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Review

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in clinical chemistry

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-Tof-MS) has recently become a popular and versatile method to analyze macromolecules from biological origin. In this paper, we will review the application of MALDI-Tof-MS in clinical chemistry and biology. MALDI-Tof-MS is used in clinical chemistry, e.g. disease markers can be identified with MALDI-MS analysis in combination with 1-D and 2-D gel electrophoresis separations thanks to either peptide mass fingerprinting (PMF) or peptide sequence tag (PST) followed by data base searching. In microbiology, MALDI-Tof-MS is employed to analyze specific peptides or proteins directly desorbed from intact viruses, bacteria and spores. The capability to register biomarker ions in a broad *m*/*z* range, which are unique and representative for individual microorganisms, forms the basis of taxonomic identification of bacteria by MALDI-Tof-MS. Moreover, this technique can be applied to study either the resistance of bacteria to antibiotics or the antimicrobial compounds secreted by other bacterial species. More recently, the method was also successfully applied to DNA sequencing (genotyping) as well as screening for mutations. High-throughput genotyping of single-nucleotide polymorphisms has the potential to become a routine method for both laboratory and clinical applications. Moreover, posttranscriptional modifications of RNA can be analyzed by MALDI using nucleotide-specific RNAses combined with further fragmentation by post source decay (PSD).

Keywords: MALDI-MS; Proteins; Markers; Microbiology; Genotyping

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-Tof-MS) has advanced from early-stage research towards applications of real clinical relevance only within the last 10 years. MALDI-Tof-MS has become a popular and versatile method to analyze a range of macromolecules from biological origin from cells to tissues. Its ability to desorb high-molecular-weight

Abbreviations: MALDI-Tof-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PMF, peptide mass fingerprinting; PST, peptide sequence tag; TFA, trifluoroacetic acid; 2-D PAGE, 2-dimensional polyacrylamide gel electrophoresis; ICAT, isotope-coded affinity tag; ICMS, intact cell mass spectrometry; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

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thermolabile molecules, its high accuracy and sensitivity, combined with its wide mass range (1–300 kDa), make MALDI-Tof-MS a promising method for the clinical chemistry laboratory for the identification of biomolecules in complex samples, including peptides, proteins, oligosaccharides and oligonucleotides.

The first reports demonstrating successful MALDI-Tof-MS biochemical analysis were published in the late 1980s from the labs of Tanaka et al. [1] and Karas and Hillenkamp [2]. Instruments have subsequently become commercially available since 1991 [3]. This implies that the general utilization of MALDI-Tof-MS has emerged as an effective analytical tool to study biomolecules only within the last 15 years. Although relatively young compared to other analytical techniques using mass spectrometry, there has been an enormous increase in the publication of MALDI-Tof-MS methods and applications in the literature [4–6]. This has occurred for two principal reasons: (1) the growth of proteomics as a discipline, which uses MALDI-Tof-MS as a tool for post-separation protein identification, and (2) the promising potential of this analytical platform as a robust tool for disease biomarkers. The present paper will focus mainly on the latter class of applications.

2. Principles of operation

The general principle of MALDI-Tof-MS revolves around the rapid photo-volatilization of a sample embedded in a UV-absorbing matrix followed by time-of-flight mass spectrum analysis (Fig. 1). The origin of ions in the MALDI process is still under discussion. Several chemical and physical pathways have been suggested including gas-phase photoionization, ion-molecule reactions, disproportionation, excited-state proton transfer, energy pooling, thermal ionization, and desorption of preformed ions [7].

