

Matrix-free and material-enhanced laser desorption/ionization mass spectrometry for the analysis of low molecular weight compounds

Matthias Rainer · Muhammad Nasimullah Qureshi ·
Günther Karl Bonn

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Abstract The application of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) for the analysis of low molecular weight (LMW) compounds, such as pharmacologically active constituents or metabolites, is usually hampered by employing conventional MALDI matrices owing to interferences caused by matrix molecules below 700 Da. As a consequence, interpretation of mass spectra remains challenging, although matrix suppression can be achieved under certain conditions. Unlike the conventional MALDI methods which usually suffer from background signals, matrix-free techniques have become more and more popular for the analysis of LMW compounds. In this review we describe recently introduced materials for laser desorption/ionization (LDI) as alternatives to conventionally applied MALDI matrices. In particular, we want to highlight a new method for LDI which is referred to as matrix-free material-enhanced LDI (MELDI). In matrix-free MELDI it could be clearly shown, that besides chemical functionalities, the material's morphology plays a crucial role regarding energy-transfer capabilities. Therefore, it is of great interest to also investigate parameters such as particle size and porosity to study their impact on the LDI process. Especially nanomaterials such as diamond-like carbon, C₆₀ fullerenes and nanoparticulate silica beads were found to be excellent energy-absorbing materials in matrix-free MELDI.

Keywords Matrix-assisted laser desorption/ionization · Laser desorption/ionization · Material-enhanced laser desorption/ionization · Matrix-free · Nanotechnology · Porosity

Introduction

This review provides a comprehensive overview of innovative techniques and methods employing nanomaterials as energy-absorbing materials for laser desorption/ionization (LDI) mass spectrometry (MS). In particular the implementation of matrix-free and material-enhanced LDI (MELDI) for enhancing the desorption and ionization process will be highlighted. The small size of nanoparticles (0.2–100 nm) is a great attraction for bioanalytical applications, as it provides the required surface area per unit mass that is crucial for improved characteristics such as reactivity, capacity and sensitivity. The characteristics are altered extremely when the dimensions (size and shape) are brought to “nano levels”, which results in unique applications in comparison with the same bulk material. Owing to their small size, the overall surface-to-volume ratio is increased enormously, which allows further improvements in sample enrichment. Moreover, nanomaterials offer unique thermal, optical and electrical properties, making them promising candidates in bioanalytical applications including MS [1, 2]. Especially in matrix-assisted LDI (MALDI) MS, nanomaterials have gained increasing interest as alternatives to conventional MALDI matrices [3]. The use of matrices such as 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid and α -cyano-4-hydroxycinnamic acid (CHCA) for the identification of low molecular weight (LMW) compounds by MALDI MS has been performed [4–7] but often faces

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M. Rainer (✉) · M. N. Qureshi · G. K. Bonn
Institute of Analytical Chemistry and Radiochemistry,
Leopold-Franzens University,
Innrain 52a,
6020 Innsbruck, Austria
e-mail: m.rainer@uibk.ac.at

difficulties such as background signals in the low mass range (below m/z 700 Da) caused by matrix molecules. These limitations make the analysis of LMW compounds very difficult [8]. To overcome these problems, matrix-assisted and matrix-free systems have been introduced.

The role of the matrix in MALDI MS

Sample preparation is one of the most important aspects in MALDI MS as every single aberration during sample preparation can affect the outcome of MALDI measurements. In general, weak organic acids such as cinnamic acids are considered to be effective energy absorbing materials (matrices) in MALDI MS. The matrix is believed to serve two functions: absorption of laser energy and isolation of the analytes from each other, preventing cluster formation [9]. The analytes and the matrix are placed on a MALDI target by putting a few microlitres (0.5–2 μL) of the analyte on the MALDI target, followed by the addition of the matrix or vice versa. A variety of matrix candidates have been evaluated. Derivatives of cinnamic and benzoic acids have been found to be good MALDI matrices [5]. In particular, DHB [10], 3,5-dimethoxy-4-hydroxycinnamic acid [11] and CHCA [12] have become favoured matrices for peptide and protein analysis. Selection of an appropriate sample preparation method is a crucial point to obtain high-quality mass spectra. Many variables influence the integrity of a good, homogeneous sample preparation and may include the concentration of the matrix and the analyte, the choice of the matrix, contaminants (e.g. salts usually disturb the desorption and ionization process), temperature, hydrophobicity or hydrophilicity character, compatible solubilities of matrix and analyte solutions and how old the prepared sample is.

