) and its application to the rapidly growing field of molecular diagnostics. In this review we will focus on applications of MALDI and ESI to the field of molecular diagnostics.**

I. Introduction to Mass Spectrometry

What is MS?

Mass spectrometry is method and set of instrumentation for sorting and detecting atoms or molecules on the basis of mass to charge ratio (m/z).[1] MS has been used extensively in chemistry from as early as the 1940s, later being adapted for biological and organic chemistry.[1] However, applications for complex biological molecules—such as nucleic acids, glycopeptides, etc–were prevented due fragmentation caused by the high energy bombardment needed to vaporise samples. This problem was overcome with the discovery of 'soft ionization' techniques with lower energy vaporisation in the late 1980s. Since then MS has become increasingly specialized for use in the biological and medical sciences.[1]

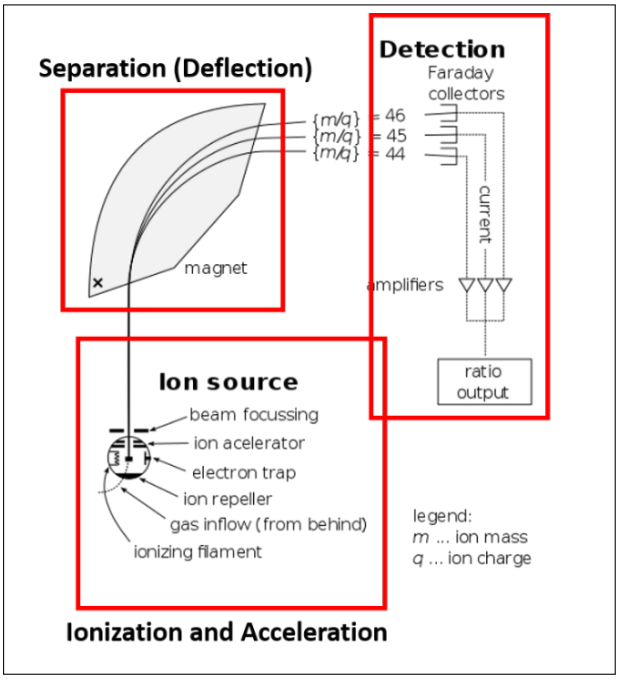

Fig. 1. Schematic of a Simple Mass Spectrometer[2]

Why is MS Relevant to Molecular Diagnostics?

Accuracy, high-throughput and multiplexing are becoming essential characteristics for all biomedical instrumentation.[1] As such MS is emerging as a powerful tool in proteomics, genomics, metabolomics and other fields of basic research. Moreover, advances in these more fields generate myriad new targets and pathways for medical intervention, diagnostic or otherwise. As information about the biological processes continues to increase, mass spectrometry has a significant role to play in both basic and applied research.

A. *MS Technical Background*[1]

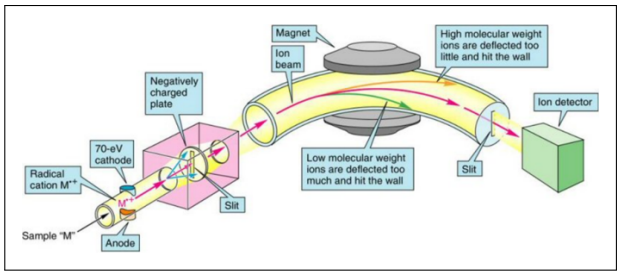

Fig. 2. Deflection Mass Spectrometer[1,2]

Mass Spectrometry is a method for sorting and detecting atoms or molecules based on the ratio of their mass to charge.The system outputs the mass to charge ratio (m/z) for each detected particle which is plotted against the intensity of each detection. The goal of mass spectrometry is to subject a sample, usually of unknown chemical composition, to five steps which will ultimately separate atoms by mass and charge. The sample is placed within a vacuum to isolate other particulates and gases, so the analysis will only run on the sample. The stages of mass spectrometry are outlined as follows:

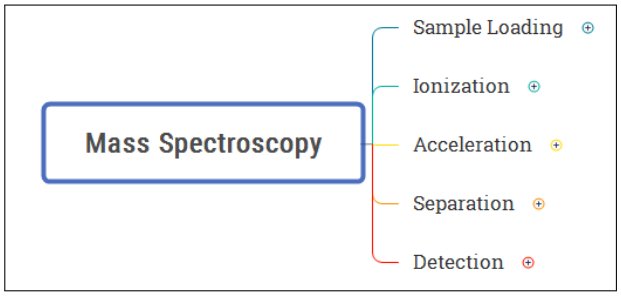

Fig. 3. Process of Mass Spectrometry

1) *Sample Loading*:

High Performance Liquid Chromatography (HPLC)

A method for physically separating components of a mixture by interaction of immiscible stationary and mobile phases.