The choice of the matrix is crucial for success in MALDI experiments. Table 1 summarizes the most common MALDI-MS matrices used. Knowledge of ion formation pathways can contribute to rational matrix selection as difficulties with MALDI analyses frequently arise from non-incorporation of analytes into the solid matrix and nonformation of suitable crystallites. Derivatives of benzoic acid, cinnamic acid and other related aromatic compounds are generally acknowledged as good MALDI matrices for proteins [8] and may also be found suitable for oligosaccharides [9]. Noncovalent complexes between antibiotics and enzymes have been characterized using 3-hydroxypicolinic acid [10]. Of critical importance is the

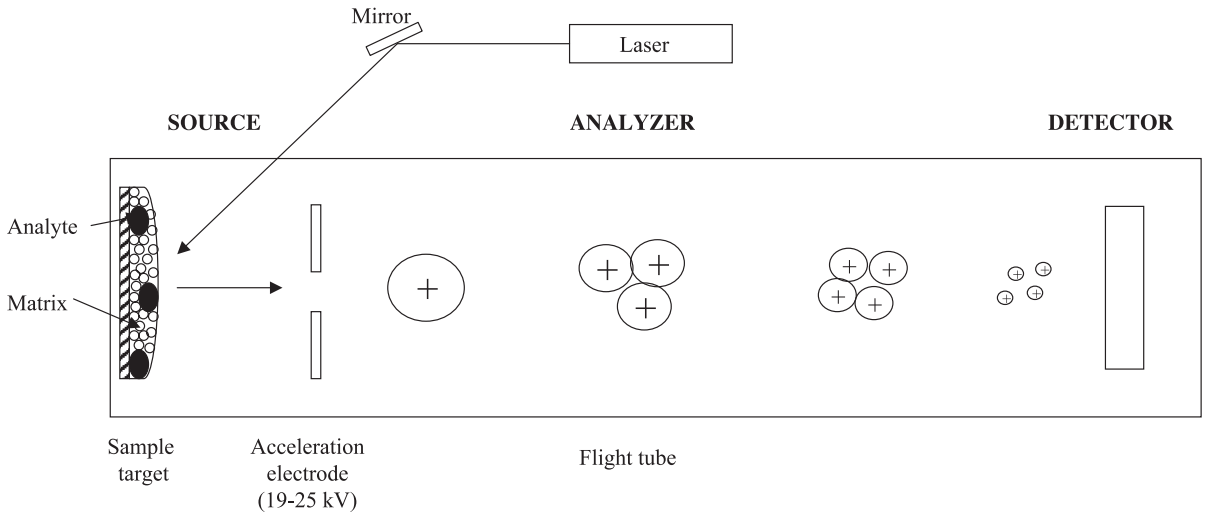
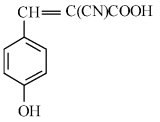
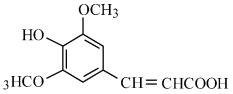
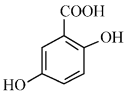


Fig. 1. Principle of matrix-assisted laser desorption/ionization mass spectrometry. The analyte mixed with a saturated matrix solution forms crystals. The irradiation of this mixture by the laser induces the ionization of the matrix, desorption, transfer of protons from photo-excited matrix to analyte to form a protonated molecule.

Table 1
Common matrices used in MALDI mass spectrometry

Matrix	Structure	Molecules
CHCA α-Cyano-4-hydroxycinnamic acid		Peptides
Sinapinic acid (SA) 3,5-Dimethoxy-4-hydroxycinnamic acidic acid		Proteins, polymers
Gentisic acid (DHB) 2,5-Dihydroxybenzoic acid		Peptides, proteins, lipides nucleic acid, saccharides
3-Hydroxypicolinic acid (HPA) 3-Hydroxy-2-pyridinecarboxylic acid		Nucleic acid, DNA

sample–matrix preparation. Although many protocols exist, the sample is usually embedded in an excess of a solid matrix, which, upon laser irradiation, assists in the volatilization and ionization of the analytes. The high excess of matrix to sample (from 100:1 to 10,000:1) is important, since the matrix serves as the primary (and highly efficient) absorber of the UV laser radiation and breaks down rapidly, expanding into the gas phase. Additionally, the high matrix/sample ratio reduces associations between analyte molecules, and provides protonated and free-radical products that ionize the molecules of interest. As the analyte itself does not absorb the laser energy directly, the method is considered a "soft" ionization technique, allowing the analysis of complex biomolecules up to several hundred kilodaltons. Typically, peptides or proteins are generally solubilized in a 0.1% aqueous trifluoroacetic acid (TFA). One microliter of this solution is mixed (usually directly on the probe tip) with a saturated aqueous solution of matrix and the solvent allowed to evaporate to form crystals.

