Fundamental Aspects of Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

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Synopsis

Matrix-assisted laser desorption ionization forms one of the major break-throughs in modern mass spectrometry. It allows today the desorption of intact ions of biomacromolecules in the mass range of up to 400,000 Dalton. Especially for peptides and proteins it is becoming a routine technique for determination of the molecular mass. Basic understanding of the underlying desorption and ionization mechanisms, however, is still very rudimentary. This can be attributed to the fact that a successful desorption of macromolecule ions is the result of a complex set of physico-chemical and experimental parameters. With regard to the matrix, these are the ability to incorporate macromolecules in the preparation step, to absorb energy upon irradiation with a short laser pulse and undergo a phase transition from solid to gas and to yield a high number of charged analyte ions by photochemical processes. Measurements of the properties of the desorbed ions show a strongly forwarded emission, a mass-independent initial velocity and an energy deficit which is increasing both with mass and laser fluence. These ion characteristics can be rationalized by a supersonic expansion of a vaporized surface layer and collisions in the acceleration step. A more refined understanding of the desorption process has to be gained by future work in order to enable systematic choice of matrix compounds and to optimize the experimental conditions for an improved mass spectrometric performance.

1 Introduction

The development of matrix-assisted laser desorption ionization mass spectrometry.

Even though lasers have unique properties, they have so far found limited use as desorption ionization sources in organic mass spectrometry. Laser light can easily be focused to yield very high power and energy densities at a sample surface, but irradiation of large fragile biomolecules with continous-wave lasers and lasers emitting pulses with durations above $1\mu s$ resulted in extensive thermal degrada-

tion rather than in the desired emission of intact molecule ions. Short-pulse lasers, either $C0_2$-lasers emitting at 10.6 μm in the infrared or frequency-multipled Nd-YAG-lasers (266 or 355nm) have been used with better results, but the analytical performance achieved either with respect to the accessible mass range, broad applicability, or sensitivity was not satisfying enough to spread out the technique. Only in specialized areas, such as in fourier-transform ion-cyclotron-resonance mass spectrometers or in laser microprobe instruments, lasers are routinely used.

The use of a matrix was proposed by the authors in the mid 80s to circumvene fundamental problems limiting the application of pulsed UV-lasers as a desorption ionization source in organic mass spectrometry. The main drawbacks were that the technique was not generally applicable to different classes of organic compounds and that the accessible mass range was limited to about 1500 Dalton in most favourable cases. Investigations into the laser desorption ionization (LDI) of small organic compounds revealed the underlying reasons. Sample absorption at the laser wavelength was found to be the most relevant factor (Karas et al., 1985); it was therefore concluded that energy coupling into the irradiated sample by resonant excitation of analyte molecules plays a key role in UV-LDI. This was in strong contrast to the general picture of the laser desorption process. Until then, a fast heating of the sample mediated by an absorbing substrate had been regarded to be the underlying mechanism, and any influence of the laser wavelength and sample absorption had therefore been disregarded.

Energy from a UV-laser beam may be deposited into a solid (or liquid) organic sample either by resonant electronic excitation - provided the sample contains a chromophore, usually an aromatic π-electron system - or at sufficiently high laser irradiances (W/cm^2) by non-linear multiphoton absorption. In a typical instrumental configuration, samples are prepared as thin layers dried from solution onto a metallic substrate which in case of a transparent sample may serve as the absorber of the laser energy. It was, however, found in a series of experiments undertaken for small organic compounds, such as amino acids and dipeptides, that - with respect to a soft and reproducible desorption of molecule ions - a high sample absorption at the laser wavelength is required (Karas et al, 1985). The necessary laser irradiance (W/cm^2) was inversely proportional to the absorption coefficient of the sample. It was furthermore found that this soft desorption ionization exhibits a steep threshold behaviour and is only possible in a narrow irradiance range at or slightly above this threshold irradiance. The value determined for this threshold was in the range of 10^7 W/cm^2. Provided the life time of the excited electronic states is short compared to laser pulse time (typically 3 to 10 ns) - which can reasonably be assumed for an organic solid-linear absorption processes are dominating and enable a controllable energy deposition into the sample by careful attenuation of the laser pulse energy. Beer's law can be used for a calculation of the energy

deposited and the penetration depth of the laser light. The energy absorbed per molecule in the uppermost layer was found to be in the range of 5 to 10 eV, corresponding to the absorption of one or more than two photons per molecule (photon energy at 266 nm: 4.6 eV); penetration depths were calculated to be 100 to 300 nm, respectively. The energy deposited per unit volume suffices to overcome the solid state cohesion forces (sublimation energy) of typical hydrogen-bonded non-volatile organic compounds. Within the given set of experimental parameters, little energy is lost from the excited volume by heat conduction. Therefore, energy is channeled efficiently into desorption. The upper limit of the usable irradiance is set by an increasing (photo)fragmentation of the organic molecules. This competing fragmentation channel is also regarded to be the reason for the observed mass range limit. With these results a qualitative model was proposed which described the desorption process as an explosive vaporization of a microvolume induced by an electronic excitation of individual chromophores and fast relaxation of the energy into vibrational excitation of the molecular lattice (exciton-phonon-coupling).