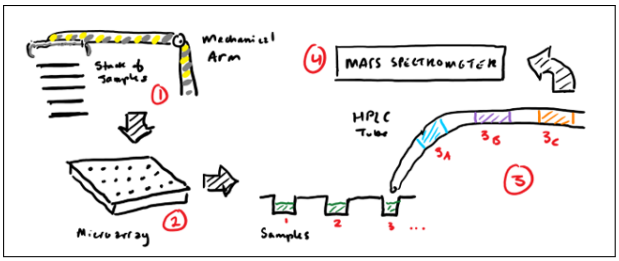

Fig. 4. HPLC System[2]

Automated Array Loading

Use micro-arrays, a mechanical arm, and a HPLC tube to yield automated, high-throughput, multiplexed sample analysis.

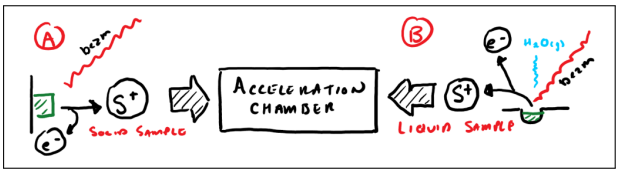

Fig. 5. Automated Array Loader

*2) **Ionization**:*

Traditional MS uses high-energy EM or particle beams to knock electrons off the sample. These high energy beams tend to fragment or destroy organic molecules, historically preventing biological applications of mS. Both solid and liquid samples can be ionized in this way. The result is a positively charged gas which passes into the vacuum of the acceleration chamber due to negative pressure.

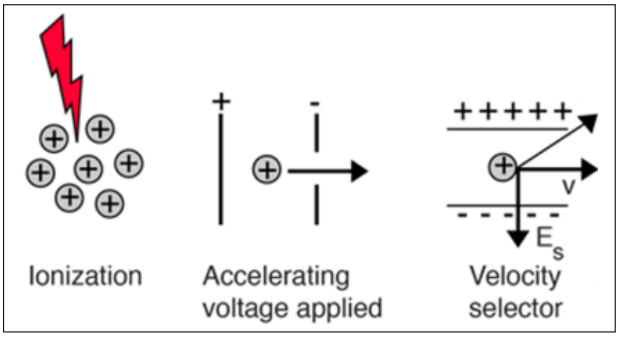

Fig. 6. Automated Array Loader

*3) **Acceleration**:*

The acceleration chamber is a vacuum thus vaporized sample ions enter the chamber due to negative pressure. A voltage is applied across two metal plates, accelerating the ions to the desired kinetic energy. A mass selector deflects ions of the wrong kinetic energy, allowing a stream of ions with identical KE to pass through a slit into the separator. Therefore, when the ions reach the mass analyzer the only properties differentiating them are their mass and charge.

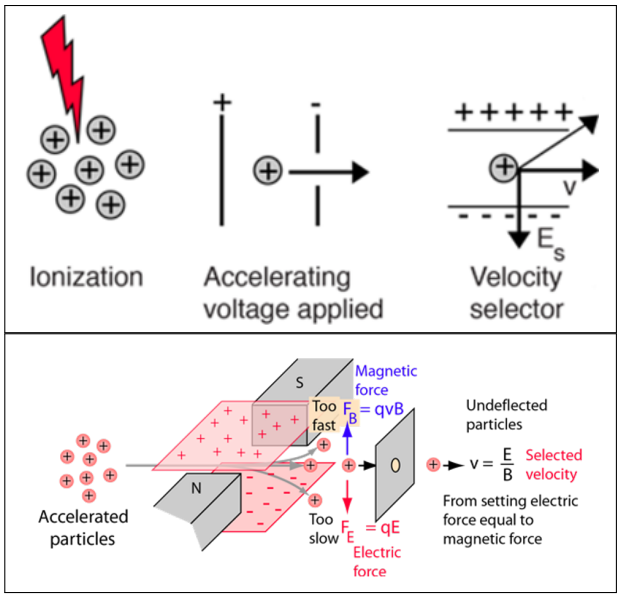

Fig. 7. Ionization (Top) and Mass Selector (Bottom)