There are different ways to prepare samples, including the “*quick and dirty*” method [13], the “*dried-droplet*” method [14] and the “*thin-layer*” method [15]. Quick and dirty preparations do not require the matrix and sample to be mixed before spotting them on a MALDI target. This method provides fast results but very uncontrolled conditions compared with other sample preparation procedures. Quite often the results are less reproducible than those obtained by the dried-droplet method, where improved results can be obtained. In this method, the matrix and the sample are thoroughly mixed in advance and a certain volume of the matrix/sample mixture is placed on a MALDI target. However, the main problem of this method is that abundant amounts of crystals are accumulated at the edges of the spot, and are not homogeneously placed [16]. A third and very frequently used technique to prepare samples on a MALDI target is the thin-layer method, which involves the use of fast solvent evaporation to form a thin

and homogeneous layer of small matrix crystals on the target’s surface. The crystal size can vary in the range of a few micrometers, depending upon the matrix and the method of sample preparation. Once prepared, the sample/matrix co-crystals deliver the best results if they are measured directly after evaporation. The solvents for matrix preparation very often include trifluoroacetic acid, which reduces the ionization of matrix ions by keeping the pH at an appropriate level. The analyte/matrix mixture can be vacuum-dried to make the crystal size smaller [17]. The small crystals obtained by vacuum drying offer another advantage in terms of mass accuracy and resolution, because of the very thin layer of the matrix. All steps of sample preparation including the technique applied, the solvents, the target and the matrix are quite critical, as they strongly influence the outcome of the MALDI measurement.

Desorption/ionization process

The mechanism of MALDI MS is based on pulsed laser irradiation of a crystallized matrix embedded with analytes to induce desorption and ionization [18, 19]. The energy absorption results in a phase transition of the matrix in combination with the analyte, followed by photoionization [20]. The ionization step in MALDI is yet not completely understood but can be explained as a two-step mechanism, the primary and the secondary ionization [21]. The primary ionization describes the formation and separation of ions during the desorption/ablation in the MALDI plume, whereas the secondary ionization concerns the formation of secondary ions due to reactions of molecular ions after the primary ionization. Studies have revealed that the analyte molecules keep their solution charges in the matrix crystal, which implies that they also retain their solvation shell [22]. This is confirmed by the observation of no change in the pH indicator of two states, i.e. the solution and the crystalline state [23]. According to the cluster model of Karas et al. [24], the preformed ions should be embedded in aggregates of various sizes during the desorption/ablation process. They should furthermore undergo various charge reductions into clusters, leading to molecular ions of lower charge. The ionization potential in the solid phase on the MALDI target can be lower but is assisted by the laser energy, supported by thermal energy created in the whole process [25]. The ionization process caused out by a UV laser can be different from that caused by an IR laser because of the characteristic differences in the nature of the lasers [26]. Matrix-free systems do not require any organic acids for laser absorption, which eliminates inhomogeneities regarding matrix/analyte co-crystallization caused by sample preparation. In LDI MS

systems, the matrix-free substrate material is responsible for absorbing laser energy and transferring it to the analytes. The energy-absorbing material works best if it absorbs in the range of the laser's wavelength.

The introduction of LDI MS

LDI MS started in the 1960 s, when it was confirmed that the irradiation of low-mass organic salts with a high-intensity laser pulse led to the formation of ions, which could be successfully mass-analysed [27]. Although this technique underwent significant improvements during the following decades [28, 29], the applicability of LDI MS was limited because of fragmentation of analytes and the restricted mass range (5–10 kDa) for analysis. A big breakthrough came with the implementation of small energy-absorbing molecules for sample preparation in 1985 by Karas et al. [4] when they discovered that alanine could be ionized more easily if it was mixed with tryptophan. The successful desorption and ionization of alanine could be attributed to the properties of tryptophan, which absorbed laser energy and helped to ionize the non-absorbing alanine. In the late 1980 s, Tanaka et al. [30] reported the first use of cobalt nanopowder and glycerol for soft laser desorption in the analysis of proteins. This was the first time that higher-mass proteins such as those of 20,000 Da were analysed by LDI MS.