Careful inspection of the UV-LDI mass spectra showed that simple models based on solution chemistry, such as (de)protonation or the emission of preformed ions, or gas-phase cationization by alkali ions do not allow for a coherent explanation of the observed ion species. Besides the expected (de)protonated molecules, often high-abundance radical molecular ions show up as well as product ions of (multiple) hydrogen addition or abstraction reactions. It was therefore proposed that the key process in ionization of strongly absorbing compounds is their photoionization. This may be initiated either by a two-photon absorption or by a bimolecular reaction of two singly-excited molecules (energy pooling). The ion species finally registered in the mass spectrum are the products of (photo)chemical reactions of usually reactive radical molecular ions and neutrals taking place in the condensed phase or in the expanding high-density material plume (Ehring et al., 1992).

2 Matrix Idea and Concept

With the above results and considerations it is obvious that UV-LDI suffers from severe and systematic restrictions which prevent its widespread use as a desorption ionization technique in mass spectrometry of bioorganic compounds. On the other hand, the well-defined desorption ionization characteristics of small highly-absorbing compounds and the characterization of the LDI-process as a collective event inducing ablation of a sample volume, gave a guideline for a new approach: provided an analyte is only present at a high dilution in a suitable compound - hence a matrix - it should be possible to set free analyte molecules and possibly molecule ions by UV-LDI of the excess matrix (Karas et al., 1986, 1987, 1988a,b,

1989a,b). Intimate mixing of matrix and analyte was therefore regarded to be a crucial point. Nicotinic acid was chosen as a test compound in the initial matrix experiments; it exhibits a high molar absorption at the wavelength of 266 nm and possesses both an acidic and basic functional group which was regarded to facilitate the embedding of polar peptide and protein molecules (Karas et al., 1988a, 1988b, 1989). The principal features of the matrix techniques have been worked out in the mid 80s in the author's lab, and its feasibility has been shown for medium-size peptides. Application to proteins above 10 kDa was initiated by reports documenting the possibility to desorb intact protein ions by a laser desorption technique; a different matrix approach had been followed, i.e. solving the sample in slurry of a non-volatile liquid, such as glycerol, and small metal particles functioning as absorption centers for the incident laser light (Tanaka et al., 1988). This matrix technique, however, has not found any further impact in analytical chemistry until now.

3 Experimental Techniques

The principal investigations for MALDI have been performed on a laser microprobe instrument, equipped with a Q-switched ND-YAG laser at a wavelength of 266 nm. Short-pulse lasers are required to induce the desired desorption process, therefore time-of-flight (TOF) mass analyzers, allowing for registration of virtually all ions formed and posing no mass limitations, are best suited. Today several dedicated laser TOF instruments are available for MALDI. These are firstly linear TOF instruments (Beavis & Chait, 1989a) using ion acceleration to high kinetic energies (up to 40 keV) to allow for a sufficiently high mass resolution also for ions of considerable initial ion energy spread (see below). Due to the conceptual simplicity, they are bench-top-type instruments. Secondly, reflectron TOF instruments are used allowing for an improved mass resolution; some of these include microscopic observation of the sample by a video-camera system and allow for a selection of the sample area irradiated which showed to be advantageous. All pulsed UV-lasers, such as Nd-YAG (266 or 355 nm) or Excimer-lasers (248, 308, 351 nm), can be used, but small, easy-to-run and inexpensive nitrogen lasers emitting at 337 nm are the most widespread ones. The laser beam is focused to the sample surface by simple quartz lenses to some 100μm in diameter, the laser intensity at the sample is controllable by fine attenuation. Ions are detected by conventional secondary electron multipliers, but due to the relatively low velocity of macromolecule ions even for high initial or post-acceleration, detection efficiency considerably decreases with mass. Furthermore, it is known today (Spengler et al., 1990; Kaufmann et al., 1992) that secondary ions formed by the impact of the large ions may considerably

contribute to ion detection, but cause time resolution problems in a TOF system. The development of improved ion detection systems thus is a highly interesting area. For signal registration fast A/D conversion by transient recorders is required. Their technical performance (sampling rate and memory) considerably improved in the last years allowing today for ns-time resolution and up to 1 million data points to be stored.

The required intimate mixture of analyte molecules in the matrix is practically achieved in a very simple preparation procedure. Small aliquots (0.5 to 1μl) of matrix (ca. 50 mM) and analyte solution (typically $\leq 10\mu$M) in widely variable solvents and solvent mixtures are mixed on a metallic support, dried in air and then transfered to the vacuum chamber of the mass spectrometer. The following matrix compounds have proven to be most useful analytically (the laser wavelengths typically used are given in brackets) :

- 2,5-dihydroxybenzoic acid (337, 355 nm) (Strupat et al., 1991)

- sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and caffeic acid (337, 355 nm) (Beavis & Chait, 1989b),

- 4-hydroxy- α-cyanocinnamic acid (337, 355nm) (Beavis et al., 1992)

Nicotinic acid has been replaced by these compounds in practical applications due to undesired features, especially formation of adduct ions between analyte and matrix. In the IR range best results have been obtained with an Er-YAG laser emitting at 2.94 μm (Overberg et al., 1990). Due to the resonant absorption into OH- and NH-stretching vibrations a large variety of compounds can be used as absorbing matrices. IR-MALDI on the other hand, is so far used only in few laboratories, but may provide some significant developments and advantages in the future.

Usually single laser shots clearly show molecule ion signals, mass spectra are typically accumulated from 10 to 100 single laser shots yielding a better signal-to-noise ratio. The whole procedure including preparation and first evaluation takes only a few minutes.