*4) **Separation**:*

The method used to isolate the ions for detection based on mass/charge ratio (m/z). Traditionally used deflection but modern MS technology have alternatives depending on the requirement of the experiment.

Magnetic Deflection[1]

The ions pass through a magnetic field and are deflected with electromagnetic forces. Different types of ions can be identified and detected, since ions with different masses would have different deflection behaviors. Sample ions enter with equal kinetic energy. Ions with smaller masses or higher charges are deflected more. The detector outputs plots the m/z value for all detected particles.

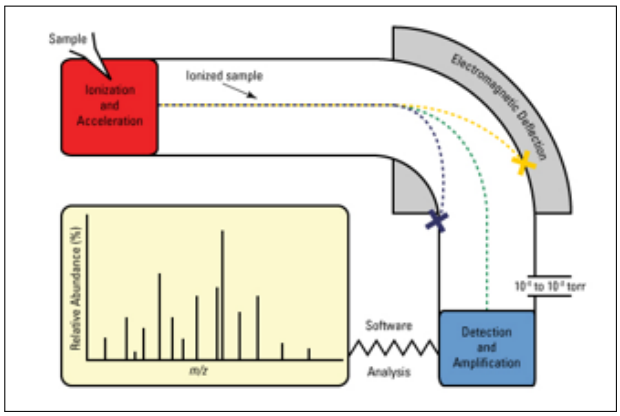

Fig. 8. A Deflection Based Mass Analyzer[2]

Time of Flight (TOF)

Ionized compounds with different mass and charge travel at speeds. Compounds can therefore be be identified by measuring the time they take to travel a known distance. Sample ions enter with equal kinetic energy. Therefore velocity is

proportional to charge and inversely proportional to mass. First particles detected are lightest, increasing proportional to time.

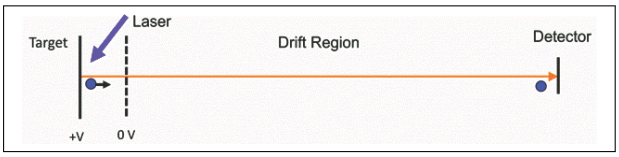

Fig. 9. A Time of Flight Based Mass Analyzer[2]

5) *Detection*[2]*:*

Records either the charge induced, current produced, or photons generated when ions pass by or collide with a surface. First generation mass spectrometers used photo-plates to make images of the detected samples. Modern systems detect voltage changes at a given location and send these to a computer to output a m/z plot. Typically, an electron multiplier is used, but Faraday cups and ion-to-photon detectors are also used depending on the MS design specifications.

Location Based

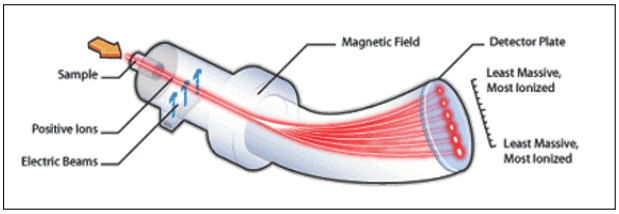

Fig. 10. Location Based MS Detector

Deflected ions hit the surface of an amplifier. The positions hit by these ions are detected, and graphed according to intensity and location. Using this information, quantity and mass of each ion in the sample can be determined, and the mass of the sample molecule can be calculated.

