MALDI and Related Methods: A Solved Problem or Still a Mystery?

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Abstract

MALDI ionization mechanisms remain a topic of controversy. Some of the major modern models are compared, with emphasis on the those of the author. Primary formation, secondary reaction and loss mechanisms are considered.

Keywords

MALDI, primary and secondary ionization, recombination, ablation

Introduction

A variety of processes have been suggested to contribute to ion formation in MALDI.[1] While many are no longer under active consideration, consensus has also not been reached. Because MALDI is a multifaceted process, it is easy to loose sight of one aspect while investigating another, but all aspects need to be taken into account, to develop models that are consistent with the available data. This paper presents an overview of key aspects of MALDI with ultraviolet laser excitation, and compares some models, with emphasis on those of the author.

Computational Methods

The rate equation model has been described in detail in refs. [2-4]. Briefly, it is a system of coupled differential equations for the evolution of neutrals, positive and negative ions, of both matrix and up to two analytes. Ionization can be either via pooling of matrix excitons, thermal, or preformed. Nonionic excited states of the matrix are treated, but the analytes are assumed not to absorb the laser light. Forward and backward ion-molecule and ion-ion reactions are treated with an Arrhenius approach, using nonlinear free energy relationships to estimate activation energies. All reactions are modulated by the temperature and pressure evolution of the plume, which is treated as an adiabatic expansion.

The breathing sphere molecular dynamics model has been described previously [5-9]. The extensions of the model to include the photophysics and ion–molecule chemistry of MALDI have been reported in refs [10-12].

Ablation and MALDI Ionization Processes

Deposition of energy in the MALDI sample by the laser is typically on the scale of a few nanoseconds, although shorter and longer pulses have been investigated. This is not very fast on a molecular scale, and ablation of the sample is largely governed by the energy density vs. depth. In UV MALDI the laser penetrates a few 100 nm, and the deposited energy decays (roughly) exponentially with depth. As a result, the top layers ablate fastest, are hottest and disintegrate most completely. Lower layers are colder, slower, and confined longer before reaching a low pressure regime. Clearly it is necessary to understand both ablation and ionization together, they are not isolated events.

The multifaceted nature of the MALDI ablation event is illustrated in Fig 1. After deposition of the laser energy, the hottest surface layer begins to evaporate individual molecules. This is quickly followed by a so-called phase explosion in which multiple subsurface nucleation events occur, leading to a frothy zone of high pressure gas and condensed matrix liquid. Further down in the material, where the energy density is insufficient for phase explosion, compression and rebound waves can exceed the cohesive strength of the material leading to spallation of larger, cold chunks of material.