Figure 1 summarizes graphically the development from LDI to MALDI; it shows the limited accessible mass range both for UV- and IR-LDI and accounts for the restriction of UV-LDI to strongly absorbing samples. The spectra show the direct UV-laser mass spectrum of a pentapeptide (left) with only low molecular ion signals besides a high unspecific background, the MALDI mass spectrum of the same peptide (top) with a prominent protonated molecule and the UV-MALDI mass spectrum of a large protein, a monoclonal antibody (IgG) of ca. 150,000 Dalton (right).

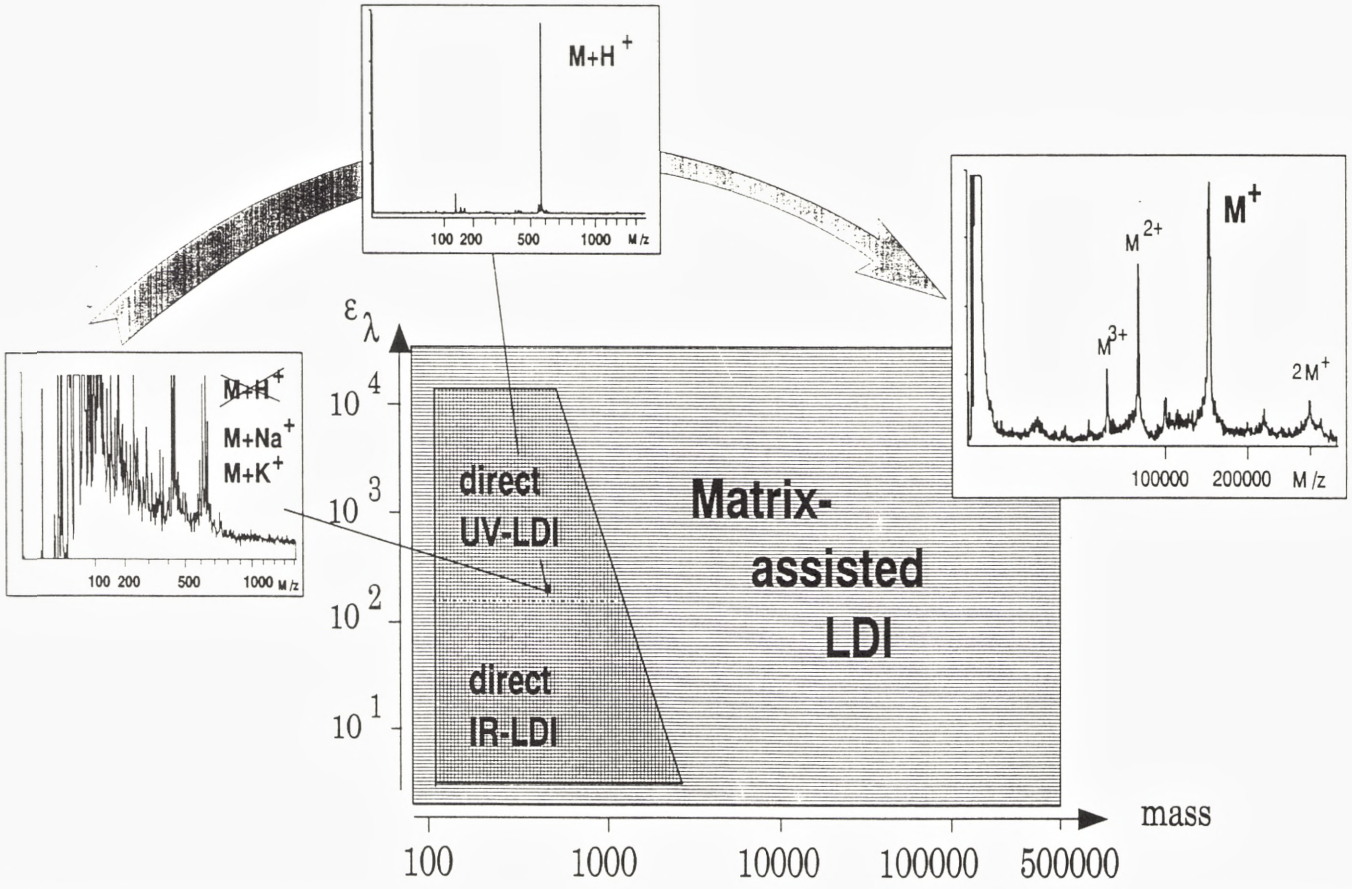


Figure 1. Schematic summary of the development from LDI to MALDI; the extention of the accessible mass range is exemplified by three mass spectra: a) direct UV-LDI mass spectrum of a pentapeptide (left), b) the improvement obtained by use of a matrix for the same

4 Current Analytical Status

Peptides and proteins can be analyzed in the mass range between 500 and 500 000 Dalton (Hillenkamp et al., 1991; Chait et al., 1992). The lower mass limit is usually determined by intense interfering matrix signals. The actual mass limit in the range of some hundreds kDa seems to be rather determined by the currently available ion detectors than by the ion desorption process. MALDI has an excellent sensi- tivity, typical sample loads are $\leq$ 1 pMol (Karas & Hillenkamp, 1989b), 1 femtomol suffices under favourable conditions (Strupat et al., 1991). Furthermore, virtually the whole sample can be regained after analysis, because the amount of material consumed for analysis is much less than the amount loaded. The greatest analytical strength of MALDI seems to be that it can be applied to proteins, independent of their primary, secondary or tertiary structure, of their widely-varying solution phase properties and functional modifications by e.g. glycosylation and phoshorylation (Beavis & Chait, 1990a). Due to the formation of essentially singly- or doubly-charged molecule ions only, mixture analysis and data interpretation is straightforward, also for rather complex natural protein mixtures e.g. milk or saliva, or for cleaved peptides obtained by enzymatic or chemical digestion of proteins (Beavis & Chait, 1990a).