Time Based

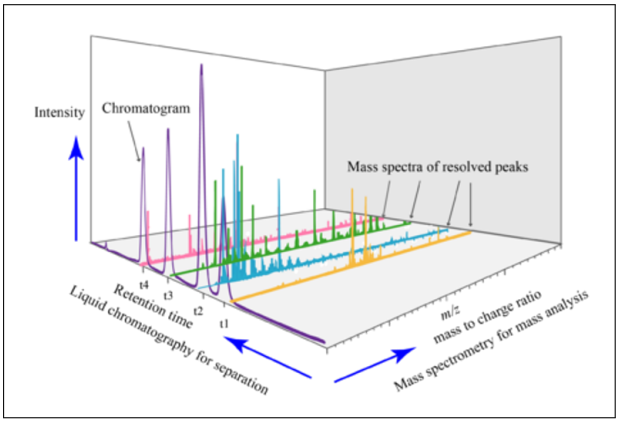

Fig. 11. Time Based MS Detector Output[2]

A detector measure voltage changes in a plate to high time resolution. This system can be multiplexed by time spacing incoming samples via liquid chromatography. The output of a multiplexed time of flight mass spectrometer is displayed in Fig 10.

B. *A Brief History of Mass Spectrometry*

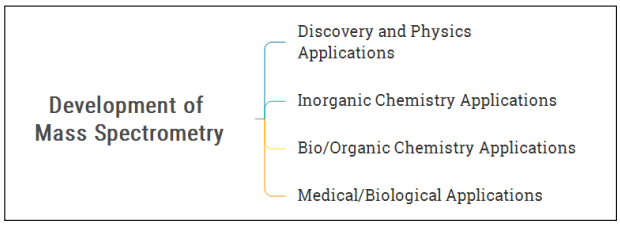

Fig. 12. History of MS Development

1) *Discovery and Physics Applications*[3]*:*

The physics necessary for MS was developed in the 1890s by Cambridge Professor J.J. Thomson. His work on cathode rays (electron beams) rewarded him with a Nobel Prize in 1906 for using EM deflection to estimate the mass of the electron! By the 1940s the technique was commonly used in physics laboratories, but were constructed by engineers on site and therefore inaccessible to other fields of study.

2) *Inorganic Chemistry Applications*[3]*:*

Electrical Engineer Alfred Nier commercialized and promoted MS use outside of the physics community. As a result, by the 1950s it became widely adopted in the field of industrial chemistry, most for quantifying the amount of each component in a mixture.

3) *Bio/Organic Chemistry Applications*[3]*:*

The work of American Chemist Fred McLafferty, Klaus Biemann, and Carl Djerassi characterized the mass spectra of known compounds. These spectra were arranged into a library of mass signatures which was used to identify unknown compounds in mixtures by matching them to known spectra! Eventually the technique advanced enough to predict the complex structures of organic compounds. By the 1980s small organic molecules were regularly being analyzed with MS.

4) *Medical/Biological Applications*[3]*:*

In 1988, novel soft ionization techniques—MALDI and ESI—were developed. These tools allow for ionization of large molecules without an excess amount of energy being deposited into the compounds. John Fenn received the Nobel Prize in Chemistry in 2002 for the development of ESI. During his acceptance speech he famously stated that his technique provided electrospray wings for molecular elephants! These techniques and their application in molecular diagnostics will be discussed in detail presently.

II. Biological Mass Spectrometry

Mass spectrometry has high accuracy, sensitivity and wide dynamic range that can be utilized in high-throughput capabilities. This has allowed MS to have countless applications

in life sciences.[4] Currently, MS has been successfully used for molecular diagnostics of microbial and viral infections.[1] Furthermore, new developments expand the application to public health evaluations and clinical fields. We will be discussing ESI and MALDI MS methods.

A recent study of a discovery of cancer biomarkers using tissue cultures took advantage of mass spectrometry-based proteomics. Discovery of biomarkers is essential in accurate diagnosis and proper monitoring of cancer patients, crucial for treatment and prevention. Almost all proteomic biomarker discovery platforms use mass spectrometry.[5] In this case, they used ESI-MS, and concluded that the capability of MS demonstrates significant potential as a promising tool for the discovery of candidate biomarkers.[5]

A. *Matrix Assisted Laser Desorption Ionization*

An ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation.[6] Ionization of the matrix allows samples to "piggy-back" into the vapor without absorbing too much of the energy applied. Thus the unstable bonds in complex biological molecules are retained, allowing analysis of compounds previously to fragile for study by MS.[6]

