Ion Formation in MALDI: The Cluster Ionization Mechanism

Michael Karas* and Ralf Krüger

Institute of Pharmaceutical Chemistry, Biocenter, JW Goethe-University of Frankfurt, Marie-Curie-Strasse 9-11, D-60439 Frankfurt, Germany

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Contents

I. Introduction

Since the end of the 1960s, a major goal has been to tear down the volatility barrier for the use of mass spectrometry and to provide the access to polar and ionic samples of increasing molecular mass. New "desorption" techniques have emerged and matured, but today all have been replaced by two new techniques originating from the 1980s, electrospray ionization and (ultraviolet-laser) matrix-assisted laser desorption ionization (UV-MALDI). On the basis of the investigation of the wavelength influence in ultraviolet-laser desorption,[1] the concept for UV-MALDI was developed between 1984 and 1986 and summarized in a 1987 paper.[2] It introduced a specific matrix consisting of small organic compounds (such as nicotinic acid) exhibiting a strong resonance absorption at the laser wavelength used. The matrix effect was described to be 3-fold: first, a controllable energy transfer to the condensed-phase matrix-analyte mixture inducing a "uniform and soft desorption", second, a promotion of ionization by chemical reactions, and third, to generate "favourable prerequisites" by isolating analyte molecules in the excess matrix. An alternative matrix approach for LDI, which uses glycerol as a solvent and a fine metal powder as an absorber for the laser energy, was reported by Tanaka in 1987 and published in 1988[3] showing singly charged protein molecular ions up to about 35 kDa. Protein mass spectra in this size range, however, had been reported before for plasma desorption[4,5] and fast-atom bombardment.[6] The inherent superiority of the UV-MALDI approach with respect to ion stability and sensitivity became soon obvious after its application to larger proteins[7] and its spread to other research groups, and finally both electrospray ionization (ESI) and UV-MALDI tore down the barriers that had until then prevented a broad use of mass spectrometry in the field of biopolymer analysis. It is interesting to note that today, despite the big attention to high molecular mass analytes, the majority of applications of both MALDI and ESI deal with the analysis of small and medium-sized peptides.

Due to the pulsed generation of ions, the MALDI technique lent itself to a combination to time-of-flight (TOF) mass analyzers which until then only played a niche role. The implementation of "time-lag focusing",[8] or "delayed-ion extraction",[9,10] respectively, which raised the performance and robustness of MALDI-TOF mass spectrometers considerably, strongly promoted the spreading of the technique. For many nonexperts, "MALDI-TOF" has become a linked synonym. However, MALDI is first an ionization technique, and today, because of some inherent advantages of the MALDI process, such as relatively low sample requirements, sensitivity, and straightforward mixture analysis, the coupling of MALDI to nearly every mass analyzer available has been either exemplified or is already in use and commercially available.[11−21] As an example, the MALDI mass spectrum of myoglobin obtained in a reflectron TOF instrument is shown in Figure 1; the extension shows the matrix region.

The broad applications of MALDI in many fields and its strong impact in the field of proteomics,[22−24] however, did not induce a vital discussion of the fundamental processes, but rather generated a set of empirical protocols for sample handling, pretreatment, and preparation depending on the class of analyte to be investigated. Investigation and discussion of fundamentals have been and still are limited to a small number of groups, and, admittedly, progress has been limited. MALDI "theory" or "models" are still crude and far away from having predictive power. There was a common agreement on the key functions of the matrix, i.e., incorporation and isolation of analyte molecules in the host matrix, their release into the vacuum upon disintegration of the matrix−analyte solid after laser-energy deposition,

* Corresponding author: Michael Karas, Institute for Pharmaceutical Chemistry, J.-W. Goethe University Frankfurt, Marie-Curie-Str. 9, D-60439 Frankfurt, Germany. Phone: ++49 69 798-29916. FAX: ++49 69 798-29918. E-mail: karas@iachem.de.

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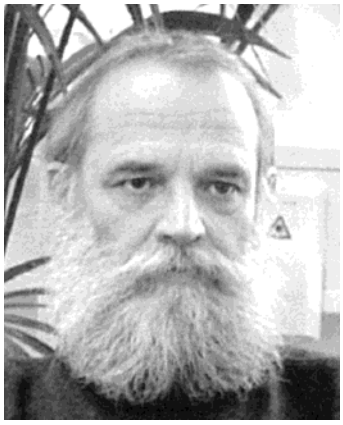

Michael Karas was born in 1952 in Wesel, Germany. In 1982, he received his Ph.D. in physical chemistry after his studies of chemistry at the University of Bonn. In 1983, he joined the research group of Franz Hillenkamp, Department of Physics, University of Frankfurt. In Frankfurt and starting from 1987 in the Institute of Medical Physics/Medical Faculty at the University of Münster the fundamental studies establishing the MALDI technique have been carried out. In 1992, he finalized his Habilitation in Physical Chemistry and became Assistant Professor at the University of Münster. Since 1995 M. K. is full professor at the University of Frankfurt, first in the Chemistry Department and since 2001 in the Institute of Pharmaceutical Chemistry in the Department of Chemical and Pharmaceutical Sciences. In 1997, he was selected for the ASMS award for a "Distinguished Contribution in Mass Spectrometry" for the development of the MALDI technique (together with Franz Hillenkamp). His current research interests are defined by a broad use of MALDI- and ESI mass spectrometry to bioanalytical problems, spanning the range from bioavailability studies for phytopharmaceuticals to methodic studies for membrane protein analysis and RNA–ligand interactions.

Ralf Krüger was born in Bonn (Germany) and studied chemistry, first in Bonn and later in Marburg, where he began to focus on analytical chemistry. Due to his interest in environmental trace analysis he moved to the Instiute for Spectrochemistry and Applied Spectroscopy (ISAS) in Dortmund, where he worked on SPE enrichment and derivatization of pesticides from drinking water for GC-MS analysis. After he had finished his diploma thesis at the ISAS and had obtained his diploma degree at the University of Marburg in 1998, he joined the instrumental analytical chemistry group of Prof. Michael Karas at the University of Frankfurt to work in the field of bioanalytical mass spectrometry. While he also spent some time on nanoESI-MS and MALDI applications, soon the fundamentals of MALDI, and in particular the elucidation of the state of the analyte in the host matrix crystal, became his major objective. Currently, he is about to finish his Ph.D. thesis on that topic and is on the move to join the group of Prof. Wolf-Dieter Lehmann at the German Cancer Research Center (DKFZ) in Heidelberg to work on the analysis of protein phosphorylation by ICP and ESI mass spectrometry.

and their ionization by ion−molecule reactions in the laser plume including matrix reactive ionic species.[25−28] However, this three-step model was neither specific enough to account for the very different experimental

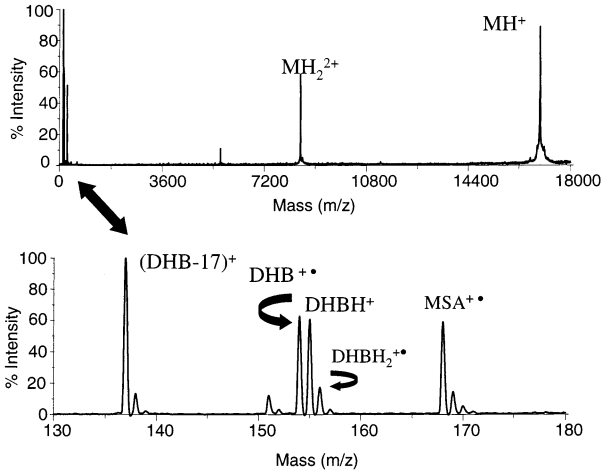

Figure 1. MALDI-TOF mass spectrum of myoglobin obtained with the matrix mixture DHBs (2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, 9:1/v:v); the lower panel shows the enlarged matrix region.

situations, analytes investigated, and preparation protocols applied, nor detailed enough to explain many of the striking characteristic features of MALDI. In many cases, they were focusing on one aspect, i.e., the transition from the solid state to the gas phase from a physical viewpoint[29−38] or the ionization from a chemical or thermodynamic one.[26,28,39−50] The key problem in investigating MALDI is that it is a complex chemical event (one-laser-shot-one-mass-spectrum); it is, moreover, happening within the time scale of a few nanoseconds. Despite the involvement of a very low absolute amount of material, a dense plume of material containing matrix neutrals as well as reactive species, such as matrix radicals, electrons, hydrogen atoms[51] is formed which is expanding into the vacuum of the mass spectrometer ion source. Within this plume regardless of a low average degree of ionization, suitable analytes can be ionized with very high efficiency and thus detected with high sensitivity.

II. Limitations of Current Models

With respect to a unified and more comprehensive picture of the MALDI mechanism, the three-step model suffered from the major limitation that each step was considered individually and separately.

The only apparent characteristic measure for the MALDI process is the laser fluence applied. The rationale to focus on it seemed to be very clear. The laser fluence is indeed the critical parameter to be adjusted practically in MALDI-TOF instruments. It turned out, however, that there is no direct correlation between fluence, deduced energy/volume data, and the internal energy of the ions generated.[35,52] It is clear today that many detrimental effects of high laser fluences are caused by secondary effects, e.g., by collisions in the acceleration phase in axial MALDI-TOF systems,[53] and do not show up in mass analyzers which effectively decouple ionization and mass analysis.[11,12] It was found that the initial ion velocity which can be straightforwardly determined in delayed-extraction MALDI-TOF systems[54,55] is a superior tool to characterize MALDI processes and to separate out boundary cases[56] (see section III).

