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REVIEW

Approaches for the analysis of low molecular weight compounds with laser desorption/ionization techniques and mass spectrometry

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Abstract This review summarizes various approaches for the analysis of low molecular weight (LMW) compounds by different laser desorption/ionization mass spectrometry techniques (LDI-MS). It is common to use an agent to assist the ionization, and small molecules are normally difficult to analyze by, e.g., matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) using the common matrices available today, because the latter are generally small organic compounds themselves. This often results in severe suppression of analyte peaks, or interference of the matrix and analyte signals in the low mass region. However, intrinsic properties of several LDI techniques such as high sensitivity, low sample consumption, high tolerance towards salts and solid particles, and rapid analysis have stimulated scientists to develop methods to circumvent matrix-related issues in the analysis of LMW molecules. Recent developments within this field as well as historical considerations and future prospects are presented in this review.

Keywords MALDI · SALDI · SELDI · Mass spectrometry · Derivatizations · Small molecules

Introduction

Maiman [1] presented the first implementation of an optical pumping technique using a synthetic ruby in 1960. Only a few years later Honig and Woolston [2] showed that a focused beam from a pulsed ruby laser was able to induce ion emissions from various surfaces, and these ions could be detected and identified in a mass spectrograph. However, laser desorption/ionization mass spectrometry (LDI-MS) was infrequently used in the following two decades. It took until the middle of the 1980s for the next big breakthrough, when Karas and Hillenkamp [3, 4] first developed the technique now known as matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). The use of a strongly absorbing organic compound as a matrix for LDI enabled a fixed laser wavelength, a reduction in parameters used in the experiments, and enhancement of the ionization yield [4]. They proposed tryptophan as a matrix to detect alanine. The experiment was performed at an irradiance of approximately one-tenth of what was required to record a mass spectrum for alanine alone.

Concurrently with Karas and Hillenkamp, Tanaka et al. [5] used cobalt nanoparticles (NPs) in glycerol instead of an organic matrix to assist the ionization. This method, often referred to as surface assisted laser desorption/ionization (SALDI), has gained popularity in recent years.

In 1993, Hutchens and Yip [6] applied modified target surfaces for LDI analysis of biomacromolecules. One strategy, when an organic compound that absorbs laser energy was covalently linked to the surface, is called surface enhanced neat desorption (SEND). This method provided high efficiency of analyte desorption/ionization without any addition of a free organic matrix. Another approach, referred to as surface enhanced affinity capture (SEAC), where the surface was modified in a way that it selectively extracted and retained analyzed compounds, was used in combination with a matrix. Both strategies are referred to collectively as surface enhanced laser desorption/ionization (SELDI) [7].

The intrinsic properties of LDI techniques, e.g., simplicity, fast analysis, high throughput, small analyte volumes, tolerance towards salts and impurities, and no or little fragmentations, attract researchers within the research fields of small molecule analysis. Henceforth, in this review, we refer to

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molecules in the mass region below 1,000 Da as low molecular weight compounds (LMW) compounds. Progress made toward various approaches for LDI-MS analysis of small molecules was extensively reviewed up to 2011. Thus, Cohen et al. [8] summarized MALDI analysis up to 2002. Peterson [9], Rainer et al. [10], and Kuzema [11] mainly focused on matrix-free LDI up to 2011, whereas Chen et al. [12] considered matrix-free LDI using nanomaterials. Law and Larkin [13] published a review focused on the latest advances within SALDI-MS techniques and bioanalytical applications in 2011, and Van Kampen et al. [14] considered the biomedical applications of MALDI-MS for LMW analysis.

This review includes most of the LDI techniques used today, and provides researchers with a fast way of gaining insight into strengths and weaknesses of different methods for small molecule analysis, thus enabling the possibility of readily choosing a suitable technique for their respective needs. Moreover, the International Union of Pure and Applied Chemistry (IUPAC)'s 2013 classification [7] of mass spectrometry definitions and terms is used to try to obtain a cohesive nomenclature for the different LDI techniques. Unless stated otherwise, LDI analysis was carried out in positive mode.

Matrix assisted laser desorption/ionization

Generally, the MALDI technique has historically been most frequently associated with analysis of large biological molecules, e.g., peptides, proteins, protein complexes in biochemistry, and analysis of synthetic polymers in macromolecular chemistry [15]. During the last decade, more focus has been directed at the analysis of small molecules using MALDI-MS [14].

It is a mild ionization technique that produces little or few fragmentations, making it suitable for fragile molecules that are otherwise difficult to analyze. During the ionization process, the matrix absorbs the laser radiation and supports the desorption/ionization process (Fig. 1) of the analyte molecules [12]. Furthermore, MALDI has several large advantages, including robustness, ease of use, fast analysis, and high selectivity and sensitivity [16]. Thus, it was possible to detect glucose at 1 amol in real samples using 1-naphthylhydrazine hydrochloride (NHHC) as a matrix [17]. Little fragmentation of the matrix in the low mass region and a high salt tolerance were among the advantages.

Several LMW pesticides have been successfully analyzed with various matrices by Madla et al. [18], whose results showed that the use of α-cyano-4-hydroxycinnamic acid (CHCA) and 2′,5′-dihydroxybenzoic acid (DHB) provided the best results. Compared to the techniques commonly used to analyze pesticides such as gas or liquid chromatography mass spectrometry, the method has several benefits: smaller

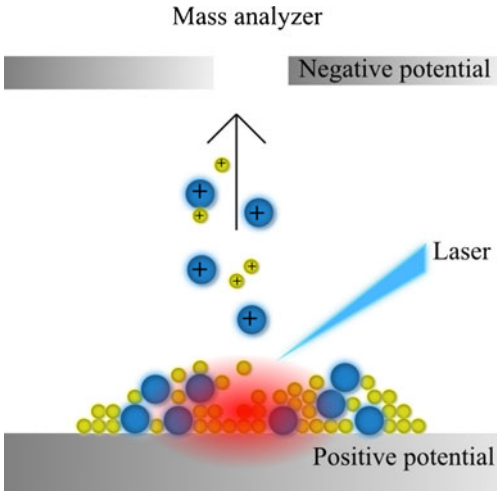

Fig. 1 Schematic picture of matrix assisted laser desorption/ionization. The *yellow spheres* represent the organic matrix, and the *blue spheres* represent the analytes

sample consumption, fast and simple analysis, and less time-consuming sample preparation.