After MALDI, the desorbed ions are mainly analyzed using a time-of-flight mass spectrometer (Tof-MS) working in the linear or reflectron mode. Early designs of Tof-MS instruments were published in the 1950s by Wiley and Mc Laren (cited in Ref. [11]) but the technology was not competitive with quadrupole or ion trap instruments, especially in terms of sensitivity and resolution. The recent surge in the use of Tof-MS has come on about following developments in bandwidth and sampling rate of the high-speed electronic recorders used to acquire Tof mass spectra. It has been these supporting technologies that have allowed Tof-MS to demonstrate the aforementioned analytical performance characteristics. It is of note that modern Tof-MS are relatively simple and inexpensive, exhibit high sensitivity, and have virtually an unlimited mass range, thereby making Tof-MS well suited to clinical and biomedical analyses when coupled with MALDI. Currently, Tof analyzers are largely used in these settings, either as a standalone instrument (MALDI-Tof-MS), coupled to a quadrupole (Qq-Tof) [12] or an ion trap (IT-Tof-MS) [13]. Recently, a Tof–Tof tandem mass spectrometer [14] has also became commercially available.

3. MALDI-Tof-MS for protein identification

MALDI-Tof-MS identification of proteins is carried out by the so-called peptide mass mapping or peptide mass fingerprinting technique. This highly effective approach of protein identification is based on the accurate mass measurement of a group of peptides derived from a protein by sequence-specific proteolysis. After proteolysis with a specific protease (e.g. trypsin), proteins of different amino acid sequence produce a series of peptides masses, which can be detected by MALDI-Tof-MS. The spectrum of identified peptide masses is unique for a specific

protein and is known as a mass fingerprint. Searching the selected masses from the fingerprint against databases of known protein sequences (e.g. SwissProt-TrEMBL) enables the identification of most proteins. An example is presented in Fig. 2 with the analysis of cytochrome *C* peptides tryptic digest by MALDI-Tof-MS. The development of large biological databases based on sequence identification has been the major driver for the application of MALDI-Tof-MS and proteomics in general.

Many proteomic data have been obtained using a combination of two-dimensional polyacrylamide gel (2-D PAGE) with MALDI-Tof-MS. In high-throughput proteomics, this process is automated using robotics to process 2-D PAGE spots (detection, cutting, proteolysis, extraction and cleanup of the released peptides). Another approach was recently described [15,16] in which the proteins are in-gel digested and then transferred by electroblotting onto a collecting polyvinylidene fluoride membrane sprayed with the matrix solution. The co-crystallization of the matrix and the peptides allowed direct MALDI-Tof-MS analysis.

4. MALDI-Tof-MS for protein quantification

Two strategies are used today to get quantitative information after MALDI-Tof-MS analysis. The first one uses the classical internal standard approach in which a known amount of an isotopically labeled analogue of the protein of interest is added to the sample. Rose and et al. showed that semi-synthetically engineered deuterated analogs can be used as internal standard to quantify insulin, proinsulin and peptide C [17–19].