Another important feature of the MALDI technique is that it tolerates even relatively high concentrations of inorganic and organic contaminants (Beavis & Chait, 1990a, Strupat et al., 1991). Salt at physiological concentration and buffers as normally used in biochemical procedures do not deteriorate signal quality, which is unique as compared to other mass spectrometric ionization techniques. This feature also enables the use of the technique in direct combination with biochemical reactions, such as enzymatic digestions. C-terminal sequencing of peptides by carboxypeptidases or determination of the carbohydrate content of a glycoprotein by enzymatic cleavage and MALDI mass determation of the intact and deglycosylated protein may serve as examples.

Mass determination accuracy obtainable is in the 0,01% range provided suitable calibration by well-defined proteins is used (Beavis & Chait, 1990b). Above 30 kDa signals of the analyte containing a matrix adduct are no longer resolvable from the protonated analyte signal. Increased mass resolution is therefore highly desirable, but due to not fully understood reasons reflectron TOF instruments, though capable to achieve sufficiently high mass resolution (up to 4000) for peptides up to 10 kDa, have not been able to yield relevant improvements in the high mass range. Nevertheless, MALDI is a valuable tool for protein analysis also in the higher mass range. For chemically heterogeneous proteins such as glycoproteins the average molecular mass is determined, the width of the molecule ion signal can be used as a semi-quantitative measure for the heterogeneity of the carbohydrate

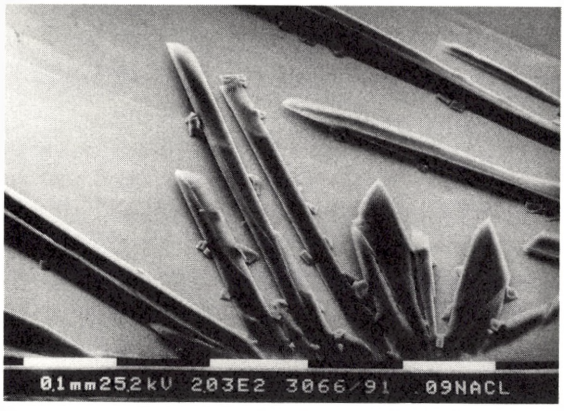
moiety. As a further example, the determination of chelator- and anticancer drug-load to monoclonal antibodies by measuring the mass shift between conjugated and non-conjugated antibody molecules has been reported (Siegel et al., 1991).

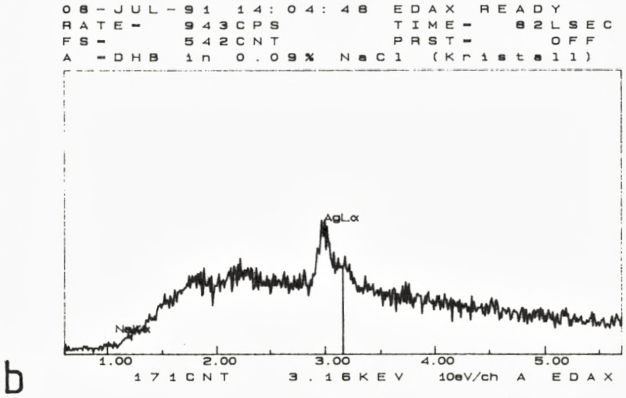
MALDI is moreover not only applicable to peptides and proteins, but also to other classes of biopolymers such as oligosaccharides (Stahl et al., 1991), glycoconjugates, e.g. glycolipids, and oligonucleotides (Parr et al., 1992; Nordhoff et al., 1992), but currently the quality of the mass spectra and the accessible mass range has not yet reached the level as routinely achievable for proteins. Further work is undoubtedly needed in these areas, focussing on new matrices and optimized sample preparation procedures.

5 Current Status of Understanding

The most obvious question is what makes a material function as a matrix. Besides some physico-chemical prerequisites, such as vacuum stability, its solubility in solvents usable also for the analytes and the lack of chemical reactivity with analyte compounds, a strong absorption at the laser wavelength is the first important issue. A high absorption cross section enables to deposit energy into the matrix and to optimize the desorption process by careful control of the applied laser irradiance. Absorption spectra usually determined in solution may be used in a practical first approach, but one has to keep in mind that the absorption coefficients and the shape of the absorption curve for a solid are expected to be different from solution; this has been exemplified for sinapic acid showing a broadening of the absorption band and a shift of the absorption maximum to higher wavelengths (Hillenkamp et al., 1992).

Two further important functions can be attributed to the matrix, but cannot yet be deduced from the chemical structure of a matrix candidate or its physicochemical properties. These are the ability to form a solid solution of analyte molecules in the host matrix and the ability to ionize analyte molecules within the desorption process. Two elegant experiments revealed the surprising ability of two matrices, 2,5-DHB and sinapic acid, to incorporate protein molecules into matrix host crystals when the solvent is slowly evaporated. For 2,5-DHB mm-size crystal were grown in a controlled way, cytochrome c was added as the analyte protein at a low concentration. The undisturbed x-ray diffraction pattern of 2,5-DHB could be observed as well as a uniform distribution of a test protein; nearly identical MALDI spectra were obtained with a laser microprobe instrument by irradiating either the surface or inner parts of the crystal (Strupat et al., 1991). Furthermore the concentration of the protein in the crystal was measured by redissolving them and using chromatographic separation and spectrophotometric determination; the