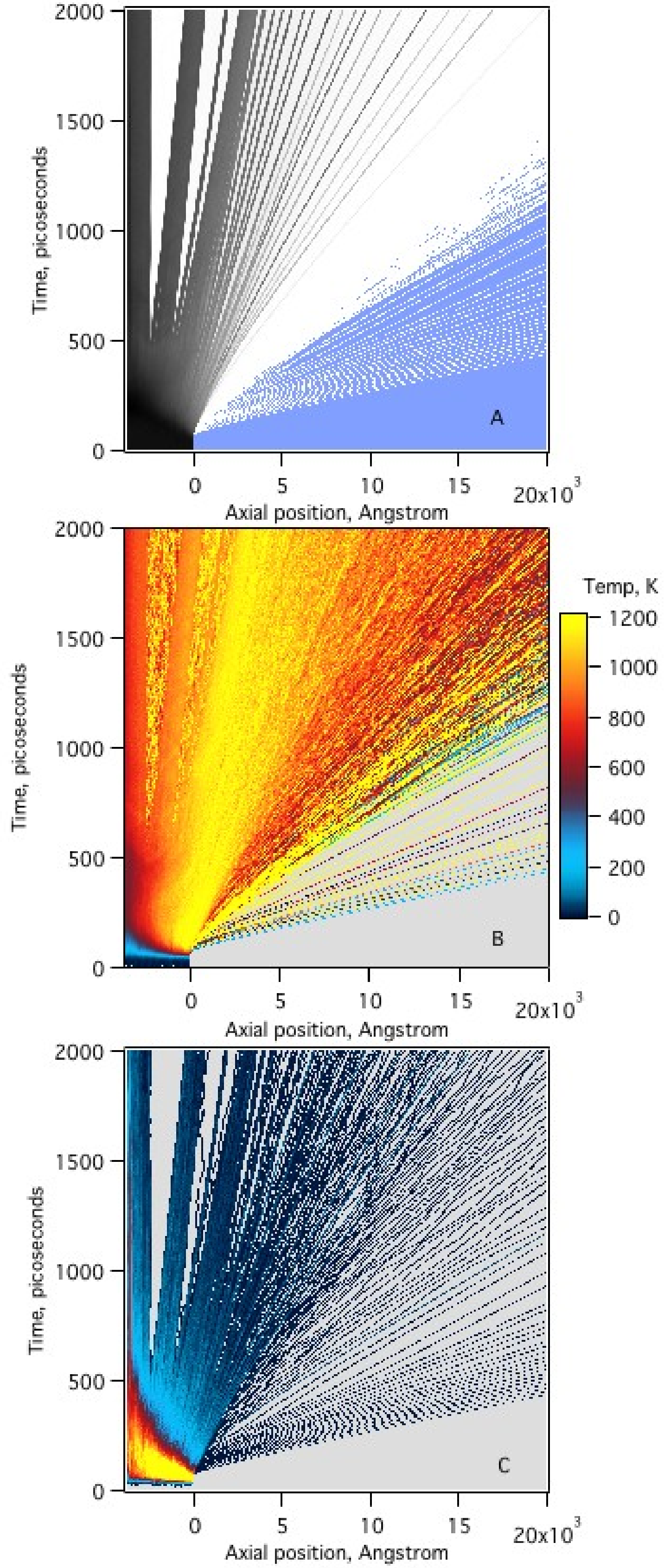
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Figure 1. Molecular dynamics simulation of a MALDI event showing total density (a), temperature (b) and charge density (c). At each time point (vertical axis), laterally averaged properties are shown. The laser is incident from the right. To the left, outside the image, the solid matrix material continues. In (a), the darkest gray corresponds to the cold solid, the lightest to single molecules per pixel. In (c) the highest charge density (dark gray) was over 150 per pixel, but rapidly decays to single charges. Evident are the widely varying speeds of the different layers, expansion cooling and temperature gradients, and the large difference between early and late charge densities.

As is evident from the molecular dynamics simulation of Fig. 1, the period of high density during and after the laser pulse is relatively short. Energy is quickly converted from electronic excitations into heat, initiating the plume expansion. The initial pressure of the plume is high, several atmospheres. Clearly, it requires a time scale orders of magnitude longer than the laser pulse for the plume to expand to a low pressure state, from which ions can easily be extracted.

This contrast between the initial period of high excitation density and the time to expand leads to a distinction between primary ionization processes that occur quickly, and secondary processes that occur later, and over a longer period. The primary mechanisms are dominated by formation processes, while secondary mechanisms are mostly charge transfer and charge recombination.

Primary Ionization

In the model developed by the author,[2-4] electronic excited states of the matrix are the key to primary ionization. In the solid before ablation, such excitations are mobile and can be treated as pseudo-particles called excitons. Exciton hopping and pooling are well known solid-state processes, and strong evidence for them exists in MALDI matrix materials[13, 14]

One or two pooling steps concentrate energy sufficient to ionize matrix molecules. The resulting radical cation and anion (after electron capture), can react with neutral matrix to form protonated / deprotonated ions. These are somewhat lower in energy than the radical ions, for many matrixes. Ionization processes do not require extremely high exciton densities. At fluences below the MALDI "threshold", electrons are already emitted from MALDI matrixes.[15] The apparent threshold is therefore more of an ablation phenomena, rather than ionization.

Direct laser excitation of analytes, or of strongly interacting matrix-analyte complexes[16-18] is not excluded. However, matrix is alway present in large excess, so any reasonably efficient matrix-only process will dominate ion formation.

