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Mechanisms in MALDI analysis: surface interaction or incorporation of analytes?

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Abstract

Since the early days of matrix-assisted laser desorption/ionization (MALDI), the definition of a unified theory on the MALDI process is a major challenge. The new results presented in this paper clearly show that the idea of a uniform MALDI mechanism that accounts for the spreading applications has to be given up. Based on different preparation protocols distinct differences in the desorption/ionization process for carbohydrates in contrast to peptides/proteins are elucidated. Although isolated/incorporated and cluster-desorbed peptides/proteins are effectively entrained and cooled within the expanding plume of matrix clusters, as shown by a low degree of metastable analyte-ion fragmentation, a laser desorption and gas-phase cationization mechanism can be confirmed as the dominant part in ionization for neutral oligosaccharides which can be initiated even for particulate analyte material or deposits onto a matrix surface. The previously presented "lucky survivor-model" on cluster desorption of preformed ions thus needs this extension. (Int J Mass Spectrom 210/211 (2001) 121–132) © 2001 Elsevier Science B.V.

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1. Introduction

Although matrix-assisted laser desorption ionization (MALDI) [1,2] has been successfully used in mass spectrometry for almost 15 years the exact role of the matrix is still not fully understood. Three key functions of the matrix have been suggested in the early papers, i.e. incorporation of the analyte into matrix crystals [3,4], a collective absorption and ablation event [5], and an active role of the matrix in ionization [6]. Until recently, it was generally agreed that incorporation of individual analyte molecules into the crystalline host matrix, the formation of a solid solution [7], is an important prerequisite for a successful MALDI analysis even though widely different macroscopic and microscopic sample morphologies are observed for different matrices and preparation protocols [3,4,8–11]. Although homogeneous bulk incorporation was shown for several matrices, such as 2,5-dihydroxybenzoic (2,5-DHB) and succinic acid and dried droplet preparations, a redissolution of the uppermost matrix layer and analyte incorporation into

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Dedicated to Professor Nico Nibbering on the occasion of his retirement.

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a shallow surface layer has been suggested for thin-layer preparations matrices such as for 4-hydroxy-α-cyano-cinnamic acid (αCHCA, [12]), prepared as a "thin layer" of microcrystals from an organic solvent and onto which an aqueous analyte solution is dispensed [13]. This concept of analyte incorporation as a prerequisite for MALDI analyses was questioned by a recent investigation [14], which reported successful desorption of cytochrome-C ions for the case of 2,6-dihydroxy-benzoic acid (2,6-DHB). In this case a true incorporation was ruled out, but the sample was microcrystalline with a large matrix-surface area, obtained in ways similar to the "thin layer preparation". The exclusive association of analyte molecules at crystal surfaces of 2,6-DHB [15] rather than incorporation, was also shown recently by confocal laser scanning microscopy (CLSM) studies [15,16]. This raises the question, if results obtained for nanoparticle suspensions matrices in glycerol by Tanaka et al. [17] and possibly also surface-assisted laser desorption/ionization (SALDI) [18] are closely related. The latter rely on energy deposition into a strongly absorbing solid material with the analyte originally dissolved in glycerol followed by precipitation on the solid surfaces [19]. The problem if and how MALDI needs to be differentiated from such surface effects will be addressed in this paper.

A series of experiments has been carried out by several authors to study the analyte incorporation into matrix crystals. For slowly grown crystals of 2,5-DHB and succinic acid a homogeneous incorporation of analyte molecules was shown [3,20]. Beavis studied the interaction of sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid, [21]) with dye-labeled proteins (horse skeletal myoglobin, noncovalently stained with the anionic dye coomassie brilliant blue) and observed an incorporation pattern, resembling an hourglass shape. The authors concluded that for sinapinic acid crystals incorporation of the protein-dye complexes exclusively occurs at the hydrophobic $(10\bar{3})$ faces [4]. Mitchell et al. have questioned these conclusions [22]. They assign the incorporation at hydrophobic surfaces to the specific structure and properties of the dye after they had shown that identical hourglass zones were also obtained for the incorporation of the neat dye [22]. In contrast to the concept of an exclusive incorporation at hydrophobic faces, incorporation into crystals without any hydrophobic domains was observed by Horneffer et al. [14,20]. Although analyte incorporation into matrix crystals is not a necessary prerequisite for analyte ion generation, the opposite seems to be true as well. Lovell et al. [23] have reported the incorporation of guest molecules into host crystals of poppy acid. Only very weak analyte ion signals of peptides were, however, obtained from dried droplet preparations of this "matrix", doped with peptides or proteins, because a high absorption at the laser wavelength. It was concluded by these authors that based on the current understanding no predictions for analyte incorporation can be derived, based only on crystal data [24–27].