A broader application for matrix-free LDI MS was reported for desorption/ionization on porous silicon (DIOS) [31, 32]. DIOS surfaces are usually manufactured by galvanostatic etching procedures and show optimal performance for molecules of less than 3,000 Da [33, 34]. It is speculated that silicon films are capable of trapping analytes in the nanopores and simultaneously absorbing the laser radiation, leading to spectra without a matrix background. Nanoporous silicon, which is usually created through etching procedures, has found many applications in LDI MS, whereas smooth silicon substrates and silicon with larger pore sizes do not support desorption and ionization [35]. Nevertheless, one should consider the limits of DIOS surfaces regarding handling and storage [36]. A main disadvantage of porous silicon surfaces is their short lifetime, which degrades the analytical utility of the DIOS plate. Porous silicon substrates have been shown to be sensitive to contamination and the shelf life is limited to 1 year after production, even when they are kept under special storage conditions [37]. Other silicon-based matrix-free materials involve desorption/ionization on mesoporous silicate [38] and silicon dioxide chips, termed “desorption/ionization on silicon dioxide” [39].

Alternative approaches are based on sol–gels, which consist of polymeric structures with siloxane backbones in

form of bulk or thin films [40]. Silica in sol–gels can be replaced with metals such as titanium and zirconium. Metals and metal oxides of titanium and tungsten in their particle forms dispersed in ethanol or methanol are also employed for LDI MS [41]. A similar approach is termed “sol–gel-assisted LDI” (SGALDI) and uses organic/inorganic hybrid films as sample supports to generate mass spectra without suppressing analyte signals or forming clusters. These silicon films are generated by plasma-enhanced chemical vapour deposition and can be successfully applied for the analysis of small organic molecules [42]. The reduced durability is also a problem that can be observed by using sol–gel-assisted substrates. The lifetime of most commonly used SGALDI supports does not exceed more than some weeks, as presumably condensation harms the surface, resulting in decreased signal intensity.

Alternative energy-absorbing materials are based on silicon nanowires 10–40 nm in diameter which are grown from silane vapours [43]. Furthermore, gold nanoparticles of 2–5 nm were reported to support the desorption/ionization phenomenon [44]. Peterson et al. [45] introduced highly porous monolithic polymers such as poly(butyl methacrylate-*co*-ethylene dimethacrylate) and poly(styrene-*co*-divinyl benzene) with micropores and mesopores as matrix-free substrates. Kinumi et al. [46] used materials such as Al, Mn, Mo, Si, Sn, SnO₂, TiO₂, W, WO₃, Zn and ZnO as energy-absorbing materials for analysing peptides and proteins.

Moreover, a lot of carbon-based nanomaterials have been shown to support the desorption/ionization process. Several devices have been designed for different applications and sample preparations. Especially carbon nanotubes (CNTs) [47, 48] have been successfully utilized for the detection of LMW compounds. In recent studies, functionalized CNTs were reported as excellent affinity probes and substitutes for conventional MALDI matrices [49] for the study of micromolecules and macromolecules. Signal intensities as well as signal-to-noise ratios could be significantly improved for a matrix of oxidized CNTs compared with simple CNTs because of removal of impurities due to the oxidation process. The ratio of analyte to CNT was optimal around 10:1, in contrast to the conventional matrices, where it is around 1,000:1 [50].

Alternative sample supports for matrix-free LDI MS include fullerenes, which also exhibit energy-absorbing properties similar to those of conventional MALDI matrices. The first LDI measurements employing C₆₀ fullerenes as energy-absorbing molecules were carried out on predeposited fullerene films [51, 52]. Unfortunately, this technique suffered from low sensitivity owing to poor mixing of non-polar fullerenes and polar analytes. However, the surface polarity of fullerenes can be significantly increased by derivatization with polar functional groups to obtain a