However, for many LMW compounds, these matrices do not work as successfully as in the previous examples owing to matrix effects, signal interference, or suppression of the analyte signal [19] in the region below 1,000 Da. The signal interference from the matrix is due to the majority of the most frequently used matrices, e.g., CHCA [20, 21] and DHB [20, 22, 23], being small organic molecules themselves. When ionized, the matrix usually form clusters at low masses, which can interfere with the detection of LMW analytes [15, 24, 25].

There are many different approaches to overcome the matrix peak interferences, and some of these intriguing concepts are described below. One of the most critical steps to obtain eligible results is the choice of matrix. This choice will have a large impact on intensities, suppression of background, and whether the analytes are detectable. The most straightforward approach is to try the matrices available and examine which work with the intended analytes and which cause too much interference and mask the signal of the analyte.

For the analysis of arecoline, the main alkaloid found in the areca nut, the matrix 7-mercapto-4-methylcoumarin was introduced by Feng and Lu [26]. This matrix was found to be suitable for the analysis of LMW compounds owing to the significantly decreased matrix interferences in the low mass region compared to CHCA.

Chen et al. [27] designed and synthesized 2,3,4,5-tetrakis(3′,4′-dihydroxylphenyl)-thiophene (DHPT) as a matrix for the MALDI analysis of LMW amines. Several LMW amines were successfully analyzed without matrix peak interferences, and the limit of detection (LOD) was in the pico to femtomole range. The method provided a

usable approach for both quantitative and qualitative analysis of LMW amines, and the authors claim that the matrix can be extended to analyze a wide range of other amines, including possible use in MALDI imaging mass spectrometry (MALDI-IMS).

Chen et al. [28] developed the matrix *N*-(1-naphthyl)ethylenediamine dinitrate (NEDN) for MALDI-TOF-MS analysis in negative mode. The matrix NEDN is an organic salt, and was used for the analysis of LMW analytes such as oligosaccharides, peptides, and explosives. Results with NEDN were very favorable compared to other ordinary matrices: higher sensitivity is obtained and fewer matrix cluster ions are formed. Moreover, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDC) can be used as a matrix for detecting glucose in a high-salt environment [27], reaching detection limits of 10 μM glucose in 126 mM NaCl.

Another approach is to use a high molecular mass matrix instead. An example is *meso*-tetrakis(pentafluorophenyl)porphyrin (F20TPP) which has the advantage of no matrix interferences in the *m*/*z* range 100–500 Da and was used to analyze, for instance, different sugars [29]. Experimental results depend on, e.g., the type of instrument used, mass resolution, accuracy, data acquisition system, sample preparation, shot-to-shot and region-to-region reproducibility [8, 24], and formation of "hot spots" or "sweet spots" caused by inhomogeneous samples (matrix and analyte mixture) [30]. Internal standards have been proven to be successful at decreasing the poor reproducibility caused by inhomogeneity [19].

A combination of the 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) matrix supported by cyclodextrin was described for small molecules, such as LMW drugs, by Yonezawa et al. [31]. The properties of the sugar prevent the analyte molecules from forming alkali metal ion adducts, and only the protonated sample molecules were visible. This reduces the background and amplifies the signal from the LMW molecules.

A target plate precoated with a hydrophobic layer and a combination of the regular MALDI matrix CHCA and nitrocellulose were investigated by Donegan et al. [32]. This combination resulted in no interferences in the LMW region and can, according to the authors, enhance the ability to detect small molecules such as verapamil, tetrahydrozoline, and haloperidol.

A different approach to suppressing matrix peak interferences was demonstrated by Guo et al. [33]. This was done by addition of cetrimonium bromide to the common CHCA matrix.

It has also been found by Suzuki et al. [34] that a combination of the matrix THAP and crystalline aluminosilicates, where the common cations are exchanged by lithium cations, makes it possible to ionize analytes such as acetylsalicylic acid and phenobarbital, which previously were not detected by MALDI-MS. For some analytes, e.g., polar compounds and hydrocarbons, alternative matrices of these types can be a superior choice. However, sensitivity using these methods can be relatively low compared to the more commonly used matrices [28].

It is also possible to suppress the matrix peak interferences by mixing common matrices with each other. This was done by Guo and He [35] by mixing CHCA and 9-aminoacridine into a "binary matrix". This matrix mixture produced less background interferences in both positive and negative mode for the analysis of LMW compounds. However, only analytes with pK_a values outside the range defined by the pK_a values of the components of the binary matrix can be detected efficiently. Furthermore, another binary matrix was developed for the analysis of small biomolecules, such as gefitinib, donepezil, and several amino acids, by Shanta et al. [36]. A combination of 3-hydroxycoumarin (3-HC) and 6-aza-2-thiothymine (ATT) was used, and resulted in less matrix peak interferences in the low mass regions.

Nevertheless, matrix signals do not always hamper, but can rather facilitate MALDI-MS analysis of LWM. The intense matrix peaks can be used for high-accuracy internal calibration. This obtained accuracy together with the high resolution of modern MALDI-TOF-MS systems is sufficient to distinguish between analyte and matrix peaks, even if they appear very close to each other. Persike et al. employed CHCA signals for internal calibration of the instrument and reached a mass accuracy below 20 ppm. This allowed the authors to separate peaks of acetylcholine at *m*/*z* 146.061 and CHCA at *m*/*z* 146.117 [37]. The LOD for acetylcholine was 0.3 fmol/μL, while the linearity of R^2=0.9996 was maintained over the range of 1–1,000 fmol/μL. In another study, Persike and Karas [38] reported on a simple, rapid, and sensitive MALDI-MS method for the simultaneous quantification of ten different phenothiazines using CHCA as a matrix. The analysis of the medicines was successfully carried out in human plasma without prior chromatographic separation, special target plates, or isotopically labeled internal standards. Good linearity (R^2>0.99), precision (RSD 7.6 %), and accuracy (mean error 8.0 %) were obtained in the range of 2–1,750 ng/mL.

Chemical modifications of target compounds

Instead of modifying the matrix and/or the target surface, small analyte molecules can also be chemically transformed. This results in analyte peaks shifted to a higher mass region. By usage of a suitable reagent, it is possible to avoid matrix peak interferences for the analyte signal. Moreover, derivatization with a reagent that can provide a permanent charge is particularly useful for non-charged compounds, which may not be possible to analyze otherwise. Another advantage of derivatization is that the signal strength can be increased, because the derivatized compound may have different

chemical and physical properties, which can provide beneficial changes in volatility and higher ionization efficiency. Furthermore, selectivity for specific analytes is significantly enhanced by a derivatization procedure.

The carbonyl group is the most frequently employed functionality for the derivatization procedure as shown in Fig. 2A and B.