The second strategy aims at determining the level of proteins expressed in a certain situation by comparison with a control condition. Several approaches have been

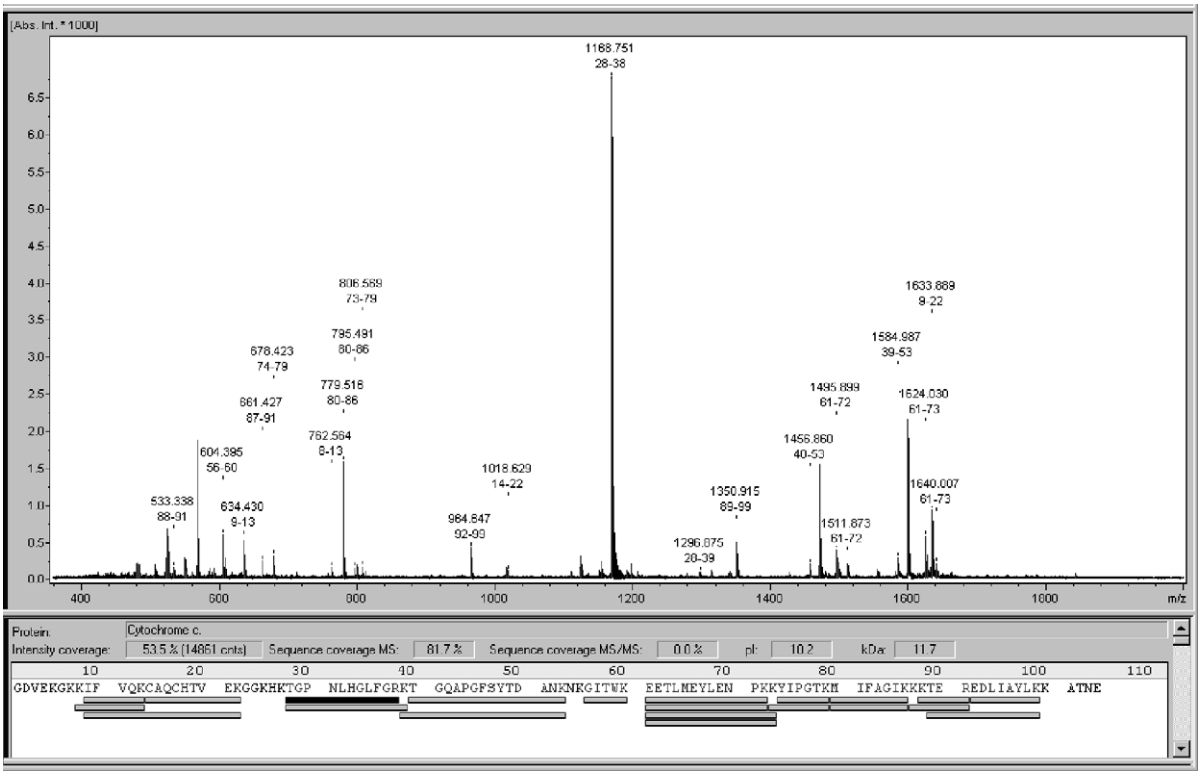


Fig. 2. Analysis of cytochrome *C* peptides digest (850 fmol/μl) by MALDI-Tof-MS. In the mass spectrum, each ion has been labeled by both the monoisotopic mass and the amino-acid position in the protein sequence. The bottom of the figure depicts the protein sequence coverage after database searching.

developed over the past years. Ji et al. [20] described a protocol in which primary amino groups in peptides from control and experimental samples were derivatized with acetate and trideuteroacetate, respectively. After mixing, the relative concentration of labeled and unlabeled peptides was determined by isotope ratio analysis with MALDI-Tof-MS and ESI mass spectrometry. Both the accuracy and dynamic range of ESI were found to be superior to that of MALDI but the latter was able to accommodate more complex samples, i.e. less sample cleanup was required.

Another approach called isotope-coded affinity tag (ICAT) was described by Aebersold et al. and gained popularity among the proteomic community [21–23]. It uses differently isotopically labeled tags that enable to selectively label two different cellular states (treated versus control) and which enable selective isolation of the labeled peptides by affinity chromatography of biotin tags. Then, the relative quantification is determined by the ratio of the labeled and unlabeled peptides.