more advanced and convenient matrix for LDI MS. Sheia et al. [53] reported hexa(sulfonbutyl)fullerene as an ion-pairing reagent for the selective precipitation of peptides. The precipitate was then directly deposited onto a target and successfully analysed without an additional matrix. Moreover, the C_{70} fullerenes were recently investigated as a matrix substitute for the mass-spectrometric analysis of sex steroids and synthetic steroid compounds in negative and positive ionization modes [54]. Besides CNTs and fullerenes, graphite also acts as an energy-transfer medium by absorbing the UV radiation, leading to thermal desorption of the analyte [55]. Sunner et al. [56] applied graphite particles and glycerol for surface-assisted laser desorption/ionization of proteins and peptides and Black et al. [57] demonstrated the use of pencil lead as an effective matrix and calibrant in LDI MS. Various groups of analytes, including peptides, polymers and actinide metals, were deposited onto a pencil lead layer before analysis. Polystyrene and poly(methylsilsesquioxane) materials derived from methyltriethoxysilane were measured in the range 100–1,000 Da by LDI MS using a graphite plate without a matrix [58]. Clean mass spectra were obtained without background signals from carbon clusters. Graphite plates were also utilized in visible surface-assisted desorption/ionization MS with a 532-nm laser for small macromolecules such as synthetic polymers and biomolecules [59].

Moreover, nanoparticles with magnetic properties such as manganese oxide magnetic nanoparticles ($d=5.6$ nm) coated with functional silicates were successfully used in LDI MS [60]. This technique was termed “nanoparticle-assisted LDI MS” (nano-PALDI MS). The silicate sheet improved the transfer of energy between the metal oxide core and the analyte, thereby causing ionization of the analytes by the nanoparticles. Analytes such as reserpine, insulin and other peptides were analysed through nano-PALDI MS and no background signals could be observed. Lin et al. [61] demonstrated the use of functionalized magnetic nanoparticles for the simultaneous extraction and detection of small molecules through MALDI MS. The authors conjugated conventional MALDI matrices such as DHB and CHCA with magnetic Fe_2O_3 nanoparticles. Salicylamide, mefenamic acid, ketoprofen, flufenamic acid, sulindac and prednisolon could be successfully analysed by MALDI MS employing these conjugated matrix–nanoparticle systems.

Matrix-free MELDI

During recent years the desire for highly sensitive matrix-free LDI substrates, which are applicable for a broad range of analytes, has become more and more important. MALDI

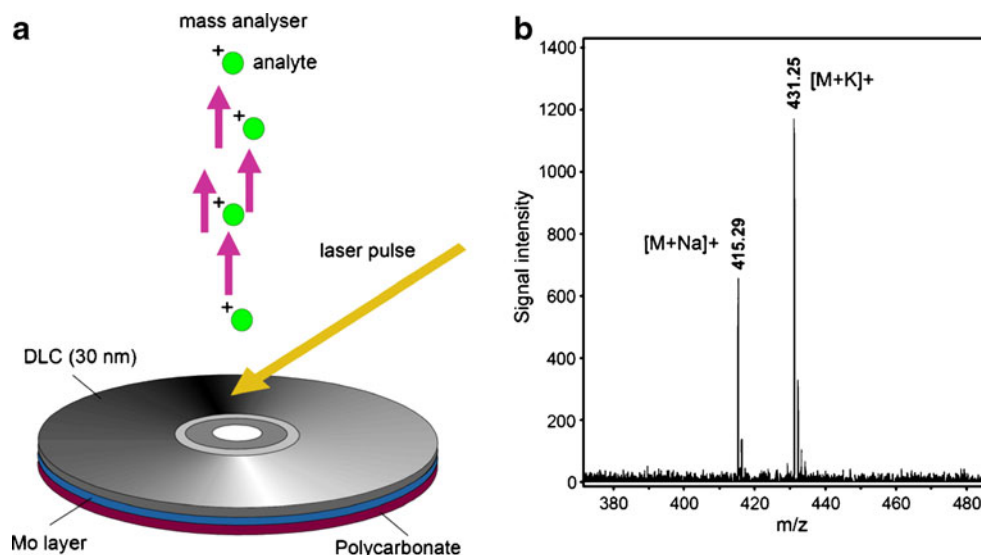
MS is often not an adequate analytical tool for detection of LMW compounds such as amino acids, carbohydrates, lipids and other metabolites, although matrix suppression can be achieved by appropriate matrix-to-analyte mixing ratios [62].