Tholey et al. [39] derivatized small biological carbonyl compounds such as pyruvate and acetaldehyde with hydroxylamine and dansylhydrazine to form oximes and hydrazones (Fig. 2Aa, b).

Brombacher and co-workers [40] analyzed steroids by employing 2,4-dinitrophenylhydrazine (DNPH) as both derivitizing reagent and matrix (Fig. 2Ac). Analysis was carried out with capillary-HPLC-MALDI-TOF-MS and MALDI-TOF, and detection limits were between 0.5 and 15 ng, and 0.3 and 3 ng, respectively. Fast analysis of oxosteroids was accomplished by using a dual matrix and derivatization reagent (naphthalene-2-ylmethyl)hydrazine [41] (Fig. 2Ad). Concentrations of 2 ng/mL were detected in human urine. There are several benefits of these types of reactive matrices: less sample handling, high throughput is possible when less time is needed, and matrix peak interferences from a complimentary matrix are avoided.

Derivatizations using Girard's reagents T and P (Fig. 2Ae, f) have been employed successfully for the

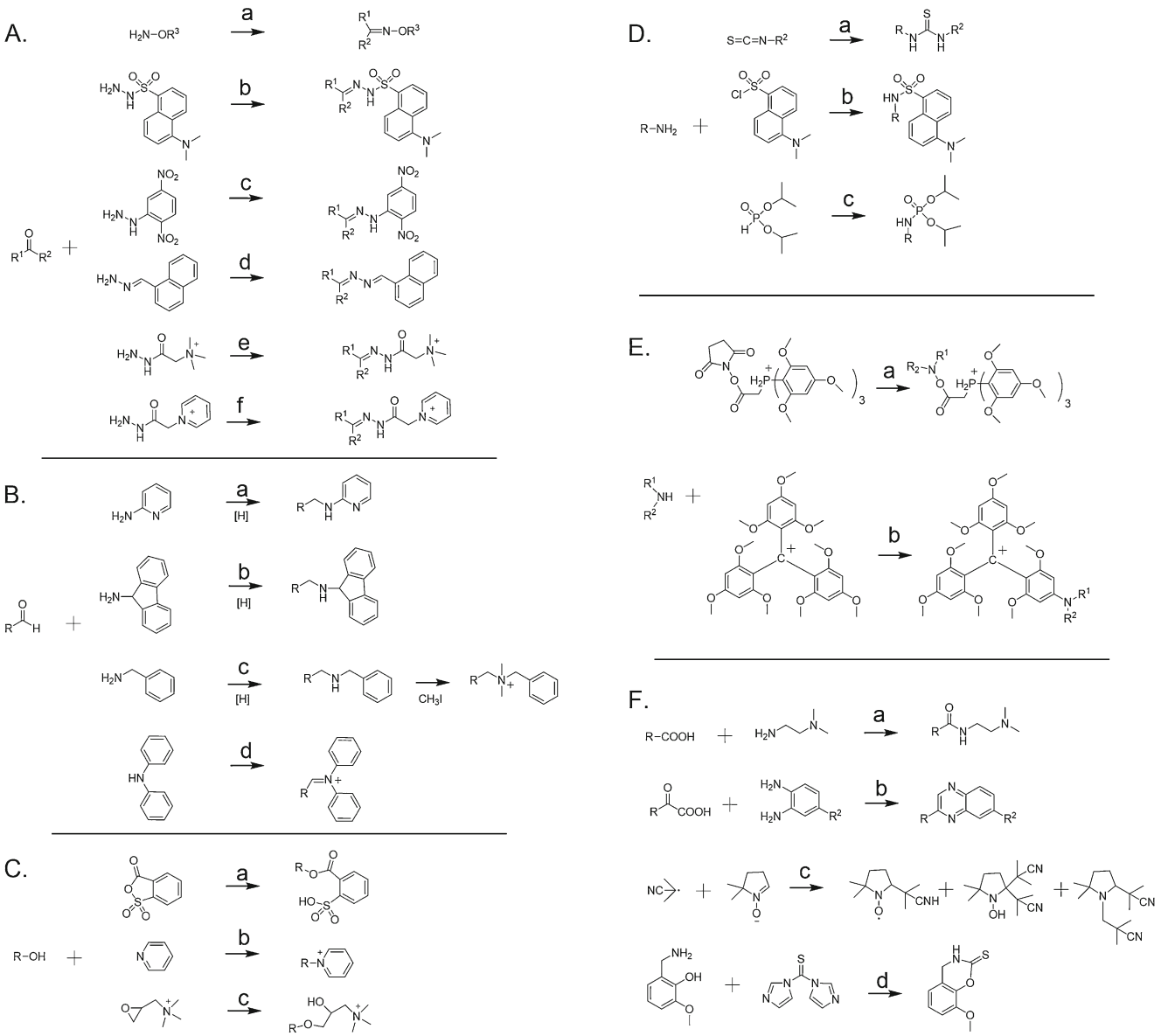

Fig. 2 Suggested reaction schemes for the derivatization of LMW *A* ketones, *B* aldehydes, *C* alcohols, *D* primary amines, *E* secondary amines, and *F* other molecules

analysis of non-charged steroids by MALDI-TOF-MS [42, 43].

Girard's reagent T is also advantageous for the analysis of small oligosaccharides [44]. Moreover, oligosaccharides have been analyzed as 2-aminopyridine derivatives [45] (Fig. 2Ba). For instance, maltopentaose showed a 100-fold improvement in sensitivity after the reductive amination using DHB as matrix. Another derivatization reagent used successfully for oligosaccharides is 9-aminofluorene [46] (Fig. 2Bb). The 9-aminoflourene derivatives have the advantage of being chemically stable and having high molar absorptivity in the UV region. The analytes were detected using MALDI coupled to Fourier transform mass spectrometry (FTMS). Furthermore, oligosaccharides have been derivatized with benzylamine followed by *N*,*N*-dimethylation (Fig. 2Bc) and then analyzed by MALDI-TOF-MS and MALDI post-source decay TOF-MS [47]. Compared to untreated oligosaccharides, the results indicated a tenfold increase in sensitivity. Small oligosaccharides have also been detected using glycidyltrimethylammonium chloride. The sensitivity increased by a 1,000-fold owing to introduction of the quaternary ammonium center into the carbohydrate molecules [44].

Analysis of small molecular aldehydes in single puff smoke was carried out by a technique called extraction and derivatization in single drop (EDSD) coupled to MALDI with Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR-MS) [48]. The extraction/derivatization reagents used were DHB and diphenylamine in methanol solution (Fig. 2Bd).