From a practical viewpoint, the analyte incorporation into matrix crystals is regarded as the reason for some of the advantages of MALDI, such as the tolerance against salts and buffers [28,29] by an in-situ clean up and separation between matrix-analyte crystals and contaminants. Depending on the matrix and the sample preparation protocol this clean up may proceed in different ways. 2,5-DHB as the most prominent example for a macroscopic separation crystallizes into a rim of crystals of hundreds of micrometers in size that all actively excludes salts, detergents, and other contaminants. These contaminants accumulate next to the large crystals and in the center of the preparation in the space between the many small matrix crystals of typically micrometers in size [3,30]. For other matrices such as sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid, SINA) [21] or 4-hydroxy-α-cyano-cinnamic acid (αCHCA) [12] a much more homogeneous, microcrystalline sample is obtained, particularly if the thin layer preparation protocol is used. These matrix crystals also exclude contaminants, but they are now distributed rather evenly across the whole preparation. Given that these matrices are usually only poorly water soluble, the contaminants can be washed away with cold water. Furthermore, the sample-, solvent- and pH-dependant incorporation [28,31] was regarded to be the reason for undesirable sample suppression effects and poor quantitative information, as well as strong spot-to-spot ion-intensity fluctuations. In gen-

eral, a sample preparation protocol resulting in homogenous matrix-analyte layers [13,29] is highly desirable for automation of MALDI-mass spectrometry (MS).

The above considerations essentially refer to the UV-MALDI analysis of peptides and proteins. Depending on the analytical intention, protocols have to be adapted, e.g. to induce a pronounced metastable fragmentation in the case of a post-source decay experiment [32,33]. For the analysis of neutral oligosaccharides [34], most successfully done with a 2,5-DHB matrix [35], best results are obtained from the inner polycrystalline area offering the cations needed for the ionization. Protocols dedicated to produce a polycrystalline homogenous matrix layer for 2,5-DHB and other matrices by distinct matrix additives [36–38] have, therefore, been developed for MALDI-MS of neutral oligosaccharides.

The goal of this paper is to expand our knowledge on the role of incorporation and state of the analyte by using new experimental approaches in addition to dried droplet and thin-layer preparation protocols. These are: analysis of doped large matrix crystals and their crushed products, of pellets of particulate matrix and analyte material, and of nanoelectrospray deposition of analyte layers onto predeposited matrix substrates; these preparation techniques yield a uniform well-defined matrix-analyte interaction, in contrast to mixed situation always resulting from standard analytical protocols. The experiments are focused on 2,5-DHB which covers a broad practical range of MALDI, since it is used for different analytes and with widely varying preparation techniques [35,39] and thus microscopic morphologies. Analytes were restricted to two, however representative, classes, peptides/small proteins and neutral oligosaccharides, known to require different preparation techniques. They represent the extreme case of analytes, which are assumed to be incorporated in the multiply protonated/deprotonated form as present in the matrix solution at its pH and also exhibiting both hydrophobic and hydrophilic domains on the one hand and a highly hydrophilic neutral compound on the other hand.

Within the frame of these experiments, it became very important to have an additional independent quantitative measure to distinguish between the different desorption/ionization behavior of different matrices, analytes, and preparation protocols in addition to the information based on the mass spectra alone. As described in [40] the initial axial velocity of the ions can serve as such a measure with high initial velocities obtained for “soft” MALDI of large peptides and proteins as opposed to small or negligible velocities in the case of laser desorption/ionization (LDI) for cationized peptides like $[\text{Gramicidin}+\text{Na}]^+$ [41]. Moreover, the ideas of a recent model for the ion formation in MALDI [42] and the desorption, respectively ablation, presented in [43,44] will be revisited and extended in the light of the experiments reported below.

The experiments addressed the following aspects:

- Item one. What is the relevance of incorporation of the analyte for the success in MALDI analysis?
- Item two. Which conclusions can be drawn for the choice of matrices and the sample preparation for the different classes of analytes?

2. Experimental setup

2.1. Samples

Peptides and carbohydrates were obtained from Sigma (Deisenhofen, Germany), matrix/additive compounds from Sigma, Aldrich or Fluka, (Germany). Samples were used as supplied commercially without further purification. As standard solvent for peptides/proteins a mixture of acetonitrile/0,1%-trifluoroacetic acid water 1:1 (v:v) (ATW) was used.