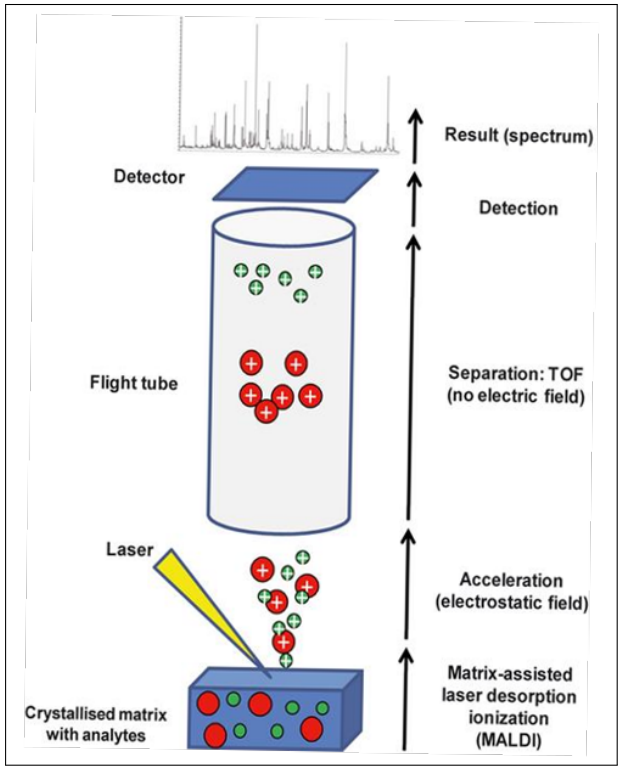

Fig. 13. Schematic of a MALDI-MS System

1) *Matrix Assisted Laser Desorption Ionization (MALDI):*

An ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. Samples are prepared by mixing with a matrix. The resulting mixture crystallizes sample within matrix, due to presence of small acid molecules. A laser irradiates the sample, triggering the release of fragment molecules from the mixture. The analyte molecules are ionized in an electromagnetic field and accelerated into the mass analyzer such that kinetic energies are all equal.

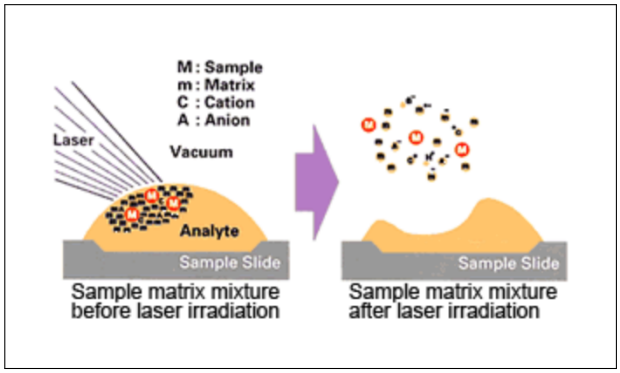

Fig. 14. Diagram of Ionizing Matrix

2) *Matrix Composition[4]:*

The key feature of this technique involves the use of a matrix to analyze samples. These matrices vary in composition and are tailored for the biomolecule to be analyzed as well as type of laser to be used. This allows for a comprehensive array of potential analytes to be tested, from intact microorganisms to large protein molecules.

Example matrices used most frequently include:

- 2,5-dihydroxbenzoic acid (DHB)
- -cyano-4-hydroxycinnamic acid (CHCA)
- Sinapinic acid (SA)
- Ferulic acid (FA)

Considerations when choosing the matrix molecule:

1) Size: Low molecular weight to allow easy vaporization but large enough not to evaporate during sample preparation.
2) Acidity: Acidic molecules act as a source of proton to encourage ionization of the analyte.
3) Absorption Range: High optical absorption range in either the UV or IR range to efficiently absorb laser irradiation. This can be achieved by molecules with conjugated double bonds.
4) Presence of polar groups: Molecules with polar groups is functional aqueous solutions.

The mixture of the organic solvent and water allows both hydrophobic and hydrophilic molecules to dissolve into the solution. CHCA, FA, SA show to be effective for detection of proteins. DHB is effective at detection of glycopeptides.