For compounds containing hydroxyl groups, several derivatizations have been utilized. 2-Sulfobenzoic acid anhydride selectively reacts with alcohols to form esters (Fig. 2Ca), while amines remain untouched during the same conditions [39]. After a derivatization with pyridine (Fig. 2Cb), alcoholic substances were analyzed in urine samples as well as in human hair [49, 50].

Amines are another class of compounds that can be chemically modified prior to LDI-MS analysis. They can be detected in the form of thioureas or sulfonamides after treatment with isothiocyanates or dansyl chloride, respectively [39] (Fig. 2Da, b).

Gao et al. [51, 52] successfully used N-derivatization with a neutral phosphoryl group with high proton affinity (-$PO(OiPr)_2$) for the analysis of amino acids and peptides by MALDI-TOF-MS (Fig. 2Dc). This method improved the ionization efficiency and significantly reduced the matrix peak interferences.

Lee et al. [53] performed derivatizations with a light and a heavy (isotopically coded) reagent, and successfully avoided matrix peak interferences while analyzing primary and secondary amines by using tris(2,4,6-trimethoxyphenyl)phosphonium acetic acid *N*-hydroxysuccinimide esters (Fig. 2Ea). The esters added 573 and 600 Da, respectively, and provided positively charged labels on the amines.

Denekamp and co-authors [54] used carbenium ions on a MALDI target plate to provide derivatization of the analytes so the derivatives obtain a permanent positive charge (Fig. 2Eb). This was done for the fast analysis of small amines, amino acid esters, and free amino acids by MALDI-TOF-MS.

In addition to the aldehydes, ketones, alcohols, and amines mentioned previously, other types of small molecules can be modified and analyzed by LDI-MS techniques. After succinimide or carbodiimide activation, volatile carboxylic acids react with asymmetric diamines (Fig. 2Fa), resulting in amides, which are easily detectable by MALDI-MS. For example, an LOD of 5 pmol was achieved for acetic acid [39]. Ketocarboxylic acids interact with 1,2-phenylenediamine causing formation of quinoxalinols [39] (Fig. 2Fb). The derivatized compounds strongly absorb nitrogen laser irradiation. Therefore they can be analyzed without any additional matrix. Thus, results of LDI-MS experiments showed that 5 pmol of pyruvate could be detected in the form of a quinoxalinol on a polished metal target.

Tian et al. [55] developed an efficient method for both detection and identification of small molecules. This was performed by MALDI-FTMS analysis of the free radical adducts with LMW compounds from hydroxyl and 2-cyano-2-propyl radicals trapped with 5,5-dimethylpyrroline *N*-oxide (DMPO) (Fig. 2Fc).

Surface assisted laser desorption/ionization

The term SALDI was first introduced in 1995 by Sunner et al. [56]. They used graphite particles with the analytes in glycerol solution, and protonated analytes and alkali cation adducts were readily detected. According to IUPAC's current (2013) definition [7], SALDI is defined to be "matrix free" and is used for biological macromolecules. In this review, we shall use the term SALDI for small molecules as well, but still require that the technique is "matrix free", meaning that, in contrast to MALDI, there is no organic matrix assisting the ionization. Instead, the ionization process is supported by either the surface of the target plate, or a nanostructure surface on particles mixed with the analyte. The SALDI technique is often based on the use of inorganic surfaces, where the surface assists the ionization process by absorption of laser energy and thermal desorption of the analyte [13] (Fig. 3). This idea is similar to the one proposed by Tanaka et al. [5] in the 1980s.

This method has been gaining in popularity, and inorganic materials containing, e.g., metal or metal oxide particles, are frequently used. Inorganic matrices have the advantage of causing few to no matrix interferences below 1,000 Da [57, 58].

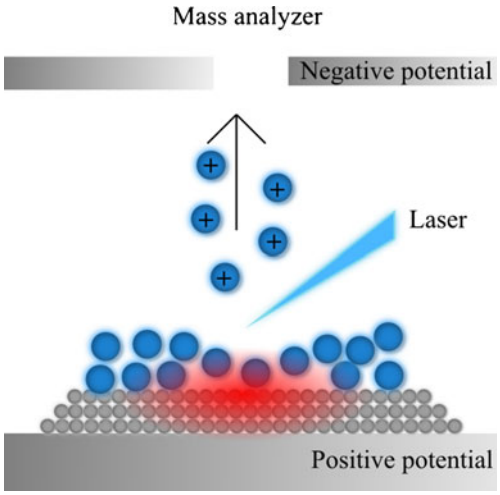

Fig. 3 Schematic picture of surface assisted laser desorption/ionization. The *gray spheres* represent the assisting surface that assists in the ionization of the analyte molecules by absorption of the laser and thermal desorption of the analytes represented by the *blue spheres*

Watanabe et al. [59] successfully ionized organic LMW analytes, e.g., polypropylene glycol with an average molecular weight of 400, testosterone, and verapamil hydrochloride. Their technique was buffer and "matrix free". They used ZnO NPs of anisotropic shapes together with the analyte solution on a stainless steel target plate. The relative standard deviation (RSD) for, e.g., the testosterone analysis, was 27 % compared to 81 % for conventional MALDI. The authors found that less sweet spots were formed, so the reproducibility was significantly better with this SALDI approach.

In 2011, Sonderegger et al. [57] used TiO_2 coated steel targets to analyze small molecules. The intensity of signal caused by the TiO_2 layer was very small compared to the analyzed sugars and flavonoids.

Moreover, the quick and simple production of the surface and the fact that the TiO_2 coating proved to be quite resilient to laser ablation, and could be washed without diminished ionization capacity, indicated that the method can be useful for many applications. Furthermore, TiO_2 in nanotube layers as a surface material has been proven to be advantageous for the analysis of small molecules such as Sutent and verapamil [58]. By electrochemical anodization, TiO_2 nanotube layer substrates were constructed. Compared to MALDI-MS analysis of Sutent and verapamil, the authors managed to obtain a tenth of the previous detection limits for these two analytes. Moreover, TiO_2 was used not only to overcome the problems with matrix peak interferences, but also for enrichment of the analytes using on-target thin layer chromatography (TLC) [60], or prior to LDI analysis [61].

Platinum NPs have been used successfully for the analysis of small biomolecules, e.g., arginine and methionine, with several advantages, among others less time-consuming sample preparation steps [62]. Three-dimensional platinum nanoflowers (NFs) have been synthesized by a new method proposed by Kawasaki et al. [63] in 2010. Examples of the small molecules they analyzed were caffeine, vitamin C, and aspirin. The analysis of caffeine was successful, and the reported LOD was 10 pmol. However, the other small acidic molecules could not be detected.