2.2. Instruments

2.2.1. MALDI-MS

The experiments were performed on a Voyager RP DE or Voyager-DE PRO time-of-flight (TOF) mass spectrometer (both from Applied Biosystems, Framingham, MA, USA). The mass spectrometers use a

Table 1
Matrices used for the studies

Matrix	Supplier	Full name	Concentration and solvents
αCHCA	Sigma	4-hydroxy-α-cyano-cinnamic acid	40 g/L aceton
2,4-DHB	Fluka	2,4-dihydroxy-benzoic acid	30 g/L ATW
2,6-DHB	Aldrich	2,6-dihydroxy-benzoic acid	saturated ATW
2,5-DHB	Aldrich	2,5-dihydroxy-benzoic acid	10 g/L ATW
3-HPA	Aldrich	3-hydroxy-picolinic acid	25 g/L H_2O
ATT	Sigma	6-aza-2-thiothymine	10 g/L acetonitrile/H_2O 1:1
ferulic acid	Aldrich	4-hydroxy-3-methoxy-cinnamic acid	8 g/L ATW
SINA	Aldrich	3,5-Dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid)	10 g/L ATW
CMBT	Aldrich	5-chloro-2-mercapto-benzathiazol	10 g/L H_2O/EtOH/THF 1:1:1
AQ	Sigma	3-amino-quinoline	20 g/L

nitrogen laser (Laser Science, Franklin, MA, USA) emitting at $\lambda=337$ nm. Some experiments were carried out using an Nd-yttrium-aluminum-garnet laser (New Wave Resarch, Sunnyvale, CA, USA) emitting at $\lambda=266$ nm. The spectra were mass calibrated with the GRAMS/386 (Galactic, Salem, NH, USA) software using internal calibration. Ion signals obtained from pellets were calibrated using external calibration with calibrants prepared on the target surface next to the sample.

2.2.2. Electrospray-MS

For nanoelectrospray (nano-ESI) mass analysis a quadrupole ion trap was used as mass analyzer (LCQ, Finnigan MAT, San Jose, CA, USA) equipped with a nano-ESI source from Protana (Odense, Denmark). The spray needles were laboratory pulled, gold-coated glass capillaries with an orifice diameter in the range of 1–2 μm. Typically 1–3 μL of the sample solution were loaded. The spray voltage was between 600–1200 V, the transfer capillary was held at temperature of 200°C.

2.3. MALDI-preparation methods

2.3.1. Large analyte-doped crystals

Analyte solutions (10^{-4}–10^{-5} M) in ATW saturated with 2,5-DHB were stored at 4°C, yielding large crystals within approximately 12 h. Such crystals can be cleaved, washed etc. as described by Xiang and Beavis [45], before being mounted in the desired orientation onto the sample holder. For the washing of large 2,5-DHB crystals ~100 μL of water was added by a pipette and sucked back immediately (3 times) to prevent substantial dissolving of the crystals. The sample holder was modified in order to adjust large crystals; these were embedded in gold sputter-coated modeling clay and their surface was aligned to the level of the standard sample holder in order to minimize fringing field effects. The same sample holder was also used for mounting pellet samples. The desorption surface of the crystals is in the same plane as are samples prepared onto normal sample plates by standard protocols, so the mass calibration and measurement of the initial velocity are identical for both cases.

2.3.2. Pellets

Pellets were prepared by grinding either crushed large analyte-doped matrix crystals or pure solid matrix material with or without solid analyte (molar ratio of matrix to analyte about 100:1 to 500:1) thoroughly in an agate mortar for 1–10 min, resulting in different grains size. Pellets of 8 mm diameter and 1–2 mm thickness were then prepared with a hydraulic press of the type applied for preparing KBr pellets for infrared absorption spectroscopy at a pressure of approximately 100 bar.

2.3.3. Dried droplet

For standard dried droplet preparations of peptides/proteins, 1 μL of matrix solution [different concentrations and solvents (see Table 1)] plus 1 μL of

Table 2
Signal quality obtained from single-crystals doped with peptide/protein before and after dissolution/drying

	Signal from crystal			Signal from redissolvated	
Analyte	Washed	Unwashed	Crunched/pellerized	Washed crystal	Unwashed crystal
cytochrome C	+++	+++	+	+++	+++
insulin	+++	+++	+	+++	+++
carbonic anhydrase	++	+++	+	++	++
maltotetraose	−	+	−	−	++

+++ = excellent (comparable to standard dried droplet), ++ = strong, + = weak, − = no ion signal observed; in the case of carbohydrates 10 mM NaCl (0.5 μL) was added prior to the drying process.

analyte solution (10^{-5} M in ATW) were mixed on the MALDI target and dried by a gentle flow of air.