The hypothesis of incorporation of analyte molecules into the bulk crystalline matrix was substantiated by generating single crystals of matrix containing proteins and yielding excellent MALDI performance.[57−63] However, new practically successful preparation protocols rather appeared to use a surface deposit of analytes.[64−66] Moreover, the spread of applications from larger polar biopolymers (peptides/proteins, oligonucleotides) to nearly every possible class of analyte (technical polymers, small polar and unpolar compounds) made it questionable whether there is one MALDI mechanism. The question of the state of the analyte molecules in the matrix, i.e., the starting conditions for any desorption and ionization event, and its role was not addressed until recently.[67]

One major consequence of the separation into single mechanistic steps was that the details of the solid−gas-phase transition were not regarded with respect to their relevance for ionization. An "instantaneous" transition from the solid to the gaseous state or the "formation of a gas jet"[68] as a result of laser excitation was used as a starting point to describe and broadly discuss gas-phase ionization processes and subsequent ion−molecule reactions. On the basis of this picture, a "photochemical" ionization mechanism was favored in the initial MALDI papers,[2,25,26] essentially considering the generation of neutral analyte species as the first step, which are then charged by proton-transfer reactions with reactive matrix species in the expanding gaseous plume. Following this gas-phase approach, every possible primary ionization step from simple cationization of neutrals to excited-state acid−base chemistry has been considered.[28,39−50] However, while the principal possibility of these different ionization mechanism was typically substantiated by energetic considerations, their practical role was often not elucidated by experiments, especially in relation to other possible and suggested mechanisms.

A first step to overcome these limitations was suggested recently.[69] The major point was to include cluster formation and the generation of charged clusters by either charge separation (deficit/excess of anions or cations) or photochemical processes as an important primary ionization step. The following will focus on this cluster ionization mechanism and it will be shown that it can (at least) qualitatively account for the complex MALDI ionization behavior. Moreover, the thorough investigation of the energetics of ion−molecule reactions in the laser plume[28] as a tool to explain the ions finally showing up in the MALDI mass spectra still keep their value and relevance when clusters (and not the gas phase) are regarded to form "chemical vials" for those reactions.

III. Characterization of the MALDI Ablation Process

Within the years, MALDI applications spreaded out considerably. Still the majority of the applications was and is in the peptide/protein area; however, oligonucleotides, oligosaccharides, technical polymers, and also low-molecular weight polar compounds were successfully investigated. In many cases, the special requirements for analysis of new compound classes prompted the development of dedicated preparation protocols. The original sample preparation protocol used a simple drying of a matrix-sample solution to a crystalline deposit ("dried-droplet"). It was hypothesized, substantiated by experiments, and generally agreed upon that the incorporation of the analyte into matrix crystals is an important prerequisite for a successful MALDI analysis.[57−63] However, new and dedicated protocols developed for and applied to certain analytical problems appeared to contradict this consensus. First, the incorporation of analyte into matrix crystals became questionable for the so-called surface preparation protocol[64−66] for which the analyte to applied from a solution on top of a predeposited matrix layer. At the extreme, a physical mixing of dry matrix and analyte was successfully applied for certain analytes.[70,71]

With the advent of delayed-extraction MALDI-TOF instruments not only the practical performance of MALDI-TOF instruments was substantially improved, but also a fundamental property of the MALDI ions became experimentally accessible, i.e., their average initial velocity.[54,55] This initial ion velocity became an extremely valuable tool to characterize MALDI processes, first to elucidate essentials of the MALDI process and second to intercompare different MALDI preparation protocols and to elucidate possible fluent transitions between MALDI and LDI with some matrix support, which would otherwise confuse the fundamental reflection.

A. Initial Ion Velocity and Cluster Formation

Characteristic for MALDI of peptides and proteins is a matrix-dependent high initial ion velocity of the analyte ions, typically between 300 and 800 m/s.[54,55] This initial ion velocity is in a first approximation independent of analyte mass and also of charge state and ion polarity. It is also not affected by the laser fluence within the analytically useful fluence range. Only those experimental variables affecting crystallization, such as solvents and additives, may result in a significant shift of v_0, with both directions occurring (some typical values of initial ion velocities are depicted in Table 1).

Different initial velocities are sometimes also observable for one matrix and can typically be attributed to areas of different crystallization state, such as a coarsely crystalline droplet rim and a microcrystalline inner part. Even though the absolute value of the initial velocity is to a certain extent depending on the method of determination, typically rather shifted to even larger values if not determined by the DE method,[68,72,73] relative differences between matrixes and crystalline deposits are consistently reproduced. The high initial velocity shows a considerable spread ($v_0 \pm 0.5v_0$) which is responsible for limitations in linear TOF analysis and can be further compensated for in reflectron configurations. The axial initial velocity is considerably larger than the radial velocity,[73−75] pointing to a strongly forwarded orientation of the MALDI plume.

This, together with the numerical simulations performed by Zhigilei and Garrison,[76−79] lead to the new picture that the generation and emission of

Table 1. Initial Ion Velocities (v_0) of Some Analyte Ions Using Different Matrices

analyte	matrix	ion	*m/z*	v_0
bovine insulin	SINA	$[M + H]^+$	5735	332 ± 29
bovine insulin	αCHCA	$[M + H]^+$	5735	291 ± 51
bovine insulin	αCHCA	$[M + 2H]^{2+}$	2868	251 ± 75
bovine insulin	3-HPA	$[M + H]^+$	5735	620 ± 87
DNA-24mer	3-HPA	$[M + nH]^{n+}$ ($n = 1,2$)	7374/3687	619 ± 16
bovine insulin	THAP	$[M + H]^+$	5735	306 ± 32
bovine insulin	THAP + THAC	$[M + H]^+$	5735	201 ± 19
bovine insulin	2,5-DHB	$[M - H]^-$	5733	535 ± 48
bovine insulin	2,5-DHB	$[M + H]^+$	5735	543 ± 40
bovine insulin	2,5-DHB + fructose	$[M + H]^+$	5735	659 ± 49
carbonic anhydrase	2,5-DHB	$[M + H]^+$	29025	599 ± 119
melittin	2,5-DHB	$[M + H]^+$	2848	530 ± 52
maltoheptaose glycosylamine	2,5-DHB	$[M + H]^+$	1154	515 ± 66
maltotetraose	2,5-DHB	$[M + Na]^+$	689	187 ± 97
human-milk oligosaccharide 1	2,5-DHB	$[M + Na]^+$	730	340 ± 10
human-milk oligosaccharide 2	2,5-DHB	$[M + Na]^+$	1242	461 ± 04
human-milk oligosaccharide 3	2,5-DHB	$[M + Na]^+$	2776	545 ± 29

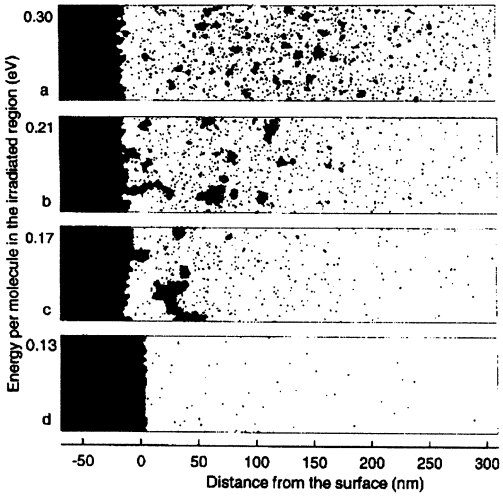

Figure 2. Snapshots of the laser plume at 500 ps as a function of the deposited laser energy. (Reprinted with permission from ref 76 Copyright 1997 Elsevier Science.)

matrix clusters is the fundamental ablation phenomenom. An illustration of the numerical simulation results giving snapshots for MALDI ablation at different fluences is shown in Figure 2, taken from ref 76. This cluster emission is accompanied by neutral evaporation, already occurring at lower laser fluences; this has been both predicted theoretically[76−79] and determined experimentally.[35]

Further evidence for the generation of MALDI ions from high mass precursors was given recently by measurements of initial ion velocities using a repulsive potential in the ion source.[80] Deviations from the linear relationship between the ion time-of-flight and the delay time were attributed to decomposition and desolvation of high-mass precursors after leaving the target. From the determined initial velocity data, an average cluster size of about $m/z = 3000$ in the case of matrix ions and about $m/z = 10.000-40.000$ in the case of peptide analytes was calculated for the precursors.

Whereas these clusters (of matrix and of analyte−matrix) do not show up directly in MALDI mass spectra, the detection of analyte ions clustered with a distribution of matrix molecules was promoted by raising the pressure in a MALDI source (to about 2 mbar) coupled to an orthogonal TOF mass analyzer.[81] This indicates that under these conditions, the cooling of the clusters in the RT bath gas is sufficient to stop the cluster decay at an intermediate stage. A series of MALDI mass spectra of myoglobin obtained at different pressures in the ion source is shown in Figure 3.

The generation of high molecular weight clusters by MALDI was shown recently by trapping these clusters with *m/z* exceeding 5×10^9 in a quadrupole ion trap.[82] Although particle detection of high mass ions was not very efficient, some clusters were nevertheless detected, and, moreover, fluorescence spectra of the clusters were obtained. But also the particles themselves can be visualized by trapping them on a sample plate and consecutive investigation of the surface using atomic force microscopy (AFM).[83] It turned out that the plate was covered with small, isolated deposits of matrix, which had diameters of about 200 nm. In addition, a small matrix film of 1 nm thickness covered the substrate. The particle production was strongly fluence dependent: Whereas no deposits were found when laser fluences below the threshold for MALDI ion production were used, the amount of ablated material accumulated to a 20 nm matrix film together with the 200 nm deposits in case of a laser energy corresponding to four times the MALDI threshold.