5. MALDI-Tof-MS for identification of protein disease markers

MALDI-Tof-MS has been developed for use in clinical chemistry as a primary investigative tool to characterize a number of cancer, Alzheimer, arthritis, and allergy protein markers of disease or susceptibility to disease. Applications in cancer and in particular colorectal cancer have led the way in demonstrating clinical usefulness of MALDI-Tof-MS, with many other applications now in research and development.

5.1. Cancer

Cancer is today one of the major human diseases. The genomic and proteomic profiles of cancer cells are enormously complex. Tumor markers are widely used for screening, diagnosis, staging, prognosis, monitoring response to treatment, and detection of recurrent disease. 2-D gel electrophoresis and high-throughput mass spectrometry (proteomics) allowed to discover serological tumor markers due to the MALDI-Tof-MS sensitivity and specificity [24]. Technologies like protein profiling of tissues by MALDI-Tof-MS brought new and powerful capabilities in providing unique protein patterns highly specific to a given tissue type. MALDI-Tof-MS is useful to identify and characterize the primary structural features of serum peptide profiles. Valerio et al. [25] used this methodology to analyze sera from patients with pancreatic diseases (pancreatic cancer and chronic pancreatitis). They identified the presence of some disease-related peptides and other normal-subject-related fragments.

Several studies have been undertaken to further characterize and identify colorectal cancer protein markers using MALDI-Tof-MS as a primary investigative tool. The abundance of proteins in epithelial cells from normal colon specimens and colonic polyps was studied to detect possible differences in expression. 2-D gel electrophoresis, immunostaining, microsequencing and MALDI-Tof-MS were used to identify these proteins [26]. The protein expressions of normal and cancerous mouse colon tissue were compared. Moreover, the protein extract obtained from the tumors was fractionated by HPLC and each fraction analyzed by MALDI-Tof-MS. Three tumor-specific protein markers were identified [27]. Other tumor-specific calcium-binding proteins were identified by MALDI-Tof-MS. The expression of these proteins, S100A9 and S100A8, were investigated in 23 matched sets of colorectal carcinoma and normal colon mucosa by 2-D gel electrophoresis. The S100A8 protein is produced in the same location as S100A9, which can lead to calprotectin formation and possible apoptopic regression of tumor mass [28,29].

5.2. Alzheimer disease (AD)

When analyzing biological samples as cerebrospinal fluid (CSF), it is desirable to avoid excessive purification steps, since a loss of material is unavoidable at each step of the sample cleanup. One feature of MALDI-Tof-MS is its ability to detect biomolecules in complex mixtures in the presence of large molar excesses of salts and buffers. For all these reasons, MALDI-Tof-MS is a high-performance tool able to perform rapid screening of chemically altered proteins in small volumes of body fluids as CSF [30,31]. Several proteins linked to AD were found at femtomolar concentrations in CSF. The identification of high and low concentrated proteins composing CSF has been performed using 2-D gel electrophoresis separation and mass spectrometry techniques as MALDI to

obtain a rapid fingerprint [32]. The comparison of 2-D gels and protein identification is helpful to analyze the standard protein variability in CSF of healthy persons and detect specific protein variations of patients with various neurological diseases such as AD. Moreover, the processing of the neuropeptides Y, galanin and somatostatin in the cerebrospinal fluid of patients with AD and frontotemporal dementia was studied using a combination of radioimmunoassay and MALDI-MS techniques [33,34].