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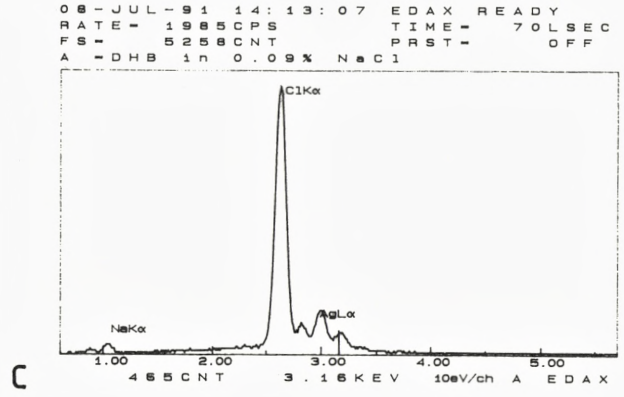

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Figure 2. a) Electron micrograph of a 2,5-dihydroxybenzoic acid preparation, containing 0.9% NaCl in the starting solution b) and c) show x- ray microanalysis spectra of b) long 2,5-DHB needles and c) a small crystalline particle sticking to a 2,5-DHB needle.

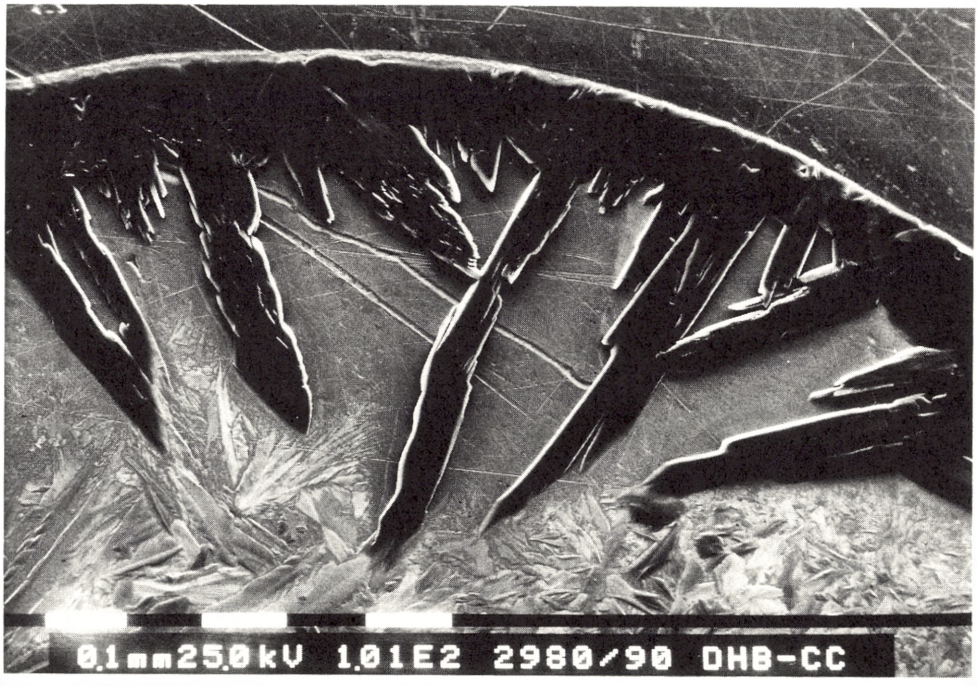

Figure 3. Electron micrograph of a part of a dried droplet of 2,5-dihydroxybenzoic acid (the matrix) containing cytochrome c as an analyte.

protein concentration was found to be directly proportional to the concentration in the starting solution. For sinapinic acid, incorporation into matrix crystals was investigated by using stained proteins. This matrix crystallizes into extended sheets and the specific staining pattern obtained showed that proteins are incorporated into a crystal by a specific interaction with one crystal surface (Beavis & Bridson, 1992). This formation of a solid solution can also explain the tolerance towards ubiquitous contaminants such as salts and buffers as an in-situ cleaning step which incorparates analyte and excludes contaminants upon crystallization. Figure 2 shows a electron micrograph of a 2,5-dihydroxybenzoic-acid-matrix preparation out of a solution containing physiological (0.9%) salt (NaCl) concentration; x-ray microanalysis revealed that the large crystal needles of DHB (and analyte) are essentially salt-free (a), whereas the small crystal particles sticking to the needles consist of sodium chloride (b).

The shape of the crystals is depending on the individual matrix used, it varies from large crystalline needles extending from the rim of the dried droplet into

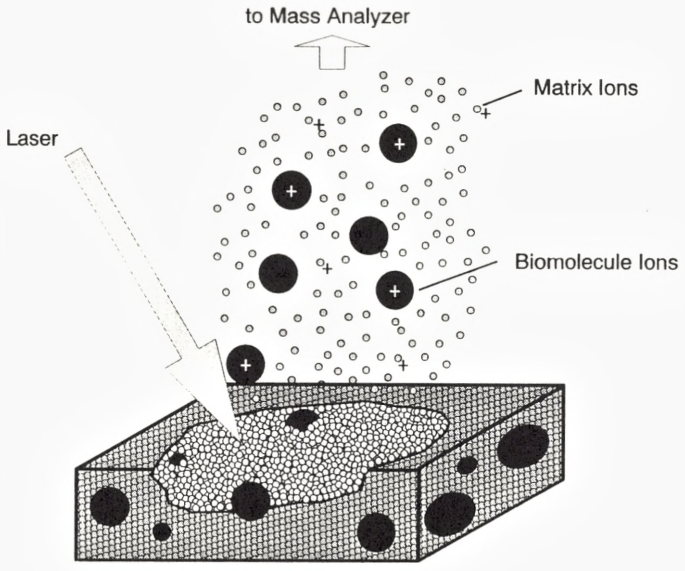

Figure 4. Schematic view on the MALDI process.