These photophysical and photochemical processes have been characterized for a typical matrix (2,5 DHB) and collected into a set of coupled differential equations. The processes are modulated by the plume expansion, which is treated as an adiabatic molecular beam. The model has proved capable of describing in a quantitative or semi-quantitative manner numerous matrix-only MALDI phenomena.[2-4]

Another widely discussed model is referred to as “lucky survivors”.[19, 20] It is based on the premise that all ions are preformed- they exist in the sample before ablation. During ablation, most ions recombine with counterions, but a few “lucky” ones escape this fate, and are detected. The conceptual basis for the model is that many analytes are present in ionic form in the preparation solution, before drying. The empirical basis is qualitative, mostly spectral changes as a function of solution conditions.[20, 20] A recent study[21] claims that “survivors” and an unspecified “gas phase” model, apparently the photochemical model above (which is not at all limited to the gas phase) can be unified by some observations using a special matrix molecule which undergoes highly atypical radical hydrogen atom transfers from a ester group, while normally labile protons are somehow unreactive.

Fundamentally, “lucky survivors” is a projection of familiar solution-phase chemical intuition onto the solid state and then onto the low temperature plasma of the plume. It is not capable of making quantitative predictions of any kind, and is not consistent with some of the most fundamental MALDI phenomena. For example, MALDI spectra of pure matrix materials exhibit a variety of matrix ions, when at most one of them might have been expected, in small quantities, in the preparation solution. Or none of them, in the case of many nonpolar matrixes. And the fact that no ionic solution dries to a solid state with extensive charge separation. Including analyte in the sample leads to even more serious contradictions. In survivors, all ions are preformed, so there is nothing which couples matrix and analyte, or two analytes. It is easy to show that analytes affect both matrix and each other, in a highly reproducible and systematic manner[22, 23] which is not readily explicable in the "lucky survivors" picture.

There has been renewed interest in some variations on thermal models. The idea goes back at least to Allwood and Dyer,[24] (though their corrected mechanism turns out to predict very low yields). Beavis and Chait proposed that the plume might offer enough solvation to allow thermal charge separation, but did not quantitatively expand on this.[25, 26] More recently very similar thermal autolysis models have reappeared.[27-30] Other authors[31] have also revived some early electrospray concepts of Vestal[32] involving charged droplets, mainly in the context of "inlet" thermal ionization.

The fundamental challenge for all thermal models is that charge separation is a very energetic process. The final products of MALDI are gas-phase, fully separated ion pairs (MALDI creates both positive and negative ions). The energetics are known for many of the ions observed. At least 500 kJ/mol is needed. For many matrix ions, it is 700-900 kJ/mol. If we assume a plume temperature of 1000 K and an ion separation energy of only 500 kJ/mol, the expected ion yield is vanishingly small, 10^{-14}. To achieve a yield even at the lowest end of experimental estimates (around 10^{-7}), the free energy of charge separation needs to be reduced to approximately 250 kJ/mol. But this is only at the peak temperature of the plume, which may not reach 1000 K, and is certainly lower in deeper layers. As the plume expands and cools, the yield will decrease exponentially. So to have a minimal final yield at a plausible final plume temperature of 500 K would require a free energy of charge separation of around 125 kJ/mol, far below the known values.

The only escape from this dilemma would appear to be a transient reduction in separation energy in the early, hot plume, followed by a kinetic limitation that prevents reequilibration as the plume cools. The means to reduce charge separation energy would presumably be solvation of the nascent ion pair by dielectric screening. The "solvent" must be matrix, since it is the only substance in sufficient excess. The dielectric constant needs to be very large to achieve the necessary screening, 35 or more. However, polar aromatics like typical matrix molecules have dielectric constants around 10 at room temperature, and all dielectric constants drop at high temperature. Even water has a dielectric constant which is much too low at plume temperatures.[33]