Recently, a material-based approach, termed “matrix-free MELDI MS”, was introduced which allows the qualitative analysis of LMW compounds [8]. Nanomaterials such as diamond-like carbon (DLC), silica and C_{60} fullerenes are selected in this regard as support materials because of their higher surface-to-volume ratios, expanded nanostructures, higher number of potential binding sites and sensitivity. The bulk characteristics of the materials change enormously when one moves to the nanolevel and LDI is facilitated enormously by submicrometre structures, especially when working matrix-free [63]. Surface roughness seems to play a crucial role in desorption/ionization, as very smooth surfaces hardly support laser absorption and energy transfer [64]. Moreover, parameters such as laser wavelength, pulse timing and intensity influence the desorption/ionization process. The exact timing of the laser pulse affects the signals, improving the desorption phenomenon for shorter laser pulses, whereas longer pulses can disintegrate the sample molecules. The exact mechanism of this efficiency is still not understood; however, one can argue that the physical characteristics of nanosurfaces (thermal, electrical and chemical properties) support the energy absorption and transfer [65]. Especially, the thermal properties contribute to this process. It is observed that local temperatures of the sample support surface are increased upon laser irradiation. This extra heat is transferred to the analytes to shift them to gas-phase ions [25]. However, the thermal reasons for desorption and ionization are not sufficient to explain the whole procedure. There is also speculation that the roughness of the sample support has an effect on the electronic states of excitation by laser energy, which increases the local electric field, promoting desorption and ionization [66].

DLC-coated DVD

Recently, nanostructured DLC was found to be an excellent support material for LDI MS [67]. DLC is a kind of amorphous carbon with mixed levels of sp^3 - and sp^2 -hybridized carbons [68] and the large numbers of vacancies, defects, and nanogrooves support the LDI phenomenon. A 1.4 GB polycarbonate DVD was first sputtered with a thin layer of molybdenum (approximately 30 nm), which sealed the DVD, preventing interference from the polycarbonate, and providing the required conductivity (Fig. 1). A rough layer (approximately 300 nm) of DLC was then deposited on top of the molybdenum by pulsed laser

Fig. 1 **a** A diamond-like carbon (DLC)-coated DVD, using a molybdenum metal interlayer for laser desorption/ionization (LDI) mass spectrometry. The DVD (1.4 GB) can be simultaneously used as a storage device for mass spectra. **b** Analysis of deoxycholic acid as Na^+ and K^+ adducts (molecular mass 392.58 Da). The spectrum correspond to 500 laser shots. (Reprinted with permission from Najam-ul-Haq et al. [67], copyright American Chemical Society)



deposition. The top layer's 10–12% hydrogen content made the DVD hydrophobic enough to hold samples in a defined targeted area. The observed absorptivity of the DLC layer was in the range 305–330 nm. The universal applicability of DLC-coated DVD targets was demonstrated through different analytes such as amino acids, carbohydrates, lipids, peptides, and other metabolites. Carbohydrates and amino acids were analysed as sodium and potassium adducts, whereas peptides could be detected in their protonated forms. The detection limit of the matrix-free target was found to be 10 fmol/ μL for [Glu1]-fibrinopeptide B (m/z 1,570.6) and 1 fmol/ μL for l-sorbose (Na^+ adduct). The inertness of DLC provides longer lifetimes without any deterioration in the detection sensitivity, allowing a broad applicability with high performance in metabolomics and peptidomics.

Modified silica gels

Another matrix-free MELDI technique is based on the surface modification of silica gel with 4,4'-azodianiline and 3-amino-4-hydroxybenzoic acid. In this method, a thin layer of derivatized silica gel particles is positioned on a conventional MALDI target, followed by the addition of the analyte [8]. The modified silica layer provides the basis for measuring LMW analytes employing MALDI MS. The MELDI material based on 4,4'-azodianiline provided an excellent signal-to-noise ratio and excellent signal intensities without background noise, suggesting great potential of this technique for carbohydrate and metabolite analysis (Fig. 2a). Silica of different porosity was functionalized to investigate the effect of the material's pore size on the LDI phenomenon. It could be observed that the pore size strongly affects the desorption/ionization in terms of the

number of signals and the signal intensity. Wide-pore silica derivatives (300 and 1,000 Å) resulted in high signal intensities with optimized signal-to-noise ratios, whereas silica materials with small pores (60, 120 Å) produced poor LDI mass spectra (Fig. 2b). Especially, the analysis of plant extracts confirmed the broad and successful applicability of the modified silica materials. The detection limit for xylose (m/z 150) was determined as 70 fmol. Moreover, 4,4'-azodianiline-modified silica could be successfully applied as a fast and reliable screening technology for the qualitative analysis of amino acids [69]. Amino acids were measured in positive as well as in negative ion mode without a prior derivatization of amino acids. The mass spectra revealed excellent signal intensities and signal-to-noise ratios. The detection limit was found to be 10 fmol/ μL in positive ion mode.