the inner area (2,5-DHB) (see figure 3) to finely dispersed microcrystals. At any rate, the light microscopic control of the crystalline structure can be used as a first inspection for a successful preparation step. A too high analyte concentration will alter the crystallization and may therefore deteriorate the MALDI performance. It is furthermore clear that the presence of contaminations which prevent the matix-analyte solution from crystallizing, such as high-vapor-pressure liquids (dimethylsulfoxide and glycerol), are detrimental (Beavis & Chait, 1990a).

The active role of the matrix in ionization has been deduced from the fact that, despite the low relative and absolute concentration of the analyte, molecular ion signals are registered at comparable or even higher intensities than those of the matrix (Ehring et al., 1992). Chemical ioniza- tion by proton transfer reactions between matrix ions and analyte neutrals is regarded to be the underlying mechanism. The ability of a matrix to function as a protonating agent is documented in the respective UV-LDI mass spectrum of the matrix compounds. The presence of both radical and even-electron species in the positive or negative ion mass spectra of all usable matrix compounds may be regarded as an indication for their inability to form stable (non-reactive) ion species and hence their photochemical reacitivity.

The currently discussed models for MALDI are still very rudimentary and far

away from giving a satisfactory description of the desorption ionization process or from giving directions for its optimization.

In the 'pressure pulse' model (Johnson et al, 1991), originally developed for fast-ion-induced ejection, a pressure gradient is set up perpendicular to the surface by the energy deposited; if that exceeds a threshold value molecules gain a net expansion velocity. The authors point out that the interpretation of ion yield as a function of fluence may be misleading, because ionization itself may be depend on fluence in a different way than the ablation. The model also predicts an increase of the expansion velocity with increasing laser fluence, which has actually not been observed in postionization experiments. In these experiments the velocity of the desorbed neutrals is determined by a variable time delay between the desorption and the ionizing laser (Dreisewerd et al., 1990). Alternatively, in a sublimation-like model molecules would leave the surface due to a local surface temperature, but an effective threshold would be observed as the yield rapidly increases over a small range of fluences. This was assumed in the 'bottleneck' model (Vertes et al., 1990) but emphasis is put on the question how, despite the high temperatures calculated, fragile large biomolecules can survive desorption to be detected as intact molecule ions in a time- of-flight mass spectrometer. Model discussion has until now concentrated on the desorption process, desorption and ionization is therefore separately discussed in the following. Figure 4 tries to visualize the MALDI process.

In the meantime, a considerable amount of data has been accumulated illuminating some relevant characteristics of the desorption process and properties of the desorbed species.

This is firstly a very steep threshold behaviour in ion desorption as a function of the applied laser pulse energy (under otherwise constant conditions, such as focus diameter) which has been reported already in the original papers and has been clearly confirmed by single-ion counting experiments (Karas et al, 1985, and 1989a; Ens et al., 1991). This holds for the neutrals, as shown in postionization experiments (Spengler et al., 1988), as well as for the ions, which for the examples examined show a somewhat higher threshold energy. These thresholds were regarded as a proof for a collective process involving a microvolume of the sample, but the true physical origin of these thresholds is still unclear. Therefore it is an open question whether a threshold irradiance (W/cm^2) or a threshold fluence (J/cm^2) is the relevant physical parameter (Karas et al. 1987; Beavis, 1992). It is furthermore noteworthy that the actual values given by the different groups differ by more than an order of magnitude. Irradiance has been used in the original papers to account for the fact that short-pulse lasers have been used and that the respective desorption process cannot be induced by conventional heating. Provided that the laser pulse is short enough and heat conduction to the surrounding sample layer or substrate is negligible (see below), fluence or irradiance can be used equiv-

alently for the calculation of the deposited energy. First results on the influence of the laser pulse time have been reported and seem to indicate that for shorter laser pulses the laser energy stays constant (and thus the irradiance increases) (Demirev et al., 1992)). Under the assumption of an essentially linear absorption process and using the absorption coefficient from solution, Beer's law can be used for an estimate of the energy deposited as well as for the penetration depth of the laser into the condensed-phase sample (Karas et al., 1985). Typical values are in the range of 2 eV (photon energy: 3.7 eV at 337 nm) per molecule in the uppermost layer. This would provide enough energy to sublime the upper layers of the irradiated volume. It is, however, interesting to note that the observed steep rise of the threshold curve can be rationalized by a simple geometric consideration, i.e. the increase of the surface area exposed to an irradiance/fluence above a threshold value in a gaussian laser beam of increasing pulse energy (Beavis, 1992). A possible explanation could be that only a very shallow surface layer (much thinner than the absorption depth) is ablated in the threshold regime. At this point in time, the threshold phenomena still need further investigation, e.g. by variation of laser pulse time, spot size and beam profile. As Johnson and Sundqvist pointed out, it may be misleading to directly interpret the data obtained from ion yield vs. fluence measurements.