Models which place emphasis on droplets[31] also suffer from difficulties with ion generation. Comparisons with electrospray are sometimes made, but this is misleading. In electrospray an external circuit is used to charge the liquid surface, and thereby the subsequent droplets. In MALDI, the plume has no net charge, so some method of droplet charge separation is needed, but has not been specified in the proposed models. Statistical fluctuations are insufficient. As Dodd concluded, even water droplets do not acquire significant charge this way.[34] Both this and several other related proposed mechanisms fundamentally rely on significant screening (they were developed for water), and are therefore not plausible in MALDI. There have been arguments that highly charged analyte ions require some special mechanism,[31] but Tabet has suggested this is a normal aspect of desolvation,[35] and highly charged analytes evolve naturally in the two step photochemical model.[11]

Secondary Ionization

After primary ions exist, they are free to react with other components of the plume. Perhaps the most important reaction is recombination, which is discussed separately below. In the models of the author, charge transfer reactions are assumed to follow the usual Arrhenius rate equation, with activation energies related to the free energy of reaction by means of nonlinear free energy relationships.[36, 37] Other models presume some plume reactions, but make no statements regarding kinetics, and hence no predictions of how ion populations should evolve in the plume, or the consequences of this for the observed result.

Although known for some time, it is worth noting some of the more dramatic MALDI phenomena which demonstrate the role of plume secondary reactions. Relative concentrations of analyte to matrix and analytes to each other have a large effect on the observed mass spectrum. A single analyte in sufficient concentration can completely suppress all matrix ions, this is the matrix suppression effect (MSE).[22, 23] In positive mode this includes matrix radical cations, proton and other adducts, as well as matrix fragment ions. The analyte may itself be of various types, radical, protonated or other adducts.[38] The effect may be understood as competition for charge. If enough analyte is present, and its ions are thermodynamically favored, other ions will be depleted to insignificance.

Clearly, this effect shows extensive reactions between all ionic and neutral species in the plume. It is strong evidence against "Lucky Survivors", because that model provides no mechanism for coupling different ions, either they exist preformed or not. Adding more or less analyte should have no effect on matrix preformed ions, and vice versa.

An analogous effect is observed between analytes. If sufficient quantities of multiple analytes are present, competing secondary reactions between them can again result in strong depletion of the energetically least favored species. This is known as the analyte suppression effect (ASE).

The quantitative models described above correctly predict and describe both the MSE and ASE. Examples are shown in Fig. 2