B. *MALDI Applications in Molecular Diagnostics*

1) *Necrotizing Enterocolitis[7]:*

NEC is a devastating disease that affects the bowels of premature infants. MALDI/TOF is used to quickly analyze fecal samples and find differences between the mutant and the functional protein responsible for NEC. It can be used

as a means of pre-symptomatic diagnosis which is needed to prevent the devastating inflammatory bowel disease of premature infants. Fecal samples were cultured, and isolates identified using MALDI-TOF, bio-markers identified with this technique assist in the development of a screening tool to allow for early diagnosis. The uniqueness of MALDI-TOF allowed for the fecal samples that normally would have been unavailable in traditional mass spectrometry due to the nature of the sample.

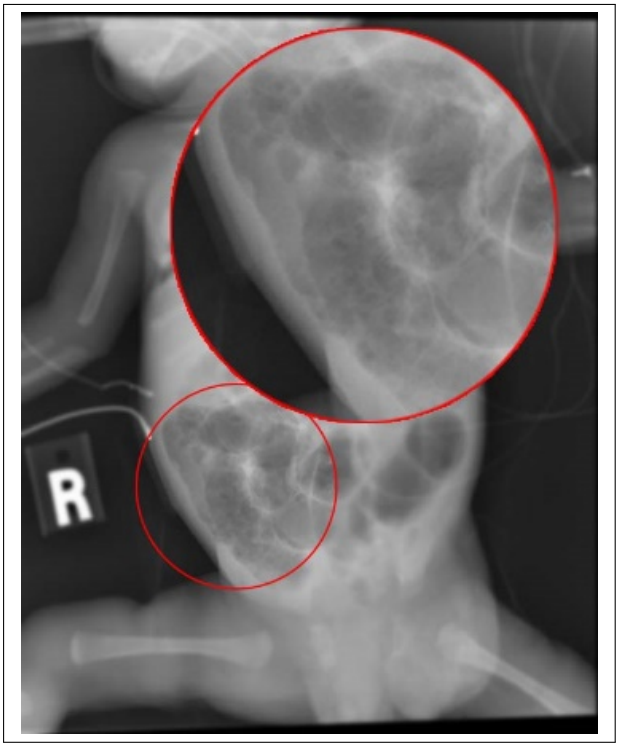

Fig. 15. Mechanism of Electrospray Ionization[2]

*2) **Pancreatic Cancer**[8,9]:*

Membrane proteins were identified as potential targets for anticancer drugs as well as bio-markers for early diagnosis. The study used MALDI-TOF mass spectrometry to analyze isolate and identify membrane proteins in pancreatic cancer cells. This allowed them to identify various proteins, which were associated with various cellular processes regulated by pancreatic cancer cells. Demonstrates the capabilities of identifying large protein molecules from samples to help in diagnosing conditions and diseases through recognizable bio-markers.

*3) **Identifying Drug Resistance in Bacteria**[10]:*

MALDI/TOF serves as a method for determining the drug resistance of bacteria, especially to -lactams (Penicillin family). It detects the presence of carbapenemases, which indicates drug resistance to standard antibiotics. In non-resistant bacteria, you would not expect to find such compounds as they facilitate cell death.

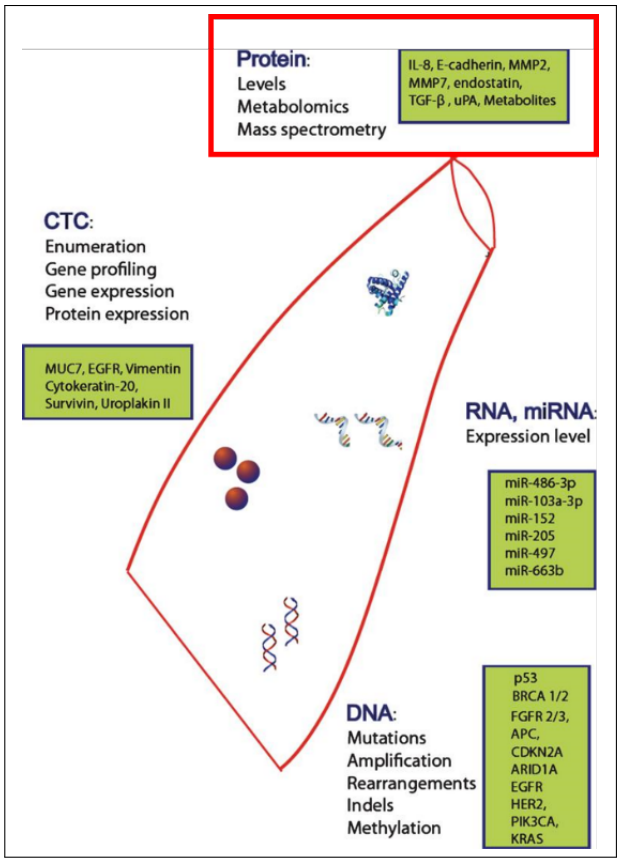

Fig. 16. Diagram of Biomarkers for Bladder Cancer[5]