5.3. Rheumatoid arthritis (RA)

Rheumatoid arthritis is a chronic inflammatory disease of the synovial joints [35,36]. RA has long been thought to be an autoimmune disease. Indeed various autoantigens have been shown to be the targets of T cells and autoantibodies are known to be present in patients' synovial fluid and/or sera. Although the autoimmune nature of RA remains controversial, these autoantibodies have received much attention because their presence might constitute a marker of the disease [37]. These autoantibodies have potential diagnostic and prognostic value for rheumatoid arthritis. The presence of autoantibodies to α-enolase, an enzyme of the glycolytic pathway, was identified in the sera of patients with early rheumatoid arthritis using 1-D, 2-D Western blotting and MALDI-Tof-MS analysis [38]. Edman degradation and MALDI-Tof-MS led to the characterization of the autoantigen p68 which has been defined as a major target for self-reactive antibodies and T cells in RA [39,40] Identification of novel autoantigen specificities can also be made with classical proteomics approach [38,41–44].

5.4. Allergy

Some of the most important allergens are coming from food from animal and plant origin. This implies a need to monitor potential allergens before, during and after food processing [45,46]. MALDI-Tof-MS is well suited to this task, having been previously used to characterize allergens; for example, in cow's milk [47]. Indeed, cow's milk protein allergy is an increasing problem for infants and results from an abnormal immunological reaction to cow's milk protein. Allergen products for allergy diagnosis and treatment are based on heterogeneous allergen extracts, which include a mixture of allergenic and nonallergenic proteins. Many efforts have been devoted in the last years to characterize the composition of these extremely complex allergen extracts. Clinical studies indicated that Bird's Nest (edible nests of *Collocalia* spp., Chinese delicacy) is the most common cause of food-induced anaphylaxis in children, which could lead to potentially life-threatening allergenic reactions. 2-D gel electrophoresis, immunochemistry, N-terminal sequencing and mass spectrometry (MALDI and nano-ESI) allowed to identify a serine protease inhibitor, the major allergen in Bird's Nest [48].

6. MALDI-Tof-MS in microbiology

MALDI-Tof-MS is employed in microbiology to analyze specific peptides or proteins directly desorbed from intact viruses, bacteria and spores. The ability of monitoring ions over a broad *m*/*z* range, which are unique and representative for individual microorganisms and which correspond to biomarker constituents, forms the basis of taxonomic identification of bacteria by MALDI-Tof-MS with clinical relevance for the identification of infectious diseases. In addition, MALDI-Tof-MS can be applied to study either the resistance of bacteria to antibiotics or the antimicrobial compounds secreted by other bacterial species. This approach offers further aid to the clinician for treating patients according to their individual microflora population and status of resistance.

6.1. Basic research

A number of different procedures have been developed in recent years that use mass spectrometry for the direct determination of protein in a complex mixture of biological origin. In particular, the application of MALDI mass spectrometry permits to obtain biomarker profiles directly from unfractionated microorganisms as viruses, bacteria and fungus cells and spores. This approach enables to detect, characterize and identify peptides and proteins from intact microorganisms and is applied in biotechnology, cell biology and pharmaceutical research [49]. As biotechnology and microbiology examples, protein expression profiles from bacterial and eukaryotic cells and cell-free extracts could be rapidly obtained by MALDI-Tof-

MS analysis [50]. The identification by MALDI-Tof-MS of intact Gram-negative and -positive microorganisms, taken directly from culture, provides a unique mass spectral fingerprint of the microorganism cell wall [51]. However, reproducible sample preparation and mass spectra are difficult to achieve in experiments that involve direct cell ionization by MALDI and large variations can be found. Indeed, the sample preparation strategy adopted in MALDI-Tof-MS of whole bacteria cells influences the detection of signals with different masses. Dried droplet method permits to detect high-mass signals (>20 kDa) compared to the bottom layer method which detects lower mass signals [52]. Saenz et al. used MALDI-Tof-MS to demonstrate the reproducibility of bacterial spectra collected on different days in order to detect specific biomarkers. This work demonstrated the validity of the direct bacteria analysis by MALDI-Tof-MS in obtaining consistent spectra from bacteria over a period of time [53].