Nitta et al. [64] investigated the desorption/ionization process of 20 amino acids by LDI using platinum NFs coated with a flourocarbon chain, and by MALDI-MS with CHCA, both in negative mode. All of the 20 amino acids were detected using their method, whereas only two amino acids were detected using MALDI in negative mode. They also performed some investigations in positive mode, and the functionalized NFs produced only protonated ions and no alkali metal adducts, providing a clearer mass spectra. Results indicated that the NFs method in negative mode was better than the corresponding MALDI method, and the authors expect better sensitivity and reproducibility compared to conventional MALDI and SALDI.

Examples of other types of inorganic matrices that were shown to be effective and have weak to nonexistent matrix interferences include other metals and metal oxides [65–67].

Carbon nanotubes were used as early as 2003 to analyze small molecules by LDI-TOF-MS. A simplified sample preparation step and less matrix interference were among the advantages. A drawback was that carbon nanotubes have low solubility. However, oxidized carbon nanotubes with higher solubility provide better efficiency and results with enhanced reproducibility [68, 69]. Montsko and co-workers [70] described the application of C_{70} fullerene as a matrix, in both negative and positive modes, for determination of five steroid hormones. Because of the high molecular mass and ability to assist ionization efficiently, these fullerenes provide good results. High sensitivity was obtained and the LOD of the steroids was between 38 and 74 pmol. However, no quantitative analysis was performed.

Graphene and graphene oxides are other examples of SALDI substrates whose special properties can enhance the desorption/ionization process. These types of substrates were first used by Dong et al. in 2010 [71]. It is possible to analyze small molecules with less laborious sample preparations, less background interference from the matrix, and improved reproducibility compared to conventional MALDI [30, 71].

Liu et al. [72] developed a fast and simple method to determine flavanoids and coumarin derivatives by using a matrix based on graphene NPs. Among the three such matrices investigated, the one that provided the best results with the highest sensitivity in their experiments was graphene oxide, which has several benefits. For instance, it is simple to prepare because it dissolves easily in aqueous solutions and the matrix peaks do not interfere with analyte signals in the mass regions below m/z 500. Moreover, there is less contamination of both

ion source and vacuum system because graphene attaches strongly to the target plate. Furthermore, graphene-based matrices have better shot-to-shot reproducibility compared to the most commonly used matrices in MALDI. Graphene flakes have been used successfully for analyzing a wide range of small molecules including amino acids, nucleosides, and nucleotides, using direct pipetting of a suspension of sample and graphene flakes on the target plate [73]. Magnetic graphene has also successfully been used for the analysis of small molecules, with low background from the matrix in both positive and negative mode, depending on the properties of the analytes [30]. Usually it is time-consuming to synthesize; however, Shi et al. [30] developed a less complex process via a hydrothermal reaction. Their analysis of several active compounds known from Chinese medicine proved to be successful using magnetic graphene as both the enrichment agent and substrate for the analysis in both positive and negative mode. Moreover, dioxins such as octachlorodibenzo-*p*-dioxin have also been analyzed with reduced graphene oxide film as a matrix with high sensitivity and detection weight as low as 500 pg [74]. Using electrospun carbon nanofibers as substrate for SALDI, Lu and Olesik analyzed small molecules such as arginine and Crystal Violet with no interferences and higher reproducibility compared to conventional MALDI techniques [75]. Boron-doped diamond nanowires were used for the analysis of small molecules such as cortisone, histidine, betaine, and verapamil. The LOD was found to be 200 zmol/μL in aqueous solution for verapamil [76], and the authors claim that the sensitivities were at least as good as for other LDI methods.

Tang et al. [77], in their mechanistic study of carbon-based SALDI, discovered the inverse relationship between the ion desorption efficiency and the internal energy. Therefore, the authors concluded that the extent of internal energy transfer in the SALDI process is not able to enhance the ion desorption efficiency, rather the weaker bonding/interaction and/or lower melting point of the SALDI probe supports the phase transition of the substrate upon laser irradiation.

Free NPs may cause contamination of the instrument. One way to avoid this phenomenon is to immobilize the particles in a polymer matrix. A clean background spectrum for low *m*/*z* and good signal-to-noise values, compared to a plain polylactide surface, were obtained for acebutolol, carbamazepine, and propranolol, using eight different NPs, namely titanium dioxide, silicon dioxide, magnesium oxide, hydroxyapatite, montmorillonite nanoclay, halloysite nanoclay, silicon nitrite, and graphitized carbon black [78].

Alimpiev et al. [79] were the first to use gas chromatography (GC) with SALDI-MS, where the SALDI substrate was amorphous silicon. This was performed for the analysis of N-alkylated phenylethylamines. Detection limits were in the range of attomoles, which the authors believe can be improved in the future. The authors also managed to obtain a linear dynamic range of over five orders of magnitude, and low chemical background.

Instead of using a regular modified target plate, LDI-MS can also be used in combination with an interface called the rotating ball [80], or the analyte and matrix mixture can be put on a target plate as an offline continuous trail [81, 82]. For the rotating ball interface, the sample passes through a capillary onto the surface of a rotating ball, and is then delivered into the vacuum and, e.g., MALDI or SALDI analysis can be performed.

Alimpiev et al. [83] designed a rotating ball interface using silicon substrates for the quantitative analysis of LMW analytes such as arginine, atenolol, reserpine, tofisopam, and chloropyramine. Results showed that sample-to-sample reproducibility was 10 % with detection limits at the femtogram level. Grechnikov et al. [84] used a rotating ball interface for reproducible and quantitative analysis without complicated sample preparation. Lidocaine, diphenhydramine, and propranolol were analyzed by SALDI using silicon substrates, and in urinary samples they obtained detection limits of 0.2± 0.5 ng/mL.

Seo et al. [85] developed a new method to detect microRNAs which, unlike the previous method based on analyzing the antigen proteins, did not require amplifications from enzymatic reactions. They used nanoengineered micro gold shells for LDI, and reached detection levels down to femtomoles. Compared to gold particles, nanoengineered micro gold shells provide lower detection limits, higher sensitivity, and higher signal-to-noise ratios [86]. Prabhakaran et al. [87] used gold to facilitate the ionization for the analysis of arginine, Irganox, polystyrene, poly(methyl methacrylate), and their fragmentations in the LMW region. The samples were coated on silicon wafers and then metallized with gold particles by physical vapor deposition. Their research showed that the ionization probability increased and that it can be an alternative to MALDI for LMW analytes, and for imaging of certain materials. They also made a comparison with MALDI analysis, and results showed that this method produces more fragmentations than MALDI.