C. Electron Ionization Mass Spectrometry (ESI-LS)

ESI A technique often used for the analysis of thermally fragile and high molecular weight polymers. A solution of the analyte is pumped through a high-voltage capillary, and the ejected droplet is transferred into the gaseous ion phase before passing through a mass spectrometric analyzer.[11] The technique is known to be the softest" ionization method (very little fragmentation) as it ionizes very fragile molecules and analyzes biological samples with non-covalent interactions.

Today, applications of soft ionization MS in biology are increasingly common with uses including classification and identification of bacteria; DNA analysis, screening and diagnostics research; multiplex genotyping; sequencing; genomics research; hospital infection control and quality testing.[4]

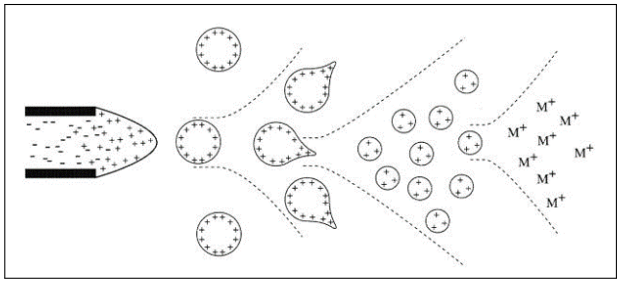

Fig. 17. Mechanism of Electrospray Ionization

A technique often used for the analysis of thermally fragile and high molecular weight macromolecules. A soft ionization technique –¿ very little fragmentation. First reported by Masamichi Yamashita and John Fenn in 1984 Later rewarded the Nobel Prize in Chemistry in 2002.

The Electrospray Ionization Mass Spectrometry (ESI-MS) Process

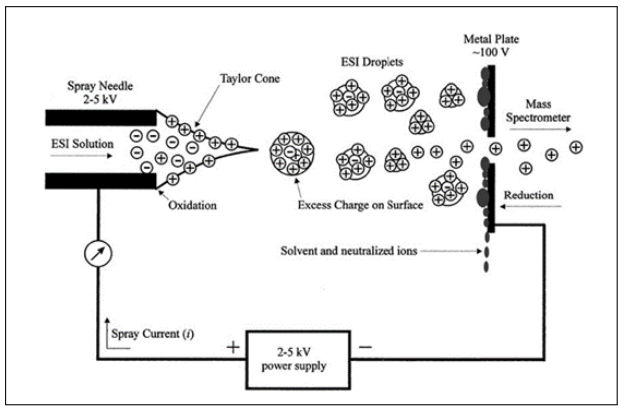

Fig. 18. **Schematic Diagram of Electrospray Ionization Process**[11]

*1) **Analyte is dissolved in volatile organic solvent (e.g. methanol)**:*

*2) **Analyte is injected into the capillary needle**:*

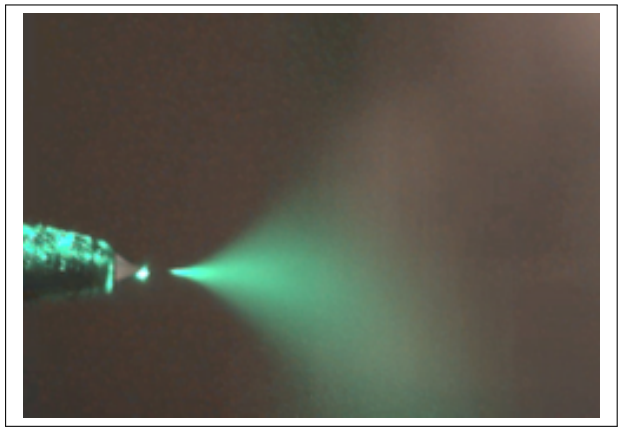

Fig. 19. Electrospray Plume Illuminated with A Green Laser[11]