Moreover, the development of MALDI-Tof-MS methods for the characterization of bacteria permits the analysis of bacterial RNA and DNA, the detection of recombinant proteins, the characterization of targeted or unknown proteins, the bacterial proteomics and the detection of virulence markers [54]. Analysis of the proteomes of virulent microorganisms is a demanding task for the elucidation of virulence factors, antigens and vaccines, all-important for diagnosis and therapy. An example, the detection and characterization of disease-associated proteins (tumor-associated proteins, dilated cardiomyopathy-associated proteins), has been performed by comparison of 2-D gel electrophoresis patterns of patients with and without infectious diseases followed by MALDI-Tof-MS analysis [55].

6.2. Application

The genus *Brucella* consists of bacterial pathogens that cause Brucellosis, which is a major infectious disease affecting humans by neurological disorders. The proteome analyzed by 2-D gel electrophoresis and MALDI peptide mass fingerprinting revealed the presence of proteins specific markers [56].

Direct analysis of Gram-negative and -positive intact bacteria cells by MALDI-Tof-MS can produce specific biomarkers that can be used for taxonomy. These markers can be generated from the characteristic mass spectra allowing identification and distinction of pathogenic bacteria from their nonpathogenic counterparts [57,58].

MALDI-Tof-MS of intact cells (intact cell mass spectrometry—ICMS) has been shown to produce characteristic mass spectral fingerprints of moieties desorbed from the cell surface [59]. ICMS is a rapid and reproducible technique, which has been successfully used for the identification and discrimination of methicillin *Staphyloccus aureus*. ICMS with MALDI-Tof-MS led to analyze media effects and inter-laboratory reproducibility [60,61]. Analysis by MALDI-Tof-MS of phylogenetically similar bacteria (family

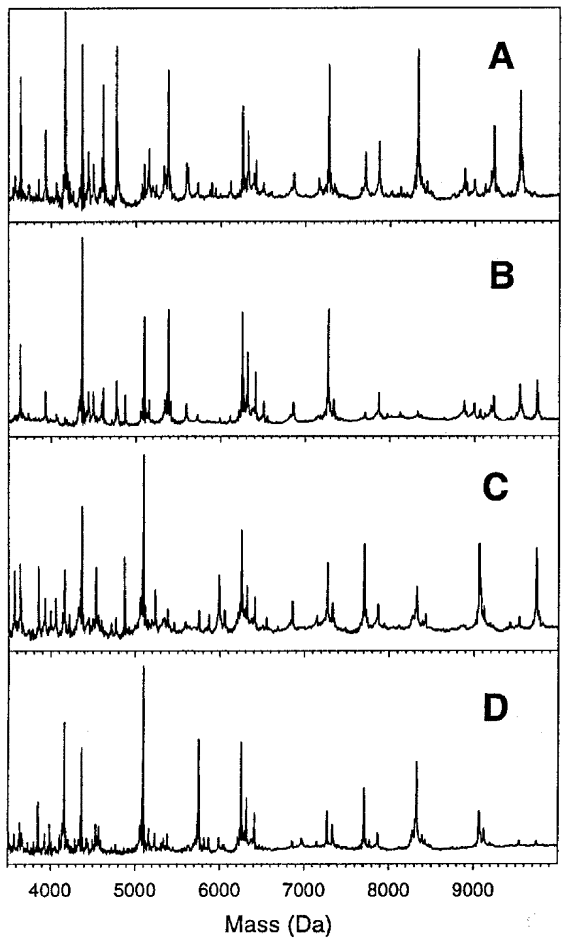


Fig. 3. MALDI mass spectra of four *E. coli* strains, (A) BLR, (B) XL1 blue, (C) RZ1032, and (D) CSH23 showing the typical mass spectral fingerprints of whole bacteria cells. [Reproduced from Arnold and Reilly [64] with the permission of John Wiley & Sons.]

Enterobacteriaceae) led to the identification of family-specific biomarkers [62]. Moreover, with this approach, small spectral differences were observed and allowed to distinguish different bacteria strains [63,64]. MALDI mass spectra of four *Escherichia coli* strains (Fig. 3) showed typical variations among the whole cell patterns [64]. Indeed, even though many peaks are in common, some unique ions led to the identification of strain-specific markers.