For carbohydrates a microcrystalline preparation with 2,5-DHB as matrix was used [36]. Carbohydrates were prepared by adding 1 μL of the 2,5-DHB solution to 1 μL of the analyte solution and 1 μL of a 10mM NaCL solution (all in water). Samples were dried by a strong stream of cold air, resulting in a microcrystalline surface of the layer.

2.3.4. Thin-layer preparation

In the case of αCHCA 1 μL of a saturated acetone solution was applied onto the MALDI target and dried. Then 1 μL of aqueous analyte solution was dispensed onto the dried matrix surface. The matrices and concentrations/solvents used are summarized in Table 1[3,12,21,46–50].

2.3.5. Nano-electrospray deposition

The analytes were deposited onto different kinds of predeposited matrix layers (standard dried droplet, pellerized pure matrices, or thin layer preparation) via nanoelectrospray, using 2–2.5 μL of a 10^{-5} M (if not otherwise noted) solution of the peptide/proteins (ATW) or 2–2.5 μL of an aqueous solution of maltotetraose (1 g/L). The capillary orifice diameter was measured by optical microscopy. For diameters of typically about 1.5 μm very small droplets are formed which arrive at the target as dry particles and thus do not wet and redissolve the matrix surface.

2.3.6. LDI

One μL of an aqueous solution of carbohydrates (1 g/L) was dried both neat and mixed with D-talose (30 g/L) or D-glucose (30 g/L) (v:v, 1:1) on the metallic sample holder plate and investigated by LDI.

3. Results

3.1. Peptide/proteins: large analyte-doped crystals

In a first set of experiments, large crystals of 2,5-DHB were investigated in order to study the incorporation of peptides and proteins into this matrix. First, identical MALDI mass spectra, signal intensities and threshold fluences for different peptides and proteins were observed from all crystal faces as well as from inner surfaces after crystal cleavage. The frequently observed variations of the signal intensity for different MALDI target spots cannot, therefore, be attributed to variations of crystal orientation on the target. The results are summarized in Table 2. These findings are in agreement with results obtained earlier [3,20] for 2,5-DHB crystals. They are, however, in contrast to the measurements by Spengler et al. [51], who reported that substance P-doped 2,5-DHB crystals exhibit analyte anisotropy in MALDI analysis [46]. In comparison to the standard 2,5-DHB dried-droplet preparation protocol a higher resolution m/Δm for the incorporated peptide and protein analytes were found for the large crystals.

For proteins and peptides spectra were recorded with and without washing the crystals before analysis. In contrast to the dried droplet preparation the (washed) large-crystal spectra are characterized by a complete absence of alkali ions and alkali-attached molecular ions, although the stock solution was not

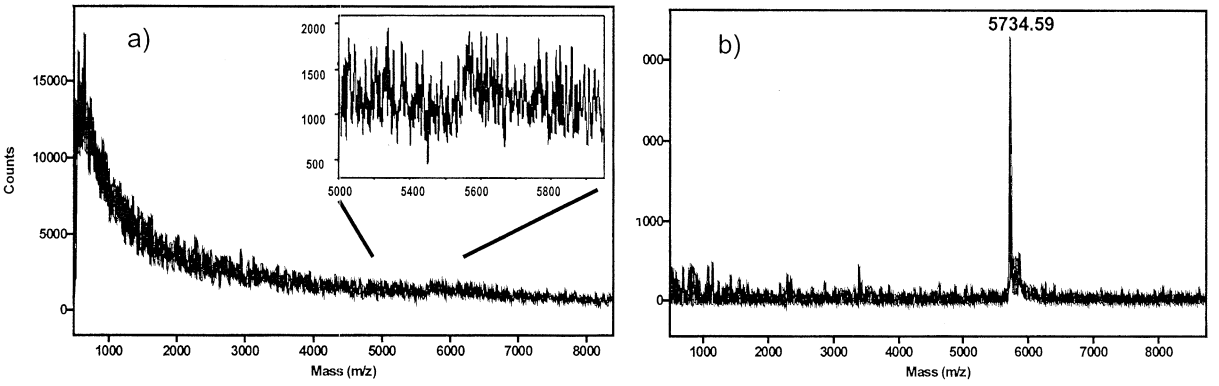

Fig. 1. MALDI mass spectra obtained from a matrix/analyte pellet (2,5-DHB/insulin), (a) directly and (b) after redissolvation (solvent ATW) of the pellet surface (same laser intensities were used for all spectra).

free of alkali impurities [3,52]. This was also observed for sinapinic acid single crystals [45]. This observation is in agreement with [53], where for 2,5-DHB dried-droplet preparation alkali-ion signals were observed from the microcrystalline inner area but not from large crystals at the rim. Moreover, the measured initial velocity of [cytochrome C+H]$^+$ ions of 550±31 m/s for all crystal faces, is in perfect agreement with the value of 543±50 m/s for insulin from a dried-droplet 2,5-DHB preparation [40]. In agreement with these results, standard dried-droplet preparation of redissolved large analyte-doped-matrix crystals showed the typical performance with strong analyte ion signal from the rim. As elucidated in [15,40], the initial ion velocity can be used as a marker to identify different incorporation and desorption behavior; for the upper case, it points to a similar situation for large crystals and the coarsely crystalline rim of 2,5-DHB.