Striking evidence for the general role of clusters in MALDI can moreover be drawn from a recent paper reporting on the coupling of an intermediate-pressure MALDI source to a quadrupole ion-trap mass spectrometer.[84] By carrying out tandem MS analyses on randomly selected noise peaks in the mass range up to 2000 Da, it showed up that the ubiquitous chemical noise, which makes up the majority of the total number of ions formed in MALDI, is to a large extent consisting of matrix clusters of the general form $[(\text{matrix})_n + (\text{matrix}-H_2O)_m + X^+]$ with X^+ forming a yet unknown and variable charged species. This is depicted in Figure 4 taken from ref 84. It was shown that these cluster species mainly consist of only weakly bound molecules and that cluster decomposition can be induced by application of moderate collisional activation over a selected mass range, leaving behind species that need more energy to fragment, e.g., peptide analyte

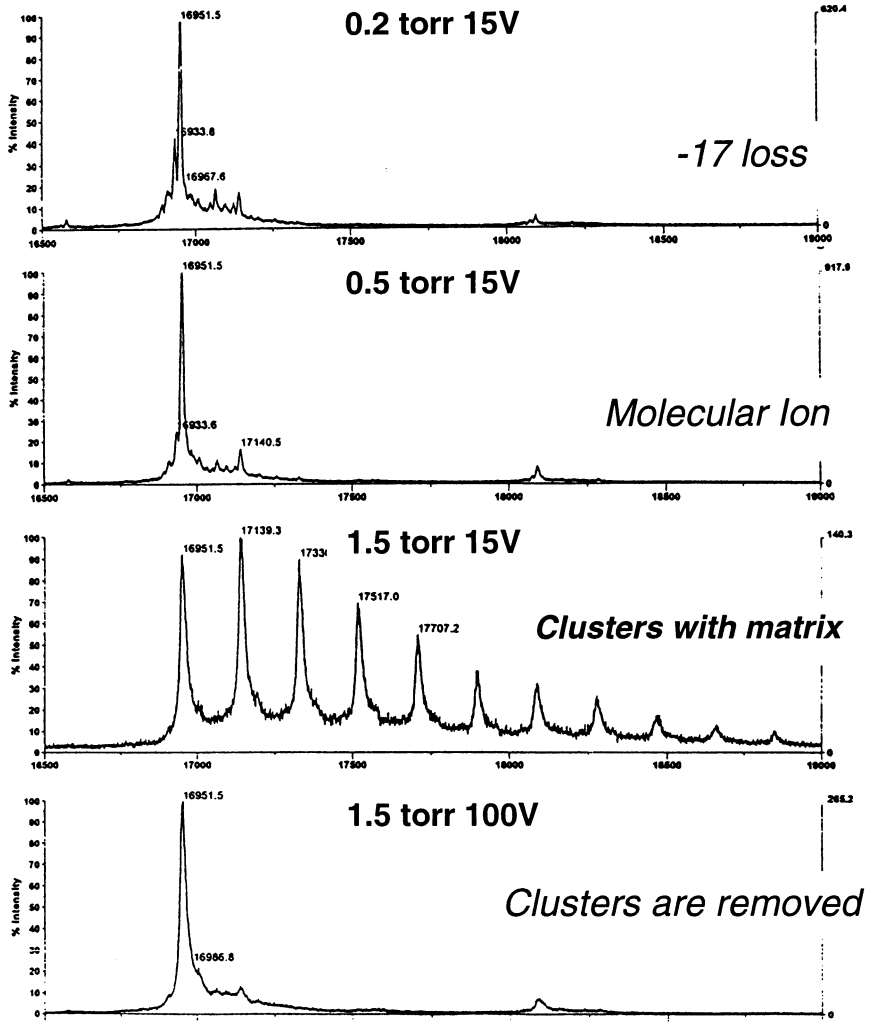

Figure 3. MALDI mass spectra of myoglobin obtained with an orthogonal TOF analyzer using different pressures in the first pumping stage. (The authors thank A. Verentchikov for the figure, which was presented on a poster at the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, Tx, 1999; see also ref 81).

ions. Moreover, the strongly forwarded MALDI plume itself was visualized by laser-induced fluorescence.[85,86]

The emission of clusters is to be understood in contrast to the idea of a dense gas jet expanding into the vacuum and entraining the analytes and accounts well for the uniform analyte ion velocities, whereas a mass-dependent slip would be expected in a case of a gas jet (consisting essentially of matrix neutrals) due to the increasingly ineffective entrainment of larger molecules. A more or less pronounced mass dependence of the initial ion velocity was indeed reported in some papers,[54,68,72,73] but field penetration into the nominally zero-field expansion region has a similar effect and can lead to an overestimation of v_0 for small molecules. As pointed out, the major qualitative influence on the initial ion velocity is determined by the matrix and the preparation conditions. Irrespective of the absolute numbers these relative differences between matrixes are reproduced for all measurements.

On the basis of this cluster approach, the threshold behavior of MALDI ionization was reinterpreted: it is now regarded as the critical point when the accumulated excitational energy density in the matrix solid reaches a critical value to induce an explosive cluster emission, and ionization is consequently linked to the occurrence of these clusters.

B. Characterization of Different MALDI Regimes Using Initial Ion Velocity Measurements

Only in certain and well-restricted cases, analyte ions of low velocity (≤ 200 m/s) are observed.[55,56] These still analytically successful cases are restricted to small neutral (noncharged) analytes typically showing up as cationized species. For small neutral oligosaccharides (and other neutral small to medium molecular mass compounds such as technical polymers)[56] a close physical mixture of matrix and analyte was found sufficient. This was either achieved by direct physical mixing of finely powdered material, by electrospraying analyte solutions on top of a matrix layer or within finely crystalline areas of a dried droplet deposit. Even though these analytes are also incorporated into matrix crystal upon conventional wet preparation—proven by redissolvation and a subsequent recrystallization by a new wet preparation—their analysis fails from (washed) bulk crystals due to the low level of cations/salts included and due to lacking (de)protonation sites.

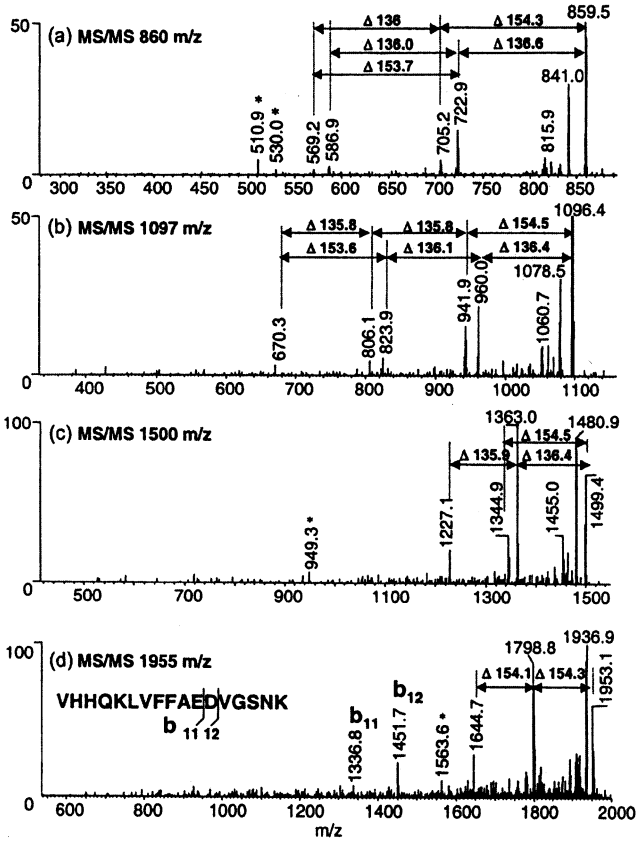

Figure 4. MALDI-MS/MS mass spectra of a peptide-containing solution obtained from a selection of *m*/*z* values. These include *m*/*z* values (860, 1097, 1500) where no peptide signals are expected as well as one *m*/*z* value (1955) where a pepetide signal is expected. Characteristic losses of intact molecules of DHB (154 Da) and molecules of DHB with eliminated water (136 Da) from the parent species are indicated. The asterisk indicates unexplained ion peaks. (Reprinted with permission from ref 84 Copyright 2002 Elsevier Science.)

It was therefore concluded that analyte evaporation is still possible in this case without destruction and that ionization is due to gas-phase attachment of codesorbed alkali cations to analyte neutrals, in agreement with earlier assumptions of a gas-phase cationization process.[27,87−90] The role of the matrix is thus restricted to facilitate a controllable energy transfer to the solid matrix−analyte mixture via the matrix particles and to provide a source for cations. However, in the extreme case a matrix is not required at all and energy transfer may be accomplished by the metallic substrate. In these cases, the initial velocity determined is close to "0", as is the initial velocity of alkaline ions generated from the metallic substrate. Only if refined protocols are applied in solvent-free preparation for the generation of fine-powder matrix−analyte composites, larger analyte (larger peptides and polysterene up to 100 kDa) can be successfully detected.[70,71] It is an open question whether this protocol finally also results in an incorporation of analyte molecules; initial ion velocities that would deliver further information have, however, not been determined until now.