To circumvent the perceived limitations of MALDI, aminopyrazine (AP) and 1-chloro-4-hydroxyisoquinoline were successfully applied as new basic matrices for the analysis of LMW carbohydrates [70]. Moreover, the newly introduced matrices were employed in combination with DHB as binary mixtures. These mixtures enhanced the matrix suppression effect and allowed the analysis of small molecules such as sugars, maltooligosaccharides and maltodextrins in standard solutions and in biological samples. A detection limit of 4 fmol/ μL was achieved by employing binary mixtures of DHB-AP, which is comparable to the detection limits of other matrix-free systems.

Fullerene silica

Another approach that has been applied for the analysis of LMW compounds is the use of C_{60} fullerene-silica as energy-absorbing material [71]. Fullerene molecules were

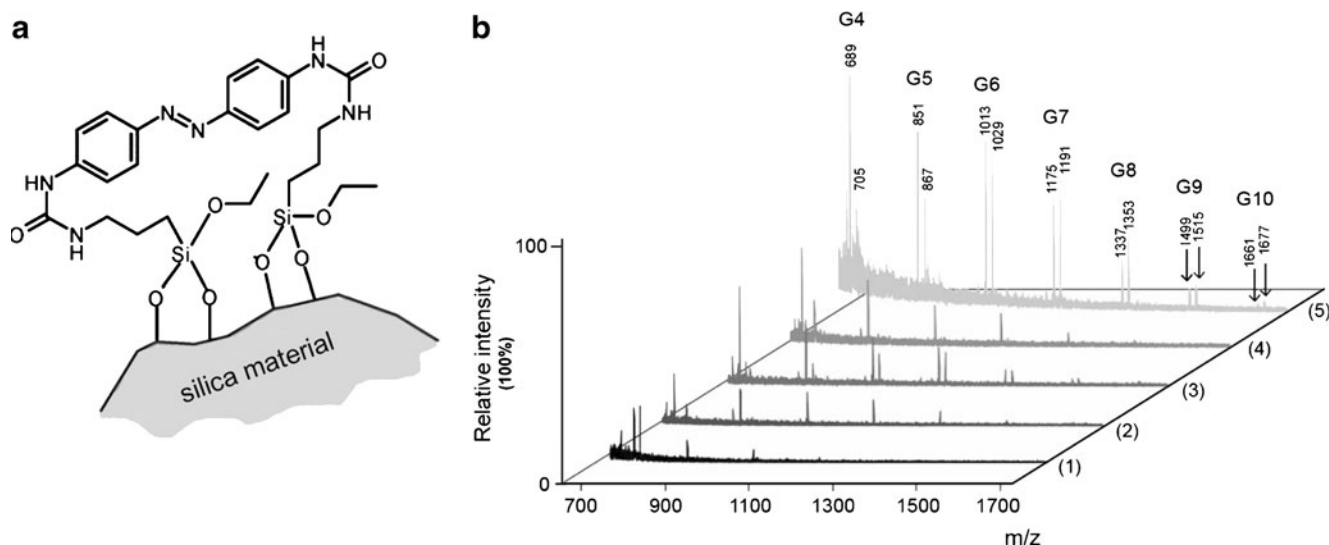


Fig. 2 **a** 4,4'-Azodianiline-modified silica. **b** LDI measurements of maltooligosaccharides G4–G10 using different silica materials: 1 silica 5 μm , 60 \AA ; 2 silica 5 μm , 120 \AA ; 3 silica 5 μm , 300 \AA ; 4 silica 5 μm , 1,000 \AA ; 5 silica 35–70 μm , 1,000 \AA . Marked signals correspond to Na^+ adducts of analytes from G4–G10 in every case. K^+ adducts of

analytes were detected only in the case of silica gel 5 μm , 300 \AA and 35–70 μm , 1,000 \AA . The spectra correspond to 500 laser shots; the amount of the samples was 0.5 μg on the target. (Reprinted with permission from Hashir et al. [8], copyright John Wiley & Sons, Inc.)