Next, large protein-doped crystals of 2,5-DHB were ground to a fine powder. The grain size, as determined by optical microscopy, was about 100 μm after 1 min of grinding and $<$10 μm after 10 min. The powder was pressed into a pellet to produce a mechanically stable sample. MALDI MS of these pellets showed a substantial deterioration of the signal quality, weak protein signals in relation to matrix background, as compared to the intact matrix/analyte large crystals. Prolonged grinding and smaller grain size improve spectra quality to some extent. However, the typical high performance was retrieved from dried droplet preparation after redissolving the pellets.

3.2. Peptides/proteins: pellets with matrices

In order to study, whether an intimate mixture/close contact of matrix and analyte - as opposed to a "solid solution" - is sufficient for MALDI of analyte ions, pellets of a thoroughly ground mixture of solid matrix and solid peptides and proteins (i.e. lyophilized cytochrom C) have been investigated (Fig. 1); the results are summarized in Table 3. No peptide and protein signals were obtained from these preparations with the exception of 2,5-DHB which yielded poor signals. Again, prolonged grinding and small grain size somewhat improved the analytical result in this case; an ion signal can be obtained for the analyte cytochrome C after 10 min of crushing. Redissolving of the pellet brings back the typical dried-droplet performance for the peptide/protein ion signal, in agreement with the fact that a high amount of analyte was present in the pellet.

3.3. Peptides/proteins: deposition of analyte via nanoelectrospray onto predeposited matrix surfaces

Electrospray was used to load a thin film of peptide/protein on top of a matrix layer. Analytes, analyte concentration, and the orifice diameter of the glass capillaries were varied as well the matrix prep-

Table 3
Signal quality obtained from pellets from lyophylized peptide/protein and solid matrix before and after dissolution/drying

Analyte	Matrix	Analyte ion signal from pellet	Concentration and solvent	Analyte ion signal after dissolvation
cytochrome C	2,5-DHB	−	30 g/L ATW	++
cytochrome C	2,4-DHB	−	saturated ATW	+
cytochrome C	2,6-DHB	−	saturated ATW	+
cytochrome C	ferulic acid	−	10 g/L ATW	++
cytochrome C	αCHCA	−	30 g/L acetone	+++
cytochrome C	3-HPA	−	20 g/L ATW	++
insulin	2,5-DHB	+	30 g/L ATW	+++
BSA-FITC	2,5-DHB	−	30 g/L ATW	++
lysozyme	3-HPA	−	25 g/L ATW	++
melittin	2,5-DHB	+	30 g/L ATW	+++

+++ = excellent (comparable to standard dried droplet); ++ = strong, + = weak, − = no ion signal observed.

aration. By using low sample concentration and small-orifice nano-ESI capillaries, two effects were achieved: first, any wetting of the surface was avoided and second, a submonolayer coverage was generated. This technique is called "dry" deposit of analytes in the following. Glass capillaries with bigger orifice diameters (12–20 μm) generate larger initial droplets; when these droplets reach the matrix surface, they still carry enough solvent to produce a continuous liquid film on the surface ("wet" deposit), which is easily detected by a change of reflectivity of the surface.

The results of these experiments are summarized in Tables 4 and 5.

For the dry deposits, no signals of peptides and proteins were detected. This is also true for submonolayer depositions, in which problems due to the formation of a thick protein layer can be excluded. In all cases and for all matrices tested, wetting of the surface by addition of a solvent droplet brings back a medium to strong protein signal, even though inferior in quality to a standard preparation. This proves that a sufficient amount of protein was available at the surface, but desorption/ionization failed. Spectra of the analytes were obtained from all wet deposits, again usually inferior in quality to standard preparation. All attempts to generate dry depositions by spraying entire matrix-analyte solutions (in the typical concentrations for matrices as described in Table 1) failed, because a stable spray without spitting could not be achieved. This indicates that successful elec-

Table 4
Signal quality obtained from "dry" and "wet" nano electrospray deposition of peptide/protein onto a dried-droplet preparation of ferulic acid[a] in dependency on the concentration of analyte solution