Niziol et al. [88, 89] suggested a method in which the target plate was treated with silver NPs before the analyte was applied to the target. The new surface allowed high mass determination accuracy and in general better results compared to previous literature methods using conventional matrices for the analysis of small molecules. Several compounds were analyzed, such as the alkaloids codeine and papaverine hydrochloride, the saccharide D-ribose, the L-amino acids (Cys, Ala, Met, Asp, Glu, Gln, Pro, Arg, Phe, Leu, Tyr, Ser, His, and Trp), the nucleoside thymidine, the nucleic base 5-fluorouracil, and the anticoagulant warfarin. Furthermore, it was shown that the use of monoisotopic ^{109}Ag NPs enabled even higher sensitivity, mass accuracy, and resolution. The

monoisotopic silver also provided very good signal-to-noise ratio.

Desorption/ionization on silicon

Desorption/ionization on silicon (DIOS) was developed in the late 1990s by Siuzdak et al. [90, 91]. It is another alternative that is "matrix free", and has been used for the analysis of a wide range of small molecules. For example, Kuzema [11] applied the method to the analysis of carbohydrates and achieved detections down to the femto to attomole range with little or no fragmentations. It is possible to analyze molecules as small as 150 Da, which are ionized by direct laser vaporization. An important development in the analysis of small molecules with DIOS was described by Kraj et al. [92] who reported a method to analyze catecholamines, which are important neurotransmitters. Moreover, porous silicon with chemically grafted cation- and anion-exchanging compounds were used for analyzing ionic dyes such as methylene blue and methylene orange [93].

Using silicon NPs from silicon nanopowder as a matrix is another approach that offers advantages for the detection of some analytes, e.g., the drugs propafenone, morphine, and verapamil; the pesticides ametryn and altretamine; dibutylphosphoric acid and adenine. Decreased matrix interference in the low mass region and tolerance to contamination from salt and buffers are some of the advantages of using this matrix [94].

A different approach was suggested by Wang et al. [95], where a silicon nanowire array was used which can be produced at low cost. This method gives no matrix interferences in the low mass region, and the array provided a signal strength of the same order as that of conventional matrices. A biomimetic antireflective silicon nanocone array was also been designed and optimized for the analysis of small molecules, such as amino acids and drug molecules, with little to no interference in the $m/z < 700$ region [96].

Silicon has most often been employed for the study of ion formation in SALDI. Alimpiev et al. [97] proposed the following order of events during SALDI: (1) formation of hydrogen bonds between silanol groups and basic analyte molecules supports the adsorption on silicon surfaces; (2) free electron/hole pairs are generated in the silicon probe as a result of laser irradiation, and positive charges are localized near the surface at the point defects; (3) positive charge enrichment of the near-surface layer causes significant increase in acidity of the silanol groups on the surface and proton transfer to analyte molecules; (4) thermal energy of 70 kJ/mol is required for dissociation of the protonated analyte from the surface. It has also been established that the efficiency of laser desorption/ionization from the surface of amorphous silicon exponentially increases with an increase in proton affinity and gas-phase basicity of the analyzed compounds [98].

Nanofilament silicon was investigated by Tsao and Devoe [99]. The idea was to use an open pore morphology able to transport the sample into the nanopores, so that the solvent could be removed more efficiently.

Surface enhanced laser desorption/ionization

The SELDI approach was first introduced as SEND and SEAC by Hutchens and Yip [6] in 1993. According to IUPAC, the term SELDI is used to describe methods involving a surface that selectively retains certain proteins and peptides. We will also use this term for small molecules.

Surface enhanced affinity capture

The SEAC approach is based on a surface that is designed to capture specific analyte molecules (Fig. 4). Usually, the surface is similar to those used in chromatography, bonded to a surface. Selective capture of analytes can be performed and, e.g., impurities can be washed away.

One way to obtain a surface that selectively retains certain compounds is to modify the target plates with thin layers of porous polymer monoliths. For example poly(butyl methacrylate-*co*-ethylene dimethacrylate), poly(benzyl methacrylate-*co*-ethylene dimethacrylate), and poly(styrene-*co*-divenylbenzene) were applied for LDI analysis of LMW drugs such as caffeine, nortryptyline, and the explosive compound 2,4,6-trinitrophenylmethylnitramine [100]. A great advantage of the polymer monoliths is that it is possible to change the chemical properties of the monomers enabling the analysis of a wider range of analytes.

Çelikbiçak et al. [101] proposed a matrix-free approach for analyzing the LMW compounds acrivastine, L-histidine, L-

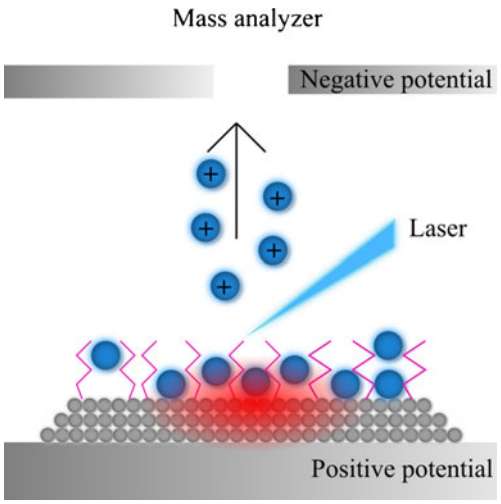

Fig. 4 Schematic picture of surface enhanced affinity capture. The *pink chains* represent the surface that captures the analyte molecules, represented by *blue spheres*

valine, L-phenylalanine, L-arginine, and L-methionine with LDI on a self-assembly monolayer (SAM), which can result in higher sensitivity compared to regular LDI-MS. Silicon substrates were prepared before analysis by cleaning them thoroughly and exposing them to solutions of different silane molecules.

The results showed clean signals and few fragmentations. Reproducible signals were obtained from each spot. The authors strongly suggest that modified surfaces with nano-overlayers should be considered for the analysis of LMW compounds.

Surface enhanced neat desorption

The SEND approach is designed to enhance the desorption by using molecules that absorb the laser energy (Fig. 5).

Lin and Chen [102] incorporated the matrix molecules into the polymer silica structure using the sol–gel approach. Figure 6 shows an example of the embedment of DHB into a silica sol–gel polymer structure.

The intensity of the background peaks was fairly low unless excessive amounts of matrix were used in the production of the sol–gel, indicating that it might be used for analyzing small molecules. Tseng and co-workers [103] developed a hybrid of immobilized silica and DHB on Fe_3O_4 NPs which produces no background.