High voltage(e.g. 2.5-6.0kV) is applied –¿ aerosols of charged droplets

*3) **Charged droplets pass down a pressure gradient and potential gradient toward the analyzer**:*

*4) **Charged droplets are continuously reduced in size with the aid of an elevated ESI-source temperature or stream of nitrogen drying gas**:*

Increase of surface charge density Decrease of the droplet radius

D. ESI-MS Applications in Molecular Diagnostics

*1) **Screening for Inborn Errors of Metabolism (ISM)**:*

Fig. 20. Newborn Blood Spot Test

Provide early diagnosis and management of IEM disorders. E.g. Phenylketonuria (PKU)An IEM that results in decreased metabolism of the amino acid phenylalanine –¿ can lead to permanent intellectual disability. In the past, PKU screening required a sufficient accumulation of phenylalanine of the infants blood sample (1 week) for testing Now, PKU screening can be performed using Day 1 blood spots –¿ Utilizing ESI-MS measurement of phenylalanine to tyrosine concentration ratio

*2) **Identification and Quantification of Haemoglobin Variants**[11]:*

ESI-MS is revolutionary in identifying large bio-molecules and proteins in biochemical research.

III. Benefits and Limitations of MS in Molecular Diagnostics

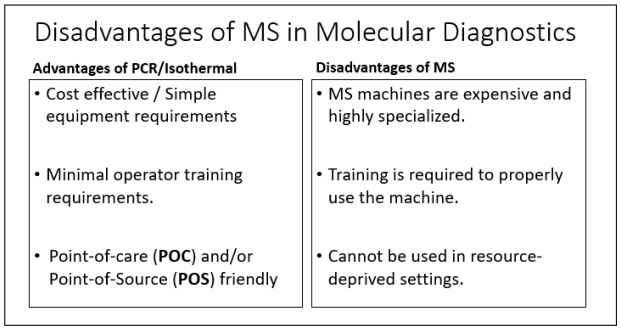

Fig. 21. Comparing MS with PCR and Isothermal for Molecular Diagnostic Applications

A. Benefits[4]

- Rapid detection: The detection process takes only milliseconds to seconds.
- Sample preparation process can be easily automated.
- Extremely high sensitivity: Sub-picomolar amounts of the analyte can be detected without fluorescence or radioactive isotope labeling or using antibodies or hybridization probes.

- MS not only surpasses electrophoresis in rapidity and sensitivity as a detection system, but allows for precise sizing of the molecule of interest and also provides information on nucleotide composition and charge.
- Contaminated samples, including fecal samples (used to study NEC) and blood samples (used to discover biomarkers) can be easily processed, unlike PCR-based diagnostics tools where DNA polymerases are often subjected to inhibition by hemoglobin or other contaminants in fecal samples.

B. *Limitations*

- MS machines are expensive and highly specialized.
- Training is required to properly use the machine.
- Cannot be used in resource-deprived settings.
- Not able to distinguish structural isomers of a compound.
- Some sample compounds are incompatible with the technology. For example, the ESI ionization technique does not work well with non-polar compounds

IV. Conclusion

MS is a highly sensitive molecular detection technology. It was initially regarded as an unsuitable technology for molecular diagnostics, but the development of MALDI and ESI has allowed MS to become an effective diagnostic tool. Despite the disadvantages, the need for the rapid, highly sensitive and easily automated molecular detection methods will remain high. Regardless of the field where it is applied, MS is a highly sensitive molecular detection technology. It can be leveraged to improve molecular diagnostics for a number of diseases including NEC.

The development of softer ionization techniques, namely MALDI and ESI has allowed MS to overcome the challenges it has faced when being applied to molecular diagnostics. Molecular diagnostics with MS have a number of advantages over the PCR-based diagnostics techniques which are more widely used in the field. Molecular diagnostics with MS also pose disadvantages, especially in terms of logistics.

Nevertheless, the application of MS in molecular diagnostics should not be disregarded, as the throughput capacity of molecular and biochemical analyses will continue to increase, and the need for the rapid, highly sensitive and easily automated molecular detection methodology will remain high.

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1,3,2,5,12,10

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