		Analyte ion signal in dependency on concentration/ volume and analyte					
		10^{-5} M/1 μ1		10^{-6} M/1 μl		10^{-7} M/10 μl	
∅ capillary		Ang[b]	Ins[c]	Ang[b]	Ins[c]	Ang[b]	Ins[c]
1,5 μm	nano-ESI deposition on ferulic acid[a]	−	−	−	−	−	−
"dry"	after addition of solvent	++	++	+	++	+	+
nanoESI	complete redissolvation	++	++	++	++	+	+
15 μm	nano-ESI deposition on ferulic acid[a]	++	++	+	++	+	+
"wet"	after addition of solvent	++	++	++	++	+	+
nanoESI	complete redissolvation	++	++	++	++	+	+

+++ = excellent (comparable to standard dried droplet); ++ = strong, + = weak, − = no ion signal observed.
[a] 2 μL solution of ferulic acid (40 g/L in tetrahydrofuran).
[b] Ang: angiotensin II.
[c] Ins: bovine insulin; the experiments with insulin as analyte were performed using both the N_2-laser and the Nd-YAG laser (λ = 266 nm).

Table 5
Signal quality obtained from "dry" nano electrospray (∅ capillary 1.5 μm) deposition peptide/protein and carbohydrate analyte solution

			Analyte ion signal intensity	
analyte	Matrix	Matrix preparation protocol	"Dry" nano-ESI deposition	After redissolvation[b,c]
insulin	2,6-DHB	thin layer preparation[a]	−	+++
	2,4-DHB	thin layer preparation[a]	−	+
	2,5-DHB	dried droplet	−	++
maltotetraose	CMBT	compare to [34]	++	++
	2,5-DHB	compare to [34]	+	++
	CMBT	pellet	+	+
	2,5-DHB	pellet	++	++

+++ = excellent ++ = strong, + = weak, − = no ion signal observed.
[a] 40 g/L in acetone.
[b] for proteins: dissolution with ATW; analyte signal intensity is comparable to standard dried droplet preparation.
[c] for carbohydrates: dissolution with ATW and fast drying.

trospray MALDI sample preparation as reported in [54,55] has been carried out by wetting the matrix surface.

3.4. Carbohydrates: large analyte-doped crystals

Large crystals doped with neutral carbohydrates were also investigated before and after careful washing. The results are summarized in Table 2. Signals of the analyte were exclusively obtained from washed crystals, which were not washed before analysis.

In order to prove that this is not because of a exclusive localization of carbohydrates on the surface and thus because of a lack of incorporation, washed crystals were redissolved in water/10mM NaCl for subsequent MALDI dried droplet and nano-ESI-MS analysis. In both cases strong cationized analyte-ion signals were detected.

3.5. Carbohydrates: pellets and LDI

Pressed pellets of a mixture of solid matrix and solid neutral oligosaccharides yielded mass spectra of high quality nearly indistinguishable (see Table 6) from optimized preparation protocols (Fig. 2). Nevertheless, the large sample amounts required make this preparation technique only useful for certain applications [56–59]. The initial velocity of sodiated maltotetraose ion of about 240 m/s agrees well with that determined for standard oligosaccharide preparations of the same matrices [40].

This prompted LDI investigations of the neat sugar in order to elucidate the role of the matrix for the above experiments. Even for high sample loads and addition of NaCl, ammonium chloride, or monosaccharides, signals of larger oligosaccharides could not be obtained within the accessible laser fluence range of the instrument.

3.6. Carbohydrates: nanoelectrospray deposition

In contrast to peptides/proteins, strong carbohydrate-ion signals can be observed, if the analytes are deposited onto the matrix surface not only by wet, but also by dry nanoelectrospray (Table 5). Again the quality of the spectra is comparable to those out of a standard preparation.

Table 6
signal quality obtained from pellets of lyophylized carbohydrate and solid matrix

Analyte	Matrix	Signal from pellet
maltotetraose	2,5-DHB	+++
maltoheptaose	CMBT	++
maltoheptaose	AQ	++
maltoheptaose	ATT	++
maltoheptaose	2,5-DHB	+++
dextran 1000	2,5-DHB	+++

+++ = excellent (comparable to standard preparation [34]), ++ = strong, + = weak, − = no ion signal observed.