Hashir et al. [104] analyzed carbohydrates with high detection sensitivity using silica gels modified with 4,4′-azodianiline. Simple sample preparation and results with very low background were produced. The detection limit for the LMW substance xylose was 70 fmol.

Mullens et al. [105] have shown the advantages of using silica modified with CHCA (Fig. 7a) for the analysis of LMW

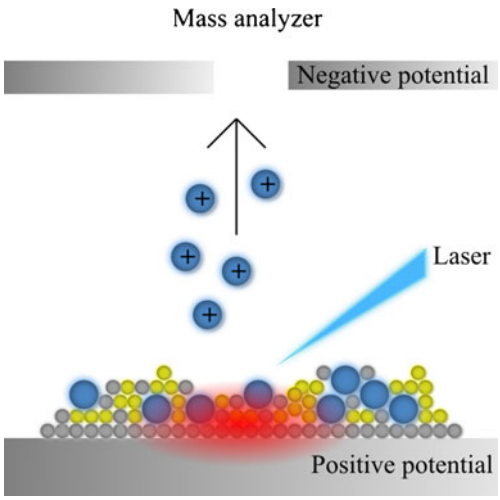

Fig. 5 Schematic picture of surface enhanced neat desorption. The *yellow spheres* represent molecules on the surface that absorb the energy from the laser and enhance the desorption process of the analyte molecules, represented by the *blue spheres*

compounds. The SBA-15-CHCA matrix retained the ionization properties of CHCA and the results showed that the noise from the low mass region in positive mode was greatly reduced. Furthermore, the signals from small molecules, such as the neurotransmitters dopamine and serotonin, had high intensities and were free from matrix interferences. Other advantages of this method are that the silica-based matrices are easy to synthesize and can be used on common MALDI target plates. Moreover, Su et al. [106] proposed a method to increase the CHCA concentration on a surface of SBA-15, and their results indicate improved ionization compared to the modification of SBA-15 suggested by Mullens et al. [105].

Functionalization of silica was also previously proposed by Li et al. [107], where an 8-hydroxyquinoline was attached to SBA-15 (Fig. 7b). Compared with the standard SBA-15, and also conventional DHB, this new matrix had significantly less matrix interference in the low mass region, and provided better signal intensity for several LMW analytes such as amino acids, metabolites such as L-carnosine and cytidine, and saccharides in honey. Furthermore, in the experiments that were carried out, no fragment ions were observed, and the detection limits for the optimized procedures were in the picomole range. Moreover, this new matrix produced better homogeneity. For the analysis of small molecule pollutants, Fe_3O_4 particles coated with polydopamine was used as a matrix and numerous pollutants such as benzo[*a*]pyrene, perfluorinated compounds, and several antibiotics were analyzed without matrix interferences [108].

Kailasa and Wu [25] synthesized dopamine dithiocarbamate functionalized gold NPs for quantitative analysis by LDI of small molecules such as the amino acid glutathione and the drugs desipramine and enrofloxacin. Results showed that this method provided high desorption/ionization efficiency for these small molecules compared to regular MALDI using DHB. They also compared their method by using the conventional matrix CHCA and proved that their method provided much clearer mass spectra for these analytes. The RSD was 6.8 % for enrofloxacin with an LOD of 0.8 nM, and 2.5 % for glutathione with an LOD of 0.2 nM.

Chiu [109] analyzed steroid hormones, such as cortisone, hydrocortisone, progesterone, and testosterone, using catechin-modified TiO_2 NPs. The experiments resulted in good reproducibility, quantitative analysis was possible because of the good linearity, and the LOD was 0.23–1.62 μM for the four steroids.

Another technique in progress is called nanostructure initiator mass spectrometry (NIMS). The technique is also "matrix free", and is based on a perfluorinated initiator incorporated into the nanopores. NIMS uses the basic concepts of DIOS but is more advanced because it uses the initiator molecules trapped in the nanostructure pores to enhance the ionization and release the molecules stuck on the surface [110]. This method was applied in the localization of lipids

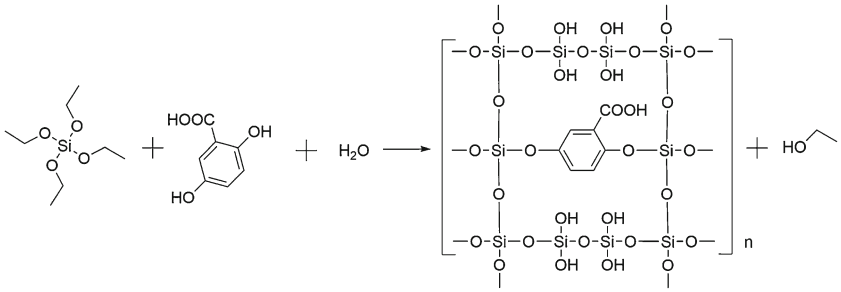

Fig. 6 Embedment of DHB molecules into the silica sol–gel polymer

in the *m*/*z* range 700–900 in *Cancer borealis* brain tissue [111].

The seed-layer method employs a quickly evaporating solvent to dissolve the matrix and deposit the mixture onto the target plate followed by fast evaporation. This forms a thin homogeneous layer of matrix on the surface, referred to as the seed layer. Using the seed-layer method, Ho et al. [19] obtained good results for the identification and quantification of the two LMW analytes morphine and 7-aminoflunitrazepam by means of matrix-conjugated magnetic NPs. The target plate was treated with DHB-encapsulated magnetic NPs prior to the application of the sample and matrix. The results demonstrated that this method was sensitive and precise with regards to quantification of the small molecules.

LDI-IMS

Imaging mass spectrometry is an excellent tool for visualization of the distribution of molecules in, e.g., tissue samples. All described LDI techniques have been employed for imaging experiments. However, most attention has been focused on MALDI-IMS. Thus, Hanrieder and Ljungdahl [112, 113] successfully mapped several LMW neuropeptides and fragments of heavier peptides in rat brain, employing DHB as a matrix.

Peukert and co-workers [114] developed a reliable method for spatially resolved analysis of metabolites in cryodissected barley grains and tobacco roots by MALDI-IMS using DHB, CHCA, 1,8-bis(dimethylamino)naphthalene (DMAN), and 2-((2*E*)-3-(4-*tert*-butylphenyl)-2-methylprop-2-enylidene)malonitrile (DTCB) as matrices.