With growing molecular mass of neutral analytes, well observable within analyte oligomer distributions (oligosaccharides and technical polymers), a gradual transition to the high v_0 level, as characteristic for peptide and proteins, is observed,[91] which is clearly not compatible with a general gas phase cationization mechanism as described above. Charged, i.e., ionic compounds, are always determined with high initial velocities, unless they are thermally stable such as quaternary ammonium compounds. This already occurs for a derivatized (aminated) tetrasaccharide. Since acidic/anionic compounds, such as sialylated oligosaccharides, are still detected in their (partially) cationized form, it was concluded that these analytes drag their cations into the matrix crystals, in agreement with earlier assumptions that certain analyte classes must be incorporated as preformed cationized ions, i.e., high-mass polymers.[92]

In contrast to small neutral analytes, ionic analytes cannot be transferred into the gas phase by evaporation without destruction. This fully agrees with the fact that typical peptides and proteins, which are highly protonated at the typically low pH used in MALDI preparation protocols, have always been observed with high matrix-specific initial velocities, as well as oligonucleotides. The gradual increase in initial velocity for oligomer-distribution samples moreover shows that the same is true also for non-ionic analytes with growing molecular mass, typically, the high peptide/protein level is reached above 3 kDa. It was therefore concluded that larger or ionic compounds need the incorporation and a true MALDI (cluster) process in order to survive the transition into the gas phase. Moreover, it was suggested that the value of the initial velocity is scaling with the degree of softness of the MALDI process and thus becomes a characteristic property of a MALDI matrix and the preparation protocol used. The rationale for this was that the initial velocity becomes a measure for the development of the cluster jet and that evaporational cooling within the decaying clusters is the reason for the softness (respectively limited harshness) of the MALDI process.

IV. The Analyte in the Matrix Crystal

The MALDI process has been related to the ability of the matrix to incorporate and isolate analyte molecules which was thus regarded as a necessary feature of the MALDI process and of the matrix.[61,93] Although there has been some debate if analyte incorporation is really needed in any case,[62] no clear contradiction of this general approach was found up to now, if one excludes the above-defined intermediate cases based on the initial-velocity measurements. In the case of surface preparation protocols, redissolvation and analyte incorporation into a thin matrix layer was suggested[64] and is further substantiated by the high initial ion velocities obtained.[55]

Despite some debate about the necessity of analyte incorporation, the state of the analyte in the matrix crystal after incorporation, e.g., their charge state or solvation state, has never been interrogated. The existence of charged peptide molecules in the matrix crystals had been hypothesized based on the assumption of a maintained pH.[69] The quenching of the fluorescence of dyes covalently bound to proteins[63] pointed clearly into that direction; however, this

important step of the model consideration, i.e., a clear definition of the starting condition of the analyte, was only addressed recently by using organic dyes as molecular probes.[67]

In a series of experiments, organic indicator dyes were selected and investigated for a choice of matrixes covering the typical pH range of MALDI experiments, i.e., a range from 2 to 9. To keep the system as simple as possible, the pH was defined by the intrinsic matrix pH with no acid or base added. As a constant feature, the matrix crystal maintained the solution color and thus made clear that the analytes are incorporated in their solution charge state. Depending on the pH and the dye, the ionic species incorporated vary from protonated to zwitterionic neutral or zwitterionic charged species to (doubly) negatively charged ions. Counterions have to be included into the matrix crystals to maintain electroneutrality; those may either be alkali cations brought in by the dyes or matrix anions (acidic matrixes) or cations (basic matrixes). Moreover, the maintained (bright) solution color is taken as an indication that around this incorporated ionic species remaining solvent is forming a pocket in which a strong association of counterion pairs is reduced by keeping ions (partially) solvated or by hydrogen-bond interaction to matrix species. The existence of residual solvents in the matrix crystals was also checked in an independent experiment: Large matrix crystals were grown from saturated solutions (acetonitril (ACN) and water, 1:1/v:v), redissolved in deuterated methanol, and the content of ACN was determined by ^{1}H NMR spectroscopy. Irrespective of the treatment of those crystals (direct determination or after heat or vacuum treatment) these crystals typically contained between 0.3 and 3% (m/m) solvent. It has to be noted, that the existence of solvent in the matrix crystals seems to be in contrast to a recently presented solvent-free sample preparation method.[70,71] The reason for that discrepancy is not yet clear, but it is obvious that interionic forces can be reduced by solvation.

While organic dyes clearly exist as precharged species in the matrix crystals, new experiments on peptides and the observation of adducts with counterions (see below, chapter V) substantiate the validity of the concept for other analytes charged in the starting solution. Moreover, even though noncovalent complexes can be detected in MALDI only under adapted sample preparation conditions (less acidic matrixes, omission of organic solvent, buffered solution), there are numerous examples published until today.[94−101] Again this needs a preservation of the native, i.e., typically charged state of the analyte complex, upon its incorporation into the matrix crystals.

V. Cluster Ionization − The Dominant Ionization Process

With the clear evidence that analytes are incorporated in their charge state as defined by the solution pH, the typical MALDI analytes (such as peptides/proteins, and nucleotides) are already precharged in the matrix crystal. In this respect, ions are "preformed" and an active ionization step by charge transfer to neutral species is not required. This should, however, not be misinterpreted in the sense that generation of free ions is facilitated and would proceed via ion evaporation, since precharged analyte ions need to be separated from their counterions, or, alternatively, counterions have to be neutralized. Moreover, most analytes bear several (oppositely) charged functional groups in solution, in contrast to the singly charged molecules generally detected in MALDI-MS, irrespective of the ion polarity recorded.

A. Primary Ionization Mechanisms: Charge Separation and Photoionization

The detailed mechanism of generation of charged clusters needs further investigation. There are mainly two pathways that are in agreement with the available data, although their individual contributions are not easily elucidated. The most straightforward means to generate charged species within a cluster ablation process is the statistical formation of clusters having a deficit/excess of ions; "ionization" is accomplished by charge separation.[69] The energy required to overcome ion-pair interactions will be easily supplied by the mechanical energy of exploding clusters and the energy requirements may be further reduced by residual solvent, i.e., partial solvation, and hydrogen-bond interaction to matrix molecules. These clusters containing an excess of matrix molecules then form the chemical surrounding (the vial) out of which the final ionic species are formed by evaporation of neutral species either directly (matrix) or of ionic species after proton-transfer neutralization (protonated cationic sites and acid anions). For typical acid−base systems, this neutralization within a shrinking cluster is energetically strongly favored due to the considerably higher proton affinity of the acid anions. This charge-separation mechanism can also be applied to neutral analytes, which are incorporated including a complexed cation. Again charge separation from the respective counteranion is needed[92] and the expected influence of the counterion on the cationization efficiency has indeed been observed recently in the case of poly(methyl methacrylate).[102]

In summary, depending on the ion pairs to be separated, different requirements exist, with the separation of tight ion pairs, such as Na^+ and sulfonic-acid anion, being more demanding. The efficiency of ionization by charge separation is determined by the acid−base properties of both analyte, matrix and additives, and their solvation and incorporation behavior; however, the starting condition is the analyte in its pH-related form in a cluster and not a neutral analyte molecule in the gas phase which is ionized by proton transfer or abstraction with reactive matrix species.

Within this charge separation mechanism, the role of the matrix is substantially reduced since an active contribution to ionization is no longer necessary. However, many common matrixes show a complex ionization behavior yielding protonated, deprotonated, and even-electron cluster ions, but also prominent radical species, molecular ions, and dehydrogenated species (Figure 1), such as the dominant

$[M\text{-}2H]^{-\bullet}$ ion in case of 2,5-dihydroxybenzoic acid (2,5-DHB). Even though there is an ongoing debate whether this (necessarily multiphoton) photoionization is possible at all at the applied photon energy of about 3.7 eV (337 nm nitrogen laser wavelength) and if energy pooling processes in clusters may facilitate this photoionization,[39,40,43,47,103] the existence and strong abundance of radical matrix species for MALDI ion generation cannot be neglected. It was also postulated that clustering might lead to reduction of the ionization potential, although only minor effects were found in the case of 2,5-DHB upon clustering and solvation.[104]

The idea of cluster photionization remains attractive, especially because experiments in molecular beams containing small analyte−matrix clusters showed that their laser ionization results also in protonated analyte species.[105−109] On the other hand, no negative ions were detected in these experiments. With respect to the subsequent ion−neutral reactions in the clusters, protonated matrix molecules will be the typical reaction product ($ma^{+\bullet} + ma \rightarrow maH^+ + (ma\text{-}H)^{\bullet}$) and these protonated matrix molecules can either proton-transfer neutralize a negative site (within the analyte or a counteranion), or form the reagent ion for a chemical ionization of neutral analytes provided gas-phase basicities are favorable as suggested early[26] when only gas-phase reactions were considered.