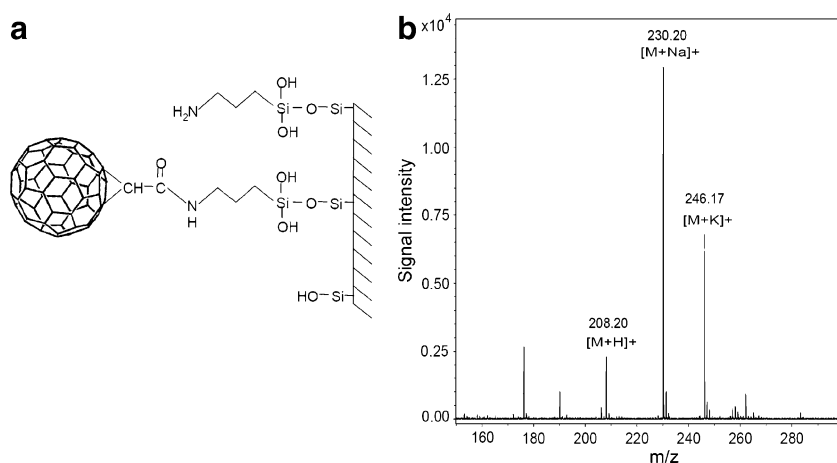
immobilized on the surface of silica gels with different pore sizes and were used as matrices for the analysis of small, biologically important molecules such as carbohydrates, amino acids and lipids (Fig. 3a). Mass-spectrometric measurements showed that both pore size and surface area are essential factors in the desorption/ionization process. From larger pores the analytes are capable of desorbing more easily; however, a high surface area can facilitate transfer of the laser energy from the fullerenes to the analytes. Fullerene–silica derivatives made from silica of 300- \AA pore size were proven to have the best properties for LDI analyses of small molecules. The size of a fullerene molecule is 70 \AA and therefore it can easily penetrate into the pores of silica of 300- or 1,000- \AA pore size. This enables attachments not only onto the outer surface of the silica particles but also inside the pores. Fullerene–silica

was proven to be an effective matrix for the analysis of small molecules in the low picomolar range. A small layer of the fullerene–silica solution was placed on a conventional steel target before the samples were added. The fullerene–silica showed almost no interferences during the measurement except in the region where C_{60} -related peaks appeared, around m/z 720 (mass of C_{60} fullerene).

Conclusion

In the low-mass region, the use of MALDI is very often limited owing to spectral interference from matrix-related ions. Although conventional MALDI matrices usually provide better results in terms of sensitivity and resolution, the spectrum below 700 Da is mainly dominated by

Fig. 3 **a** C_{60} fullerene–silica. **b** LDI time-of-flight analysis of miglitol (molecular mass 207.22 Da, 5 nmol) using fullerene–silica of 30-nm pore size. The spectrum was summarized from 500 shots. (Reprinted with permission from Szabo et al. [71], copyright by John Wiley & Sons, Inc.)



fragments, molecular ions, clusters and matrix adducts that obscure the signal for small molecules of interest. The high abundance of matrix-related ions in the laser plume causes strong space-charge effects in the ion source and deteriorates the ion optical performance. Moreover, the excessive amount of matrix ions saturates the detector, resulting in a loss of sensitivity for the analyte signal. To avoid the generation of interfering peaks caused by the use of conventional organic matrixes, the investigation of new and highly sensitive matrix-free LDI substrates has become more and more important. Especially, nanostructured materials are regarded as excellent candidates in LDI MS owing to their unique thermal, optical and electrical properties. Substantial improvement in handling and performance might increase the applicability of nanomaterials in LDI MS. In general, layer-based matrix-free systems such as DLC-coated discs or graphite plates are preferred in comparison with suspension-based systems (e.g. silica or metal oxide powders), which generally suffer from mass shifts resulting from sample preparation on the target. However, this can be overcome by the implementation of internal standards for mass calibration.

Recently, a material-based approach termed “matrix-free MELDI” was introduced for screening LMW compounds by MS. One of the main benefits of matrix-free MELDI is the adjustment of different morphological parameters such as particle size and porosity to directly enhance the LDI, resulting in improved mass spectra with minimal interferences. The broad applicability of matrix-free MELDI allows the analysis of amino acids, metabolites, flavonoids and carbohydrates on the same sample support.

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