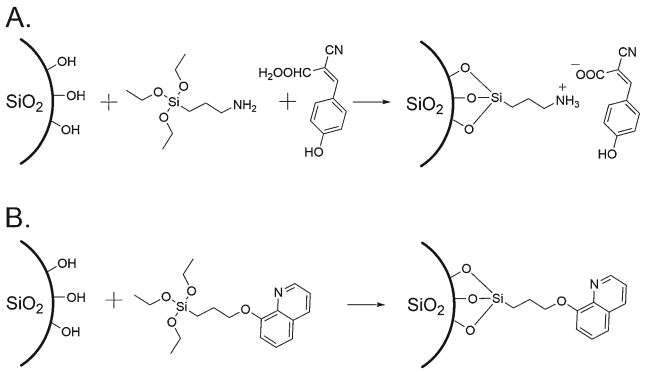

Fig. 7 Synthetic pathways to silica modified with **a** 3-aminopropyl residues and CHCA molecules and **b** 8-propoxyquinoline groups

The first MALDI-IMS method with high resolution in mass and space was recently developed by Römpp and Spengler [115]. The distributions of lipids, neuropeptides, and drug compounds were mapped in a wide range of biological samples at a spatial resolution of 5–50 μm. As an example, the aniticancer drug imatinib $[M+H]^+$ at m/z 494.2662 was imaged in mouse kidney at 35-μm pixel size with overall mass accuracy 2 ppm. [116].

In 2011, Chacon et al. [117] performed MALDI-IMS on-tissue chemical derivatization of the small molecule 3-methoxysalicylamine with the reagent 1,1′-thiocarbonyldiimidazole (Fig. 2Fd), where the resulting compound—an oxothiazolidine derivative—was easier to detect. Their results show that it is possible to make an on-tissue chemical derivatization and detect the analyte as a derivative. Therefore, the authors concluded that this method can be beneficial in the future for the analysis of small molecules such as drugs.

The issues concerning the low mass region are similar to those in regular LDI analysis.

An interesting approach to remove the matrix peak interferences was presented by Shariatgorji et al. [15], where they synthesized D^4-CHCA, which shifts the matrix cluster peaks. These peaks were then compared to the peaks of the ordinary CHCA. This allowed the authors to detect analytes in the lower mass regions without interference of the matrix and still keep the advantages from the CHCA matrix such as the good ionization properties. Their results proved it possible to analyze endogenous small molecules and pharmaceutical compounds that were previously difficult to detect owing to the matrix peak interferences using both MALDI-MS and MALDI-IMS.

Two specific problems with MALDI-IMS are that the washing step often is omitted to avoid removing the analyte, and applying a matrix in liquid form can cause analyte spreading. Goodwin et al. [118] proposed a method whereby the matrix is applied as a finely divided dry powder. Good imaging results were obtained using CHCA for, e.g., clozapine and

4-bromophenyl-1,4-diazabicyclo[3.2.2]nonane-4-carboxylic acid in rat liver and rat brain, respectively.

For the MALDI-IMS of small molecules in tissues, the target plate can be coated in advance. Grove et al. [119] developed a precoated MALDI target plate of gold. Normally, when analyzing tissue samples by MALDI-IMS, the matrix needs to be applied over the tissue sample. There are several different techniques such as spray coating, sublimation, dry-coating, and microspotting [120, 121]. To make the sample preparation step easier, faster, cheaper, and more reproducible, they developed precoated MALDI targets for imaging. The matrix DHB was used for precoating of both target plates and the glass slides. They showed that precoated target plates offer a homogeneous layer of small crystals, and can be used for MALDI-IMS directly, are fast, and are to be preferred for the analysis of small molecules like pharmaceutical compounds, lipids, and peptides [119].

Ronci et al. [122] performed imaging of small molecules by contact printing the hypobranchial gland from marine mollusc tissue to analyze biologically active brominated precursors to Tyrian purple, using fluorocarbon functionalized porous silicon chips. From the tissue sample, the chip extracted and trapped the small hydrophobic molecules. After the tissue was removed, the chip was analyzed. The authors found the method to be not only easy and reliable, but even to be favorable in several cases compared to MALDI-IMS for small molecules. Some benefits are its high lateral resolution and possibility to analyze thick tissue samples.

Kawasaki et al. [123] investigated platinum vapor deposition, which was used for SALDI-IMS without solvents, to get a homogeneous layer of NPs for direct detection of small molecules such as saccharides, the pigments crystal violet and rhodamine B, and the drug verapamil hydrochloride on printed paper and several analytes separated by TLC. Regarding the reproducibility, the authors suggest further experiments are necessary.

Conclusions

Different approaches for LDI analysis have been an active research subject for many years owing to their attractive features like ease of use and fast analysis. These methods have historically been most commonly associated with large molecules, although a wide range of small molecules such as biological samples, pharmaceuticals, and environmental pollutants, which have previously not been easily detected by other methods, have proved to be susceptible to LDI techniques. However, there are many analytical parameters, such as sensitivity, selectivity, reproducibility, and also variables like cost and time consumption to consider. Usually LDI analysis is dependent on an assisting agent in the ionization/desorption process. For MALDI, small organic molecules are most commonly employed, but owing to matrix peak interference, care is needed when choosing an appropriate matrix.

By mixing commercially available matrices together, matrices sometimes referred to as binary matrices, one can remove matrix peak interferences and make the analyte signals easier to distinguish.

Chemical modifications of the analyte provide significantly increased selectivity. One disadvantage is that this can be expensive and might involve complicated sample preparation steps. Hopefully, there will be more developments focusing on simplifying the sample preparations, to make derivatizations easier and faster.

Quantitative MALDI-MS analysis is an expanding research field. However, there are difficulties regarding the reproducibility and repeatability, e.g., shot-to-shot and batch-to-batch, which makes it hard to obtain precise results. These issues can be solved by using isotopically labeled internal standards, which provide the best results. These internal standards are expensive and frequently unavailable. Therefore, cheaper unlabeled compounds with similar chemical structure can be used instead and still provide valid results.

Different surface modifications of the target plates are also constantly evolving to overcome the problem of matrix peak interferences in the low mass regions. While MALDI has the advantage of high sensitivity and SALDI has the advantage of eliminating so-called sweet spots, future prospects lean towards a combination of these two approaches. Employing a technique such as SELDI allows researchers to benefit from the two aforementioned LDI methods, namely decreasing the matrix peak interferences, while still maintaining the high sensitivity.

To conclude, there is no easy way to find which method will work best with which analyte. The most straightforward way is to read about similar compounds and try a method that has been proved successful previously, or to use a more "trial and error" approach. While reviewing the literature, it is clear that several efficacious methods have been suggested by thinking "outside the box".

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