B. Interionic–Proton Transfer

Once charged clusters are produced, formation of the final ions is accomplished by desolvation of matrix and/or residual solvent. Ionic adducts trapped at charged functional groups have to be neutralized by proton transfer before evaporation of small neutrals can proceed. Schematically, only some representative cases are depicted and discussed in the following (M: analyte, ma: matrix, A: anion, B: base, {..}: cluster):

$$\{(M + nH)^{n+} + (n-1)A^- + x\ ma\} \rightarrow MH^+ + (n-1)AH + x\ ma$$

$$\{(M - nH)^{n-} + (n-1)BH^+ + x\ ma\} \rightarrow (M - H)^- + (n-1)B + x\ ma$$

While the first reaction describes the typical case of peptide/protein analysis from acidic solutions, the second case represents that of an oligonucleotide. In the first case, anions are either matrix anions or those of the added acid. For the second case, one practically relevant boundary condition is already obvious. Would the cations be metal cations (and not a protonated base), a neutralization by proton transfer and evaporation of the neutral base is not possible; the ionic species show up as full or partial salts which seriously affects indeed the analysis of oligonucleotides.[110] Dedicated sample preparation protocols have been developed to fully exchange metal cations against protonated bases, such as ammonium.[111−114]

Within the above approach, also the detection of ions with reverse polarity is easily explainable:

$$\{(M + nH)^{n+} + (n+1)A^- + x\ ma\} \rightarrow (M + A^-) + x\ ma\ [\rightarrow (M - H)^- + AH]$$

$$\{(M - nH)^{n-} + (n+1)BH^+ + x\ ma\} \rightarrow (M + BH^+) + x\ ma\ [\rightarrow MH^+ + B]$$

The final reaction to the analyte ion species is clearly controlled by gas-phase basicities and will therefore not always yield analyte ions with high abundance. This would also account directly for intensity differences observed between positive and negative-ion mass spectra; however, neutralization of negative sites by proton transfer may also occur (see also below). For example, for the case that A^- is the typical deprotonated carboxylic acid, either added or matrix, charge separation into the neutral analyte and anion may be energetically favored; the anion−adduct intermediates that are detectable under optimized electrospray conditions are not observed, indicating a too high level of excitation in the MALDI ion generation process. For peptide or oligonucleotide analytes, the situation may be more complicated due to a mixed situation of protonated and deprotonated sites, depending on matrix and solution conditions; however, this does not change the general picture. In the case of positive clusters, the most basic cluster component will finally show up as an ion, regardless of the detailed reactions. Vice versa, this holds also for the negative ion mode, leading to deprotonation of the most acidic compound. This gives also a straightforward explanation for the very efficient ionization of many (highly basic or acidic) analytes and the nearly complete matrix suppression in case of high analyte concentrations,[115−118] because no matrix ions should appear if all clusters contain at least one easy-to-ionize analyte.

The thermochemical considerations discussed above, i.e., the energetically favorable proton-transfer neutralization within the clusters and the consecutive evaporation of the neutral species, however, point to a possible experimental verification by using extremely strong acids, i.e., weak bases respectively, as solution additives. The selected one were: perchloric acid (gas-phase basicity/GB= 1180 kJ/mol), bis(trifluoromethanesulfone)imide (HTFSI, GB = 1221 kJ/mol); these low GB values are in contrast to more typical values between 1325 kJ/mol (trifluoro acetate) and acetate (1429 kJ/mol). Even though the gas-phase reaction bilance even with strong bases (GB = 1007 for arginine) is still negative, Coulombic attraction in the ion pair may make the entire reaction endergonic depending on both acid and base properties. For the case of positively precharged analytes, this leads to the following modification of the reaction schemes

$$\{(M + nH)^{n+} + (n-1)A^- + x\ ma\} \rightarrow [(M + nH)^{n+} + (n-1)A^-]^+ + x\ ma$$

$$\{(M + nH)^{n+} + (n+1)A^- + x\ ma\} \rightarrow [(M + nH)^{n+} + (n+1)A^-]^- + x\ ma$$

and to the formation of stable ionic products on the

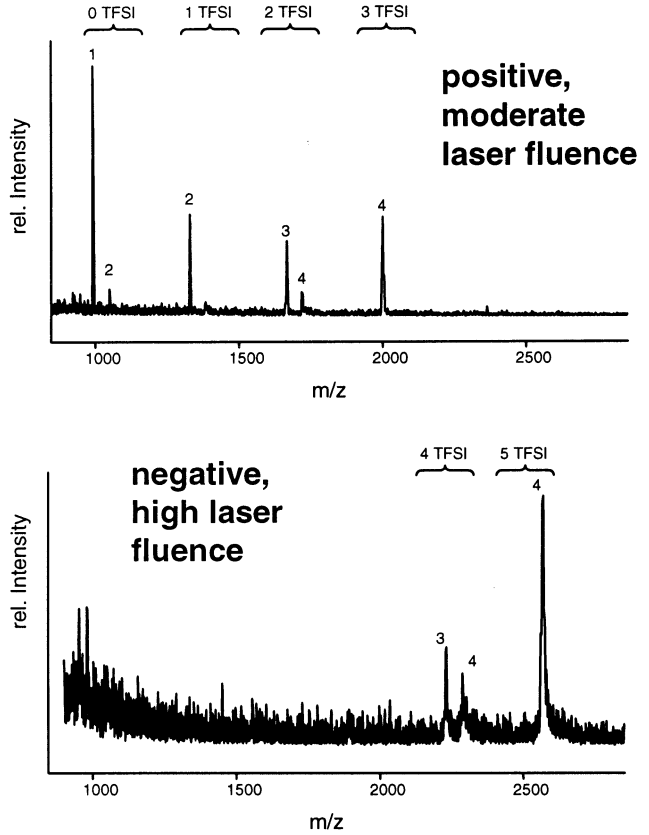

Figure 5. Mass spectra of a peptide mixture obtained with 4-methoxy-α-cyanocinnamic acid (αCMCA) and addition of HTFSI (bis(trifluoromethylsulfonyl)imide). The peptides have the sequence AXG AXG AXG AXG with X = Arg or Thr, and the peak labels refer to the number of arginines in the peptide. The peaks are grouped according to the number of TFSI adducts.

stage of the ion pairs (after evaporation of the neutral matrix). This extreme case is indeed observed for a neutral matrix (4-methoxy-α-cyanocinnamic acid amide). Figure 5 shows the MALDI mass spectra obtained for a mixture of four peptides (AXGAXGAX-GAXG) with X being either a threonine or arginine residue using HTFSI acidification.

Nearly exclusively the salt ions are detected being charged by deficit or excess of one TFSI anion. Only minor signals formally due to one proton-transfer neutralization step are observed. The proton neutralization is enhanced at higher laser fluences (data not shown), pointing to the fact that a higher level of excitation may overcome the energetic barrier for neutralization. The high noise level in the negative-ion mass spectrum and the lack of the peptides containing less than three arginines points to the problems to form stable anion-adduct negative ions. A more pronounced shift to ions containing a reduced number of anions is observed for acidic and more common matrixes, such as 2,5-DHB, 3-hydroxypicolinic acid (3 HPA), and 4-hydroxy-α-cyanocinnamic acid (αCHCA) (Figure 6). This can, however, be attributed to the competition between TFSI and matrix anions for the counterion site and the complete neutralization of the latter ion pairs by proton transfer. This is discussed in detail in another paper.[119]

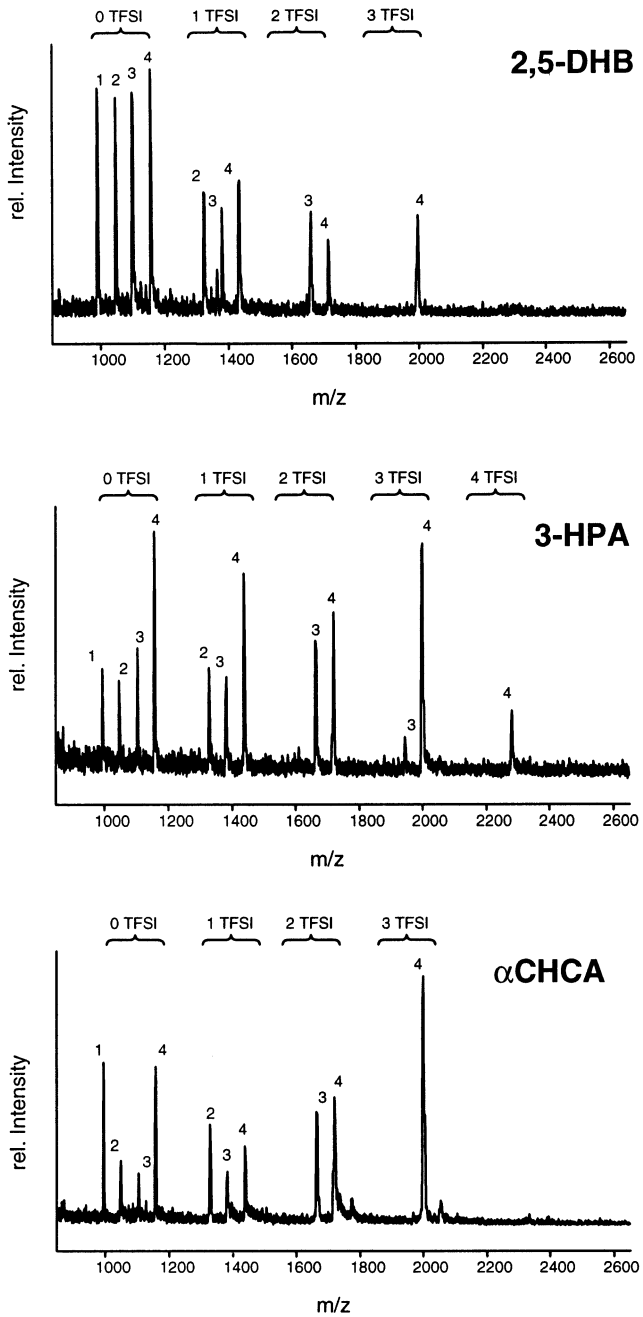

Figure 6. Positive mass spectra of a peptide mixture obtained with different matrixes and addition of HTFSI (bis(trifluoromethylsulfonyl)imide). (2,5-DHB = 2,5-dihydroxybenzoic acid, 3-HPA = 3-hydroxypicolinic acid, αCHCA = hydroxy-α-cyanocinnamic acid. The peptide sequences and the peak labels are explained in Figure 6).