Initial velocities of desorbed matrix and analyte ions have been investigated by measuring the flight-time differences between prompt and a 'two-stage' extraction with an initial field-free region (Beavis & Chait, 1991; Zhou et al., 1992). Results show a high initial velocity in the range of 700 m/s for analyte ions, irrespective of their mass, and hence an initial energy proportional to the ion mass, and a slightly higher velocity for matrix ions. To account for these high initial velocities, the phase transition of a matrix layer to a high-pressure fluid and its adiabatic expansion into the vacuum, forming a supersonic jet, has been proposed (Beavis & Chait, 1991). The strongly forwarded emission characteristics has been confirmed by measurement of the radial kinetic energy which is the range of 2.4 eV for insulin compared to 17 eV (Ens et al., 1991) of axial energy.

Despite this directional emission and high initial velocities, ions exhibit kinetic energy deficits, a typical value for positive molecule ion of insulin was determined to be 24 eV. The size of the deficit increases both with the intensity of the signal and the molecule ion mass; it is also larger for negative ions (Zhou et al., 1992). Two processes may be responsible for this feature of the desorbed ions. The first is an impediment of the ion acceleration by collisions in the expanding material plume above the surface, the second is a delayed ion formation by charge(proton)-transfer reactions taking place by collisions of neutrals and ions above the surface and thereby at lower electrical potentials. It is expected that both effects contribute; and it can be anticipated that further research into these processes will become

highly relevant for an improvement of matrix-LDI and will have strong influence on optimized instrumental concepts.

The time scale of the desorption event is essentially confined to the laser pulse length of some ns. This can be deduced from the minimal peak width of the observable ion signals which was found to be in the range of 5 ns for peptides in a reflector instrument (Ingendoh et al., 1993). Prolonged ion emission which would not be compensated for in a reflector TOF instrument can thus be excluded.

The electron-microscopic inspection of the irradiated matrix/analyte preparations confirms that only a very shallow surface layer is ablated in matrix-LDI with UV-lasers (Strupat et al., 1991). Visible ablation and the formation of craters happens only at considerably higher irradiances, far above the useful irradiance range. On one hand, the observation of a minute sample ablation agrees well with the small penetration depth of the laser light, as estimated with the assumption of linear absorption processes. This also explains the very high number of LDI mass spectra obtainable from one sample spot. On the other hand, ablation does not proceed uniformly on a microscopic scale. Slight local roughening can be observed within the irradiated area, as well as small spherical structures which indicate melting of the sample; the latter accumulate at edges or cracks in the matrix-analyte crystals. These observations have been done with 2,5-dihydroxybenzoic acid which crystallizes into large structures. Whether these observations can be applied also to other matrices needs to be investigated.

The initial statement that MALDI produces exclusively intact quasimolecular ions has in the mean time been replaced by a more refined picture. Fragment ions have indeed been observed for peptides and proteins (Spengler et al., 1991; Hill et al.,1991). For proteins, they mainly arise from loss of small neutral molecules, such as ammonia, water and CO_2. A strong influence of the residual gas pressure indicates that bimolecular collision-induced fragmentation is a major source for these fragment ions. Furthermore, a smaller, matrix-dependent 'thermal load' resulting in metastable fragmentation is stemming from the desorption process (Spengler et al., 1992a). The strong effect of gas phase collisions indicates that also the 'matrix contribution' is due to collisional activation in the dense desorbing material plume and not to direct laser excitation. If time spreads due to postacceleration to the detector can be neglected, these fragmentations do not show up in a linear TOF instrument, and result in resolved peaks or tailing respectively in reflectron instruments. If confirmed by further investigations, these effects set clear boundary conditions for the ion source design. If controllable, the decay of ions may, on the other hand, be a further and highly-useful feature of MALDI, because it provides structural information, e.g. for peptide sequencing (Spengler et al., 1992b).

With regard to a more refined description of the LDI process, the energy deposited into the matrix is the first important factor. Because of the moderate

irradiance values in the threshold regime, linear (classical) absorption processes seem to give a satisfying number for the energy absorbed per absorbing molecule; due to the usually short lifetime of the excited states both multiphoton absorption or bleaching should be negligible. The incident laser energy has to be corrected for reflection losses - which may be hard to do because of the microscopic structure of the sample. The same holds for the energy reemitted by fluorescense. Heat conduction into the surroundings has to be considered as a further loss mechanism. For typical irradiation conditions, i.e. penetration depth is much smaller than the focus diameter, the contribution of the essentially one-dimensional heat conduction can be estimated by the determination of the thermal diffusion depth, $dt_L = \sqrt{4kt_L}$ [k: thermal diffusivity, t_L: laser pulse time][1]. This value describes the thickness of the layer to which heat is transported within the laser pulse time. For typical UV-MALDI conditions this value is in the range of 100 nm, which is comparable to the laser penetration depth. On the other hand, experiments using shorter laser pulses showed that the pulse energy stays essentially constant - which indicates that energy loss due to heat conduction is not essential already at ns-pulses -, longer pulses where this condition is no longer valid have not been investigated yet. It is moreover important to keep in mind, that the intensity profile of the laser at the sample may be a further critical parameter. It is usually assumed to be gaussian, but irradiance or fluence values are calculated for a homogenous (=flat-top) profile.

Within the relatively long laser pulse time, energy deposited into electronic excitation of matrix molecules is expected to be thermalized leading to heating of the organic molecular lattice with a temperature distribution both laterally and in-depth. Two main questions arise:

1) Is a direct phase transition solid-gas taking place or is intermediate melting possible and essential for the process ?

2) Does the MALDI ablation process proceed uniformly, more comparable to an adiabatic expansion of a vaporized surface layer, or is the ablation confined to highly localized areas within the irradiated spot.