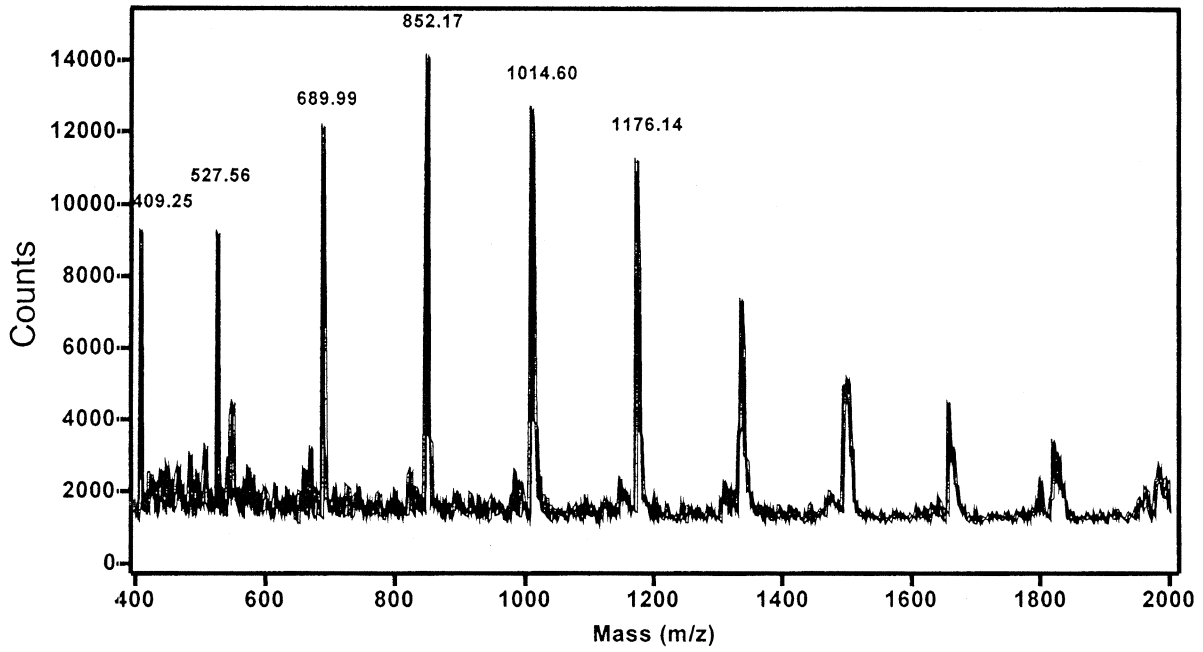

Fig. 2. MALDI mass spectrum obtained from a matrix/analyte pellet surface (2,5-DHB/carbohydrates, lyophilized dextran 1000), same laser intensity as for spectra in Fig. 1 was used. Please note that the mass spectrum is indistinguishable from the one obtained for an optimized wet preparation protocol.

4. Discussion

The results clearly prove that incorporation into matrix crystals is not the privilege of peptides and proteins. Hydrophilic oligosaccarides get embedded into 2,5-DHB upon drying as well. This rules out that incorporation is driven by hydrophobic interactions. However, salts are strongly excluded from the organic molecular lattice [3,4,53] and since neutral oligosaccharides are not easily protonated under MALDI conditions, their sodiated ions are only detected from contaminated (unwashed) surfaces of large crystals, whereas no ion signals are recorded after washing. Moreover, it is reasonable to assume that at least some of the many organic compounds, which have been tested and were found to not constitute good matrices, do nonetheless incorporate analytes.

Based on the above results, it is proposed that those matrices, which yield ions of large analyte molecule ($>$ about 30 kDa), incorporate analytes in all wet preparation protocols. This incorporation can either take place into the bulk of macroscopic crystals, as is the case for 2,5-DHB, or at least into shallow surface layers of smaller crystallites as one assumes, e.g. for thin layer preparations. In this view, because of the fast precipitation of αCHCA during a dried-droplet preparation, the final state will not be strongly different from a true thin-layer preparation procedure. Even though this incorporation and solid-solution formation is unavoidable in wet preparation at least for many common matrices, it is not required since some matrices show analyte ions up to about 30 kDa in mass without incorporation, provided a large specific surface is offered [14]. Unexpectedly, the major differences appear to be associated with the different classes of analytes rather than the widely varied preparation protocols examined in the experiments. Whereas for peptides and proteins sample preparation protocols not including a wet crystallization step failed or yielded strongly inferior results, oligosaccharides delivered results, often indistinguishable from optimized preparation protocols, even in those cases

where no peptide ions were detected. The relative differences between peptides and oligosaccharides, as reported in this paper for the pellet preparation, are highly significant, but it cannot be ruled out that a further variation and optimization of the grinding and pelleting protocol will significantly improve the detectability of peptide and protein ions. A more thorough grinding of the lyophilized material (matrix and peptide/protein) in the motar did indeed result in smaller particles sizes and improvement of the peptide/protein signals from these pellets. The results do, however, not allow to conclude, whether the increase of the specific surface simply increases the number of accessible analyte molecules within the desorption area, or whether this also improves the analyte isolation on the surface.