As a second example, a positive- and negative-ion mass spectrum of insulin is depicted (Figure 7). In this case, 3-HPA is used as a matrix which is known to be a very soft matrix (also reflected in the high initial velocity, compare Table 1). For insulin, the number of adducted anions agrees with the number of all basic sites (arginine, lysine, histidine, and N-terminus) with one lacking or one in excess for positive and negative ions, respectively. This provides evidence that also anion pairs with less basic sites can survive the ablation step provided that the internal excitation of the clusters and ions is minimized due to the matrix used. Earlier reports of anion

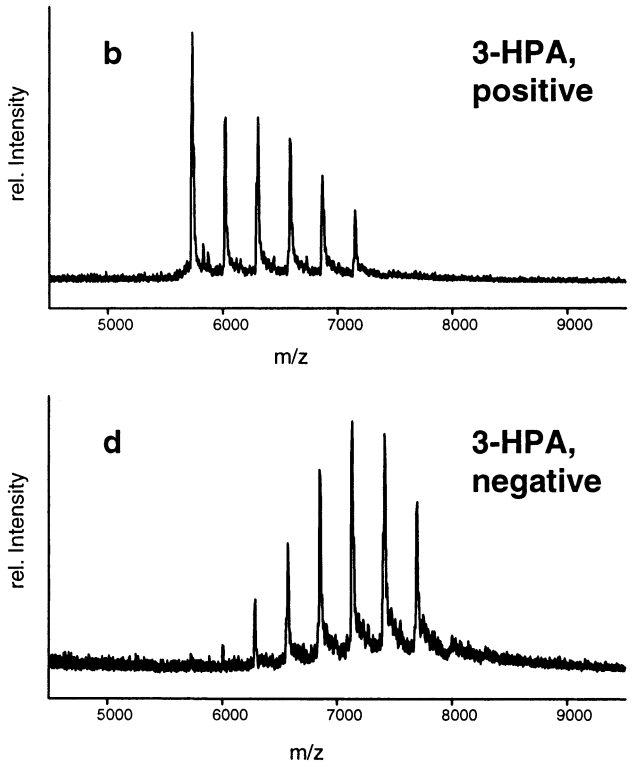

Figure 7. Mass spectra of bovine insulin in positive and negative ion mode obtained with 3-HPA (3-hydroxypicolinic acid) and addition of HTFSI (bis(trifluoromethylsulfonyl)-imide).

adducts, e.g., of sulfonate-containing dyes, which were attributed to specific noncovalent interactions,[120−122] would fully agree with a simple cluster ionization process.

Many typical matrixes can only form negatively charged (counter) anions, making possible the neutralization of protonated sites, but the reverse reaction should be possible in case of matrixes with basic functional groups. It has been reported that the matrix 3-HPA is advantageous for oligonucleotide analysis especially in the positive ion mode.[123] Besides the already mentioned softness oft this matrix, represented by its high initial velocity, a second feature of this matrix can also be important: 3-HPA exists at least partially in the protonated form (in contrast to other acidic or neutral matrixes) and can thus work as counterion for the phosphate group and subsequently lead to enhanced anion neutralization. Already, in the early days of MALDI, initial experiments with oligonucleotides did not show the complete salts as to be expected by the cluster model, but partial proton neutralization of phosphate groups resulting in a distribution of molecule ion species containing different numbers of sodium and potassium.[112] It was found by investigations of modified oligonucleotides, that 3-HPA interacts with the phosphodiester backbone,[124−126] which further substantiates the above picture. For practical applications to oligonucleotides, the cation problem is typically solved by exchange of metal cations and addition of high excess of a "proton source", such as ammonium,[111−114] which is easily understood in the frame of the cluster ionzation model.

C. Charge Reduction and Other Secondary Reactions

Finally, the problem of more highly charged ions must be addressed. Within the frame of the cluster ionization mechanism, cluster charging is accomplished by ion pair separation or photoionization. Until now only separation of one ion pair has been considered leading to singly charged ions which typically make up the majority of MALDI analyte signals; any further charge separation by excess/deficit of more than one counterion will be both statistically less favorable and energetically more demanding due to coulomb attractions.

Nevertheless, with increasing analyte-ion mass (and thus increasing number of ion pairs) the formation of also more highly charged positive ions by separating more than one ion pair will become more probable. This effect is indeed observed in positive MALDI mass spectra, but typically the charge state of negative ions is lower. Since photoionization processes can also happen in clusters, which are already singly positively charged by the cluster mechanism, the observed shift of the charge state between positive and negative ions may be induced by the photoionization pathway. It is, moreover, not unlikely, that the photoionization pathway is at least partially responsible for the intensity differences between positive and negative ion mode.

The detectability of more highly charged analyte ions is, however, also affected by a possible neutralization in the laser plume. Since this plume contains also electrons, hydrogen atoms[51] and all those matrix ionic species showing up the mass spectra in high abundance, electron- or proton-transfer charge reduction and neutralization, which will become more effective for higher charge states, have been considered.[69] Electron neutralization was experimentally proven to occur for a diquaternary nitrogen compound (ethyl viologene) and for a polynuclear metal complex.[127] However, it cannot be easily determined to which extent electron capture or electron transfer, respectively, is responsible for charge reduction in MALDI of biopolymers, since other charge reduction mechanisms lead to the same products. Recently, it was reported that most electrons stem from the matrix-covered metal surface, and it was discussed that the matrix reduces the work function of the metal.[128,129] Moreover, the efficiency of electron production was found to depend on the thickness of the matrix layer, and both a high matrix coverage and no matrix at all lead to decreased electron production.

It has been postulated[69] that the so-called "in-source" fragmentation,[130−134] which mainly produces c and z fragments of peptides and proteins, in contrast to other fragmentation techniques such as CID or PSD, is induced by a similar mechanism as proposed for electron capture dissociation (ECD) of electrospray generated ions.[135−138] This reaction involves hydrogen atom loss from hypervalent species originating from electron capture or electron transfer and the attachment of these hydrogen atoms at specific analyte sites, e.g., amide bonds, and following molecule decomposition. Another possible hydrogen

source can be the (ionized or neutral) matrix, and these two possibilities are not easily sorted out. Hydrogen transfer from excited matrix to amide bonds was recently proposed as explanation for the observed ISD fragmentation.[139] Direct hydrogen transfer from the matrix to UV-absorbing species was also shown using photochromic dyes, i.e., spirooxacines.[140,141] The reduction of these dyes by addition of up to three hydrogen atoms was only detected with MALDI and not with matrixless LDI, and it has been shown that hydrogen transfer follows excitation. Hydrogen addition was also observed at some azo dyes used in the studies to determine the analyte charge state in the MALDI crystals[67] and was, moreover, also found to occur at oligonucleotides, presumably at cytosine.[48]

D. Role of the Matrix

Definitely, the matrix is an indispensable element of the MALDI process; its important role on energy absorption and analyte isolation is generally accepted and has only been partially covered in this review. Within the cluster ionization model presented, a direct role of matrix reactive species in ionization, as postulated and used for every model regarding charge-transfer reactions,[26,28,39−50] is no longer an indispensable part of the matrix function. Even though it may be attractive to completely give up the original photochemical ionization picture, the contribution of photoionization within clusters cannot be excluded, since the subsequent chemical reactions, which will usually result in anion neutralization, lead to the same ionic products. The obvious similarities between spectra obtained with IR- or UV-lasers[142,143] may also suggest that there is no important role of matrix photoionization, although both techniques must not necessarily have the same mechanism. On the other hand, a decision whether a contribution of cluster photoionization occurs or to what extent both possible pathways are responsible for ion production is actually not possible due to the lack of unequivocal experiments. A contribution of cluster photoionization may, e.g., be responsible for the differences in positive and negative ion mode from one sample at one laser irradiance which show up both in analyte ion intensities and the relative intensities of singly and more highly charged ions.

The major advantage of the new picture is its inherent simplicity and straightforward explanantion of the MALDI phenomenology by solution acid−base chemistry, as long as preparation and crystal formation is concerned, and by gas-phase thermochemistry when considering the formation of the ions finally observed. Moreover, most of the bioanalytical applications deal with analytes which contain charged functional groups and would therefore fit into the above scheme. The only exception are neutral oligosaccharides for which the above ionization path is excluded. Consequently, they are detected as cationized species, for small oligomers via gas-phase adduction, as documented by the low initial velocity. Larger oligomers, which need the (softer) matrix cluster emission process to survive the transfer into the gas phase, trap and include contaminant salt into the matrix crystals in order to find their way of charged-cluster ionization; again this is connected to the high initial ion velocity as observed for a typical MALDI process. Only for a small class of analytes, i.e., those with a low ionization potential and lacking functional groups which promote proton-transfer reactions, a direct photoionization or a (radical) charge transfer from matrix to analyte is feasible.