The latter is supported by the EM inspection, which shows changes within the irradiated areas on the μm- and sub-μm scale, such as individual minicraters and regions which appear to have melted into spherically shaped structures. An intermediate liquid phase - presumably at highly localized regions in the irradiated spot and preferentially formed at local protrusions and crystal imperfections and boundaries - would become unstable when heated up close to the critical temperature and undergo a phase explosion (Sunner et al., 1988). This may furthermore

[1] Note from your editor: This is inconvenient notation because t cannot be a length and a time at the same time. I suggest you replace dt_L by a length such as x_L. Note also that the differential is inappropriate.

give a new access to the explanation of the threshold behaviour. Intermediate formation of a liquid and the reduced heat conduction of a liquid would result in a steep rise of the deposited excitation energy and favor localized emission centers.

If the fundamental process is a fast laser heating of a defined volume, the next question would be: how can large fragile macromolecules survive? Is there a mechanism which would supply a lower excitation of analyte molecules compared to the absorbing matrix molecules and/or a source of cooling and confining the high temperature to a very short time period? The first case has been discussed proposing a bottleneck for the energy flux within the matrix/analyte system (Vertes et al., 1990). On the other hand, one has to consider that these large molecules have a huge amount of degrees of freedom and may be stable even if heated to relatively high temperatures at least for the time period to be detected in a time-of-flight instrument (McKeown & Johnston, 1991). Adiabatic expansion will result in a cooling of internal degrees of freedom, even though cooling is much more pronounced for the translational energy. The phase explosion as an instantaneous phase transition of a liquid volume is expected to result in the emission of a broad distribution from gas molecules to larger clusters. The shrinking of the clusters by evaporation of matrix molecules would provide a cooling of a 'hot' analyte molecule ion. However, some of these considerations may be futile and misleading. The main objection is that they are based on the strongly enhanced fragmentation observed for the ions with increasing irradiance and simply assume that the same holds for the neutrals. This can only be done if ions and neutrals are excited by the same source of energy, i.e. heating. As discussed above, there is, however, strong evidence that fragmentation is induced by collisions within the field-free drift region and reasonably also in the ion acceleration region. This is further substantiated by an observation done in a laser (neutral) desorption/postionization experiment; postionized intact molecules were still registered at irradiances up to a factor of 10 higher than those applicable for direct laser desorption of molecule ions (Spengler et al, 1988).

All the above-mentioned experimental results and their discussion show that the basic understanding of the desorption (ablation) event is still at a very early stage. Moreover, the above discussion focuses on one part of the desorption ionization process, i.e. the ablation. It is obvious that the ionization of the analyte molecules forms the second equally important step of the LDI process. For the ionization, however, a more elaborate model is available today which covers qualitatively most of the features of the observed ion spectra (Ehring et al., 1992). The starting point for this model has been the careful inspection of the UV-LDI mass spectra of numerous small organic compounds. An active role of the matrix ions is deduced from the fact that high-intensity analyte ions are observed, even though they are present only at a high molar dilution in the matrix-analyte mixture. Cationization, i.e. the

adduct ion formation between alkali ions and neutral molecules, is only of minor importance for peptides and proteins. Desorption of preformed ions would result in a strong influence of the chemical properties of the peptides which is actually not observed. Formation of ion pairs by a disproportionation reaction is energetically unfavorable as can be deduced from simple thermodynamic considerations.

The basic idea of the ionization model is that ionization is initiated by photoionization of the matrix molecules, either by absorption of a second photon, by an excited-state molecule, or by reaction of two singly-excited molecules. Thus, a radical (positive) molecular ion as well as a solvated electron or a radical anion ,respectively, are formed initially. These reactions will occur at the early phase of the desorption ionization event, i.e. within the laser-pulse time and still in the solid or the high-density expanding material plume. The ionic species finally observed are products of (photo)chemical reactions between ions and neutrals and reflect the chemical reactivity of the radical ion precursors. Compounds which are able to form stable radical or protonated or deprotonated species should therefore have a low tendency to transfer their charge to analyte molecules. The ionizing reactivity of a matrix is expressed in the positive and negative ion mass spectra of the matrix compounds by the presence of both radical and even-electron species and further uncommon species such as high-abundance $[M + 2H]^+$ - or $[M - H]^+$-ions, and indeed all matrix compounds found usable so far show this behaviour. These reactions may furthermore become highly probable if they happen within desorbed clusters of matrix containing one analyte molecule.

6 Conclusion

MALDI is a relatively young desorption ionization technique which has considerably extended the useful mass range for mass spectrometry. It has already found widespread analytical use. This is also expressed by the fact that several commercial instruments have become available in a short time after the introduction of the technique. It is noteworthy that with the introduction of the technique new fundamental problems and questions arise; the most important are the phenomena of large-ion impact on surfaces which are highly relevant for the detection of high-mass ions by conventional ion detectors. It is typical that progress has so far been mainly obtained empirically. Improved basic understanding of the LDI process will not only improve practical aspects such as the choice of the new matrices, but is expected to have direct impact on the instrumental design and its optimization. IR-MALDI whose feasibility has been shown in the author s lab both for Er-YAG (2.94 μm) (Overberg et al., 1990) and for CO_2-lasers (10.6 μm) (Overberg et al., 1991) and which yields mass spectra of comparable characteristics will open

a new field with regard to the elucidation of the underlying fundamental processes. Preliminary results indicate that the energy deposited by vibrational excitation is below that required for vaporization or even melting. It therefore appears that new concepts both for the ablation process and for the ionization have to be developed.

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