The observed strong differences between peptides/proteins and oligosaccharides for "dry spray" raise another important question. Does this difference reflect mostly the different (gas phase?) (cat)ionization, or does it signal a different interaction between the analytes and the matrix molecules particularly at/near the surface and a different desorption mechanism as a result of this? Gas-phase cationization was identified as one possible mechanism of ionization in MALDI by Wang et al. [60]. Liao et al. [61] also proposed this mechanism among other possible ionization mechanisms, which were summarized by Zenobi et al. [62]. For LDI of apolar polymers it is widely assumed, that cationization takes place in the gas phase [63]. Further MALDI experiments [58,64,65] supported a gas-phase mechanism.

Vaporizing ("exploding") grains of matrix material may be sufficient to generate neutral gas-phase oligosaccharide molecules and salt cations for gas-phase cationization. Codesorption of these two components possibly of only a very superficial sample layer may be sufficient for a high-quality MALDI mass spectrum. The agreement in initial velocities for carbohydrates between the different preparations including standard analytical protocols may even point to the fact that all protocols result in very similar starting conditions. The failure to obtain spectra of oligosaccharides by LDI from simple metal surfaces, however, clearly demonstrates that even for carbohydrates the matrix serves more functions than just energy absorption and transfer. The significant decrease in spectral quality of peptide and protein desorption-ionization for matrix-analyte pellets or their complete failure in the case of "dry sprayed" analytes points to the important effect of the matrix to overcome/reduce analyte clustering and ion-pair (protein counterion) interactions upon incorporation of these analytes [42] as well as to the role of cooling in the cluster ablation process [40,42,44]. The softest possible desorption of proteins, i.e. their detectability with low metastable fragmentation in a reflector configuration or in a linear TOF for larger ones [66] is only observed for matrices which exhibit incorporation into the bulk of large crystals, and is practically only achieved for matrices like 2,5-DHB. It is not surprising that small and stable cyclic peptides, gramicidin S in particular, form intermediate cases. In the latter case even LDI of the neat component is possible and has thus often been used as a misleading test sample for soft desorption. The detection of intense signal of sodiated species in this case gives further evidence for a "LDI-like" desorption process.

5. Conclusions

The main result of this paper is that the idea of a uniform MALDI mechanism may have to be given up. There seems to be a gradual transition between processes more related to LDI with a matrix "support" effect and "true MALDI" which is needed for formation of large protein ions with minimized internal excitation. It is suggested that this gradual transition is based on two different desorption processes, sublimation and ablation, as described in the molecular dynamics model from Zhigilei et al. [43]. This model of desorption has been connected with the results for the initial velocity [40] and was included in a recently proposed model for ionization via clusters [42]. In sublimation, analyte neutrals are transferred into the gas phase and cationized by gas-phase ion-neutral

reactions with coevaporated ions. In ablation, the matrix-analyte solid is expelled into the gas phase via small clusters, which decompose and shrink by matrix neutral evaporation. These clusters may be charged by excess or deficit of ions or by matrix photochemical ionization processes and the final ions observed stem from those cluster components exhibiting the largest affinity for the available charge (e.g. protons). In this view, the ablation process is characterized by a large initial velocity of the ions with a strong forward component (high axial-to-radial velocity ratio) [67] whereas sublimation results in lower initial velocities and less pronounced forward characteristics; thus the initial velocity acts as an independent control measure. Even though still a model, the distinct differences between the initial velocities for cationized analyte ions and protonated peptide ions can easily be explained. Within this hypothesis, three important effects are connected with ablative MALDI, first the incorporation of the analyte in a solid organic host crystal with the reduction of ionic interactions for charged analyte (such as peptides and proteins) and cooling within the jet of cluster. This is why large proteins need larger matrix crystals in order to produce ions with a low internal excitation and thus a sufficiently large lifetime to be detected in a reflector TOF or even linear TOF for larger ones.

The results, discussion, and models presented in this paper focus on the unexpected strong differences for different analytes, proteins, and carbohydrates. Since the model is hypothetical and in the view of the results presented, new questions have to be addressed in more specified experiments. These are: Which is the initial velocity of modified carbohydrates which can be protonated, what is the accessible mass range and spectral quality for “nonincorporating” matrices like 2,6-DHB or thin-layer preparation techniques. The mass spectral changes below and above ionization threshold have to be carefully reinvestigated to distinguish between sublimation and ablation as the dominant processes in desorption. Further experiments on the crushing of solid matrix and analyte material with respect to the grain size and applied pressure are in preparation, also.

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