VI. References

(1) Karas, M.; Bachmann, D.; Hillenkamp, F. *Anal. Chem.* **1985**, *57*, 2935.
(2) Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Proc.* **1987**, *78*, 53.
(3) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151.
(4) Hakansson, P.; Kamensky, I.; Sundqvist, B.; Fohlmann, J.; Peterson, P.; McNeal, C. J.; Macfarlane, R. D. *J. Am. Chem. Soc.* **1982**, *104*, 2948.
(5) Sundqvist, B.; Kamensky, I.; Kakansson, P.; Kjellberg, J.; Salehpour, M.; Widdiyasekera, S.; Fohlmann, J.; Peterson, P. A.; Roepstorff, P. *Biomed. Mass Spectrom.* **1984**, *11*, 242.
(6) Barber, M.; Green, B. N. *Rapid Commun. Mass Spectrom.* **1987**, *1*, 80.
(7) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299.
(8) Colby, S. M.; King, T. B.; Reilly, J. P. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 865.
(9) Brown, R. S.; Lennon, J. J. *Anal. Chem.* **1995**, *67*, 1998.
(10) Vestal, M. L.; Juhasz, P.; Martin, S. A. *Rapid. Commun. Mass Spectrom.* **1995**, *9*, 1044.
(11) Krutchinsky, A. N.; Loboda, A. V.; Spicer, V. L.; Dworschak, R.; Ens, W.; Standing, K. G. *Rapid. Commun. Mass Spectrom.* **1998**, *12*, 508.
(12) Loboda, A. V.; Krutchinsky, A. N.; Bromirski, M.; Ens, W.; Standing, K. G. *Rapid. Commun. Mass Spectrom.* **2000**, *14*, 1047.
(13) Castoro, J. A.; Wilkins, C. L. *Anal. Chem.* **1993**, *65*, 2621.
(14) Castoro, J. A.; Köster, C.; Wilkins, C. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 239.
(15) Amster, I. J. *J. Mass Spectrom.* **1996**, *31*, 1325.
(16) Schwartz, J. C.; Bier, M. E. *Rapid. Commun. Mass Spectrom.* **1993**, *7*, 27.
(17) Jonscher, K.; Currie, G.; McCormack, A. L.; Yates, J. R. *Rapid. Commun. Mass Spectrom.* **1993**, *7*, 20.
(18) Qin, J.; Steenvoorden, R. J. J. M.; Chait, B. T. *Anal. Chem.* **1996**, *68*, 1784.
(19) Krutchinsky, A. N.; Kalkum, M.; Chait, B. T. *Anal. Chem.* **2001**, *73*, 5066.
(20) Laiko, V. V.; Baldwin, M. A.; Burlingame, A. L. *Anal. Chem.* **2000**, *72*, 652.
(21) Laiko, V. V.; Moyer, S. C.; Cotter, R. J. *Anal. Chem.* **2000**, *72*, 5239.
(22) Aebersold, R.; Goodlett, D. R. *Chem. Rev.* **2001**, *101*, 269.
(23) Lahm, H.-W.; Langen, H. *Electrophoresis* **2000**, *21*, 2105.
(24) Gevaert, K.; Vandekerckhove, J. *Electrophoresis* **2000**, *21*, 1145.
(25) Karas, M.; Bahr, U.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Proc.* **1989**, *92*, 231.
(26) Ehring, H.; Karas, M.; Hillenkamp, F. *Org. Mass Spectrom.* **1992**, *27*, 472.
(27) Liao, P.-C.; Allison, J. *J. Mass Spectrom.* **1995**, *30*, 408.
(28) Zenobi, R.; Knochenmuss, R. *Mass Spectrom. Rev.* **1998**, *17*, 337.
(29) Vertes, A.; Gijbels, R.; Levine, R. D. *Rapid Commun. Mass Spectrom.* **1990**, *4*, 228.
(30) Johnson, R. E.; Sundqvist, B. U. R. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 574.
(31) Ens, W.; Mao, Y.; Mayer, F.; Standing, K. G. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 117.
(32) Beavis, R. C. *Org. Mass Spectrom.* **1992**, *27*, 864.
(33) Sundqvist, B. U. R. *Int. J. Mass Spectrom. Ion Proc.* **1992**, *118/119*, 265.
(34) Vertes, A.; Irinyi, G.; Gijbels, R. *Anal. Chem.* **1993**, *65*, 2389.
(35) Dreisewerd, K.; Schürenberg, M.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion. Processes* **1995**, *141*, 127.
(36) Dreisewerd, K.; Schürenberg, M.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion. Processes* **1996**, *154*, 171.
(37) Chen, X.; Caroll, J. A.; Beavis, R. C. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 885.
(38) Sadeghi, M.; Wu, X.; Vertes, A. *J. Phys. Chem. B* **2001**, *105*, 2578.
(39) Tang, X.; Sadeghi, M.; Olumee, Z.; Vertes, A. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 968.
(40) Karbach, V.; Knochenmuss, R. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 968.
(41) Land, C. M.; Kinsel, G. R. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1060.