7. Genotyping/pharmacogenetics

Recently, MALDI-Tof-MS was successfully applied to DNA sequencing (genotyping) as well as screening for mutations (Fig. 4) [4,65–67]. Mass spectrometry of DNA is more complex than protein analysis due to the formation of sodium and potassium adducts which complicate mass spectra interpretation. Thus, the introduction of 3-hydroxy-picolinic acid as a matrix for DNA together with extensive washing procedures made oligonucleotide analysis possible. High-throughput genotyping of single-nucleotide polymorphisms has the potential to become a routine method for both laboratory and clinical applications [68,69]. Moreover, posttranscriptional modifications of RNA could be analyzed by MALDI using nucleotide-specific RNAses combined with further fragmentation by post source decay (PSD).

MALDI-Tof-MS offers the potential for reliable high-throughput screening of known point mutations and single-nucleotide polymorphisms. Application of MALDI-Tof-MS genotyping to the diagnostics of thrombotic risk alleles may provide health care benefits [70]. Homogeneous fluorescence-based PCR assays and MALDI-MS analysis were used to evaluate the presence of Toll-like receptor4 polymorphisms in patients to predict susceptibility to bacterial infection [71]. Moreover, candidates nucleotide polymorphisms of coronary artery disease-related genes were diagnosed by MALDI-Tof-MS using a MassARRAY system [72].

8. Conclusion/future development

After surveying the literature, it is now apparent that after a significant incubation period in analytical research circles, MALDI-TOF-MS has matured into an analytical technique capable of confidently delivering information of clinical and industrial importance. Currently, it is principally a tool of rapid identification of large biomolecules. Therefore, the applications that now emerge are those which have particular needs in high-throughput, highly accurate identification, and low sample cleanup. Examples of applications meeting these criteria that have been reviewed here include, diagnostics for cancer, allergy and allergen detection, rheumatoid arthritis, bacterial detection and speciation, and genotyping. As these analytical characteristics are highly desired in other clinical areas of application, testing labs are likely to see a number of additional applications developed in the near future. Additionally, as the field of application increases, demand for affordable instrumentation will increase, and a host of manufacturers will begin to offer dedicated MADLI systems for clinical use.

One of the most important recent developments is the MALDI-Tof imaging mass spectrometry [73–76]. This technique generates profiles and two-dimension-

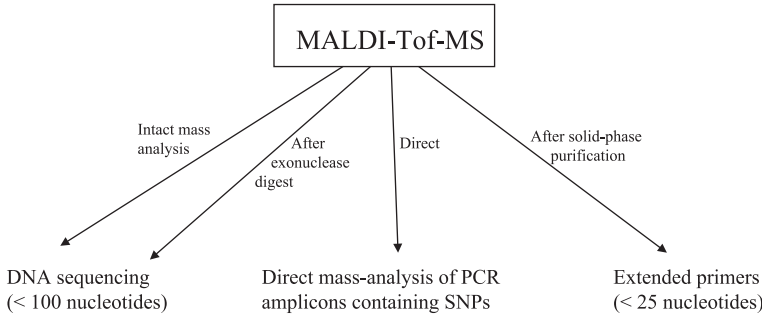


Fig. 4. Application overview of MALDI-Tof-MS analysis applied to DNA.

al ion intensity maps of peptide and protein signals directly from the surface of thin tissue sections. Tissue sections are attached to the sample plate and the laser is used to raster over the section. MS image maps can be calculated from the mass spectra for hundreds of proteins [77,78]. MALDI-MS imaging can be used to map the distribution of targeted compounds in tissue, providing important molecular information in the area of human health. For instance, this new technology was applied to the study of amyloid β peptide distribution in brain sections from mice, showing features reminiscent of Alzheimer's disease [79].

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