(42) Meffert, A.; Grotemeyer, J. *Ber. Bunsen-Ges. Phys. Chem.* **1998**, *102*, 459.
(43) Knochenmuss, R.; Lehmann, E.; Zenobi, R. *Eur. Mass Spectrom.* **1998**, *4*, 421.
(44) Olumee, Z.; Vertes, A. *J. Phys. Chem. B* **1998**, *102*, 6118.
(45) Wong, C. K. L.; So, M. P.; Chan, T.-W. *Eur. Mass Spectrom.* **1998**, *4*, 223.
(46) Breuker, K.; Knochenmuss, R.; Zenobi, R. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 1111.
(47) Knochenmuss, R.; Stortelder, A.; Breuker, K.; Zenobi, R. *J. Mass Spectrom.* **2000**, *35*, 1237.
(48) Koomen, J. M.; Russell, D. H. *J. Mass Spectrom.* **2000**, *35*, 1025.
(49) Knochenmuss, R.; Vertes, A. *J. Phys. Chem. B* **2000**, *104*, 5406.
(50) Meffert, A.; Grotemeyer, J. *Int. J. Mass Spectrom. Ion. Process.* **2001**, *210/211*, 521.
(51) Scott, C. T. J.; Kosmidis, C.; Jia, W. J.; Ledingham, K. W. D.; Singhal, R. P. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 829.
(52) Ingendoh, A.; Karas, M.; Hillenkamp, F.; Giessmannn, U. *Int. J. Mass Spectrom. Ion Proc.* **1994**, *131*, 345.
(53) Zhou, J.; Ens, W.; Standing, K. G.; Verentchikov, A. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 671.
(54) Juhasz, P.; Vestal, M. L.; Martin, S. A. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 209.
(55) Glückmann, M.; Karas, M. *J. Mass Spectrom.* **1999**, *34*, 467.
(56) Glückmann, M.; Pfenninger, A.; Krüger, R.; Thierolf, M.; Karas, M.; Horneffer, V.; Hillenkamp, F.; Strupat, K. *Int. J. Mass Spectrom.* **2001**, *208*, 121.
(57) Strupat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion. Process.* **1991**, *111*, 89.
(58) Beavis, R. C.; Bridson, J. N. *J. Phys. D: Appl. Phys.* **1993**, *26*, 442.
(59) Xiang, F.; Beavis, R. C. *Org. Mass Spectrom.* **1993**, *28*, 1424.
(60) Dai, Y.; Whittal, R. M.; Li, L. *Anal. Chem.* **1996**, *68*, 2494.
(61) Strupat, K.; Kampmeier, J.; Horneffer, V. *Int. J. Mass Spectrom. Ion. Process.* **1997**, *169/170*, 43.
(62) Horneffer, V.; Dreisewerd, K.; Lüdemann, H.-C.; Hillenkamp, F.; Läge, M.; Strupat, K. *Int. J. Mass Spectrom. Ion. Process.* **1999**, *185/186/187*, 859.
(63) Horneffer, V.; Forsman, A.; Strupat, K.; Hillenkamp, F.; Kubitscheck, U. *Anal. Chem.* **2001**, *73*, 1016.
(64) Vorm, O.; Roepstorff, P.; Mann, M. *Anal. Chem.* **1994**, *66*, 3281.
(65) Xiang, F.; Beavis, R. C. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 199.
(66) Dai, Y.; Whittal, R. M.; Li, L. *Anal. Chem.* **1999**, *71*, 1087.
(67) Krüger, R.; Pfenninger, A.; Fournier, I.; Glückmann, M.; Karas, M. *Anal. Chem.* **2001**, *73*, 5812.
(68) Beavis, R. C.; Chait, B. T. *Chem. Phys. Lett.* **1991**, *181*, 479.
(69) Karas, M.; Glückmann, M.; Schäfer, J. *J. Mass Spectrom.* **2000**, *35*, 1.
(70) Przybilla, L.; Brand, J.-D.; Yoshimura, K.; Räder, H. J.; Müllen, K. *Anal. Chem.* **2000**, *72*, 4591.
(71) Trimpin, S.; Rouhanipour, A.; Az, R.; Räder, H. J.; Müllen, K. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1364.
(72) Berkenkamp, S.; Menzel, C.; Hillenkamp, F.; Dreisewerd, K. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 209.
(73) Spengler, B.; Bökelmann, V. *Nucl. Instrum. Methods B* **1993**, *82*, 379.
(74) Zhang, W.; Chait, B. *Int. J. Mass Spectrom. Ion Proc.* **1997**, *160*, 259.
(75) Ayala, E.; Vera, C. C.; Håkansson, P. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 792.
(76) Zhigilei, L. V.; Kodali, P. B. S.; Garrison, B. J. *Chem. Phys. Lett.* **1997**, *276*, 269.
(77) Zhigilei, L. V.; Kodali, P. B. S.; Garrison, B. J. *J. Phys. Chem. B* **1997**, *101*, 2028.
(78) Zhigilei, L. V.; Garrison, B. J. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1273.
(79) Zhigilei, L. V.; Garrison, B. J. *J. Appl. Phys.* **2000**, *88*, 1281.
(80) Fournier, I.; Brunot, A.; Tabet, J. C.; Bolbach, G. *Int. J. Mass Spectrom. Ion. Process.* **2002**, *213*, 203.
(81) Verentchikov, A.; Smirnov, I.; Vestal, M. L. *Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics*; Dallas, TX, June 13−17, 1999.
(82) Cai, Y.; Peng, W.-P.; Kuo, S. J.; Sabu, S.; Han, C.-C.; Chang, H.-C. *Anal. Chem.* **2002**, *74*, 4434.
(83) Handschuh, M.; Nettesheim, S.; Zenobi, R. *Appl. Surface Sci.* **1999**, *137*, 125.
(84) Krutchinsky, A. N.; Chait, B. T. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 129.
(85) Puretzky, A. A.; Geohegan, D. B. *Chem. Phys. Lett.* **1998**, *286*, 425.
(86) Puretzky, A. A.; Geohegan, D. B.; Hurst, G. B.; Buchanan, M. V.; Luk'yanchuk, B. S. *Phys. Rev. Lett.* **1999**, *83*, 444.
(87) Spengler, B.; Karas, M.; Bahr, U.; Hillenkamp, F. *J. Phys. Chem.* **1987**, *91*, 6502.
(88) Wang, B. H.; Dreisewerd, K.; Bahr, U.; Karas, M.; Hillenkamp, F. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 393.
(89) Rashidezadeh, H.; Guo, B. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 724.
(90) Belov, M. E.; Myatt, C. P.; Derrick, P. J. *Chem. Phys. Lett.* **1998**, *284*, 412.
(91) Karas, M.; Bahr, U.; Fournier, I.; Glückmann, M.; Pfenninger, A. *Int. J. Mass Spectrom.*, in press.
(92) Lehmann, E.; Knochenmuss, R.; Zenobi, R. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1483.
(93) Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1193A.
(94) Juhasz, P.; Biemann, K. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4333.
(95) Rosinke, B.; Strupat, K.; Hillenkamp, F.; Rosenbusch, J.; Dencher, N.; Krüger, U.; Galla, H.-J. *J. Mass Spectrom.* **1995**, *30*, 1462.
(96) Moniatte, M.; Lesieur, C.; Vécsey-Semjén, B.; Buckley, J. T.; Pattus, F.; van der Goot, F. G.; van Dorsselaer, A. *Int. J. Mass Spectrom. Ion Process.* **1997**, *169/170*, 179.
(97) Cohen, L. R. H.; Strupat, K.; Hillenkamp, F. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 1046.
(98) Thiede, B.; von Janta-Lipinski, M. *Rapid. Commun. Mass Spectrom.* **1998**, *12*, 1889.
(99) Vogl, T.; Roth, J.; Sorg, C.; Hillenkamp, F.; Strupat, K. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 1124.
(100) Jespersen, S.; Niessen, W. M. A.; Tjaden, U. R.; van der Greef, J. *J. Mass Spectrom.* **1998**, *33*, 1088.
(101) Farmer, T. B.; Caprioli, R. M. *J. Mass Spectrom.* **1998**, *33*, 697.
(102) Hoberg, A.-M.; Haddleton, D. M.; Derrick, P. J. *Eur. J. Mass Spectrom.* **1998**, *4*, 435.
(103) Allwood, D. A.; Dyer, P. E.; Dreyfus, R. W. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 499.
(104) Lin, Q.; Knochenmuss, R. *Rapid. Commun. Mass Spectrom.* **2001**, *15*, 1422.
(105) Krutchinsky, A. N.; Dolguine, A. I.; Khodorkovski, M. A. *Anal. Chem.* **1995**, *67*, 1963.
(106) Meffert, A.; Grotemeyer, J. *Eur. Mass Spectrom.* **1995**, *1*, 594.
(107) Land, C. M.; Kinsel, G. R. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1060.
(108) Land, C. M.; Kinsel, G. R. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 726.
(109) Meffert, A.; Grotemeyer, J. *Int. J. Mass Spectrom.* **2001**, *210/211*, 521.
(110) Nordhoff, E.; Kirpekar, F.; Roepstorff, P. *Mass Spectrom. Rev.* **1996**, *15*, 67.
(111) Stults, J. T.; Marsters, J. C. *Rapid. Commun. Mass Spectrom.* **1991**, *5*, 359.
(112) Nordhoff, E.; Ingendoh, A.; Cramer, R.; Overberg, A.; Stahl, B.; Karas, M.; Hillenkamp, F.; Crain, P. F. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 771.
(113) Cheng, S.-W.; Chan, T.-W. D. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 907.
(114) Zhu, Y. F.; Taranenko, N. I.; Allman, S. L.; Martin, S. A.; Haff, L.; Chen, C. H. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1591.
(115) Chan, T.-W. D.; Colburn, A. W.; Derrick, P. J. *Org. Mass. Spectrom.* **1990**, *26*, 342.
(116) Chan, T.-W. D.; Colburn, A. W.; Derrick, P. J.; Gardiner, G. J.; Bowden, M. *Org. Mass Spectrom.* **1992**, *27*, 188.
(117) Knochenmuss, R.; Dubois, F.; Dale, M. J.; Zenobi, R. *Rapid. Commun. Mass Spectrom.* **1996**, *10*, 871.
(118) Knochenmuss, R.; Karbach, V.; Wiesli, U.; Breuker, K.; Zenobi, R. *Rapid. Commun. Mass Spectrom.* **1998**, *12*, 529.
(119) Krüger, R.; Karas, M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 1218.
(120) Salih, B.; Zenobi, R. *Anal. Chem.*, **1998**, *70*, 1536.
(121) Friess, S. D.; Zenobi, R. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 810.
(122) Friess, S. D.; Daniel, J. M.; Hartmann, R.; Zenobi, R. *Int. J. Mass Spectrom.* **2002**, *219*, 269.
(123) Chou, C.-W.; Williams, P.; Limbach, P. A. *Int. J. Mass Spectrom. Ion. Process.* **1999**, *193*, 15.
(124) Butler, J. M.; Jiang-Baucom, P.; Huang, M.; Belgrader, P.; Girard, J. *Anal. Chem.* **1996**, *68*, 3283.
(125) Gut, I. G. *Int. J. Mass Spectrom. Ion. Process.* **1999**, *169/170*, 313.
(126) Gut, I. G.; Jefferey, W. A.; Pappin, D. J. C.; Beck, S. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 43.
(127) Schubert, U. S.; Lehn, J. M.; Hassmann, J.; Hahn, C. X.; Hallschmid, N.; Müller, P. *ACS Symposium Series* **1997**, *704*, 248.
(128) Frankevich, V.; Knochenmuss, R.; Zenobi, R. *Int. J. Mass Spectrom.* **2002**, *220*, 11.
(129) Gorshkov, M. V.; Frankevich, V. E.; Zenobi, R. *Eur. Mass Spectrom.* **2002**, *8*, 67.
(130) Brown, R. S.; Lennon, J. J. *Anal. Chem.* **1995**, *67*, 3990.
(131) Brown, R. S.; Carr, B. L.; Lennon, J. J. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 225.
(132) Katta, V.; Chow, D. T.; Rohde, M. F. *Anal. Chem.* **1998**, *70*, 4410.
(133) Takayama, M.; Tsugita, A. *Int. J. Mass Spectrom.* **1998**, *181*, L1−L6.
(134) Reiber, D. C.; Brown, R. S.; Weinberger, S.; Kenny, J.; Bailey, J. *Anal. Chem.* **1998**, *70*, 1214.

(135) Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1998**, *120*, 3265.
(136) Zubarev, R. A.; Kruger, N. A.; Fridriksson, E. K.; Lewis, M. A.; Horn, D. M.; Carpenter, B. K.; McLafferty, F. W. *J. Am. Chem. Soc.* **1999**, *121*, 2857.
(137) Kruger, N. A.; Zubarev, R. A.; Carpenter, B. K.; Kelleher, N. L.; Horn, D. M.; McLafferty, F. W. *Int. J. Mass Spectrom.* **1999**, *182/183*, 1.
(138) Kruger, N. A.; Zubarev, R. A.; Horn, D. M.; McLafferty, F. W. *Int. J. Mass Spectrom.* **1999**, *185/186/187*, 787.
(139) Takayama, M. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1044.
(140) Calba, P. J.; Muller, J. F.; Hachimi, A.; Laréginie, P.; Guglielmetti, R. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1602.
(141) Calba, P. J.; Muller, J. F.; Inouye, M. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1727.
(142) Niu, S.; Zhang, W.; Chait, B. T. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1.
(143) Nordhoff, E.; Kirpekar, F.; Karas, M.; Cramer, R.; Hahner, S.; Hillenkamp, F.; Kristiansen, K.; Roepstorff, P.; Lezius, A. *Nucl. Acid Res.* **1994**, *22*, 2460.

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