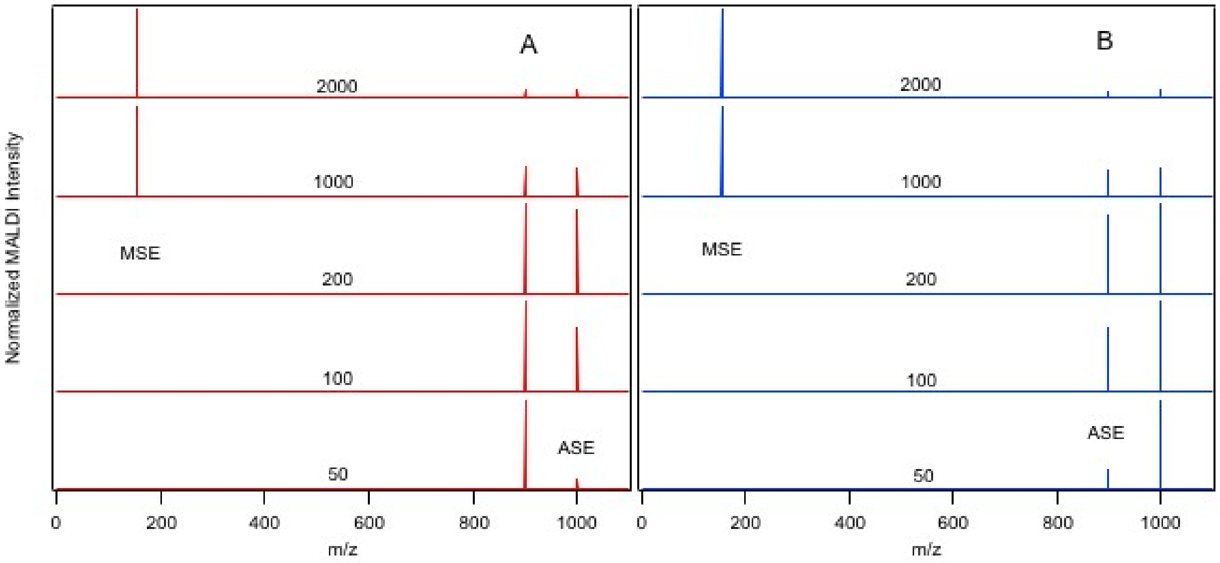

Figure 2. Rate equation calculations of MALDI ion intensities for DHB matrix and two peptidic analytes, with molecular weights 900 and 1000. Panel (a) shows positive ion spectra; panel (b) negative. The matrix-analyte charge transfer reaction free energies were: analyte 1: -150 kJ/mol (positive) and -50 kJ/mol (negative). Analyte 2: -75 kJ/mol in both polarities. The matrix:analyte mole ratios in the sample before ablation are indicated for each spectrum. Both analytes were present in equal amounts. Between M/A=1000 and 200, the matrix suppression effect (MSE) becomes apparent. At higher analyte concentrations, the analyte suppression effect (ASE) occurs. These effects have also been demonstrated experimentally, as noted in the text.

While simple preformed models like "Lucky Survivors" make weak predictions and fail to account for some MALDI effects, the topic of preformed ions should not be left without a closer examination. It is, of course, true that some widely used matrixes are carboxylic acids, with corresponding degrees of ionization in aqueous or polar organic solution. Stronger acids are also sometimes added to the solution (e.g trifluoroacetic or formic acids). Certain very important analyte classes, such as peptides and proteins have well established and highly predictable acid-base characteristics, from which it is clear that they are often charged to a significant extent in these solutions.

What happens to these ions when the solution is dried to make a MALDI spot? As solvent is lost, ionic materials become ever less soluble, pKa values decrease. Like common salt, materials which are highly dissociated and ionic in aqueous solution dry so as to pair positive and negative ions to the highest degree possible.

MALDI matrixes and the analytes in a MALDI sample are not different. Acidic matrixes crystallize as molecular solids- not as separated positive and negative charges. An analyte which has been protonated by a strong acid in solution will pair in the solid sample with the conjugate base, as a salt. This is normal behavior, there is no reason why a MALDI sample should be any different.

Of course, defects and dislocations remain in any real sample, and these will be more common in a heterogeneous MALDI sample than in a carefully prepared pure single crystal. But even if large amounts of separated ions exist, they are insignificant if the exciton/pooling model is correct. Consider the predicted results when there are no preformed ions and all are preformed. In both cases, the model predicts are identical. The preformed ions are overwhelmed by the laser-generated ions, and the ion yield is determined by the race between recombination and plume expansion.

Local Thermal Equilibrium and Kinetic Limitations

As seen in Figure 1, and is intuitively obvious from the way laser energy is absorbed by the matrix, the sample is not uniformly heated. Because top layers are both most strongly energized, and the least hindered in their expansion, they ablate with the highest speeds. Deeper layers are progressively slower. This has the important consequence that the axial scale on which there is heat or mass exchange is short. Exchange becomes ever more hindered as the plume expands and layers are less dense and more separated. Effectively, sample layers rapidly become independent of each other.

Since the plume never comes even close to being a homogeneous fluid, with uniform pressure, temperature and density, equilibrium cannot be reached within it. However, as the molecular dynamics simulations make visible, there are many collisions in the early plume, so over distances of thermal communication, layers might approach what is known as local thermal equilibrium (LTE).

In LTE, enough collisions take place that local concentrations of chemical species may be approximated by their respective equilibrium constants, at a locally defined temperature. Depending on the kinetics of different reactions, some might be in LTE while others are not. The LTE at one place in the plume is not the same as in another, and is not constant over time.

MALDI spectra often give the impression of reaching a kinetic steady state, which has been (rather casually and incorrectly) labelled as equilibrium,[39] in some cases. Breuker et al [40] showed that ion ratios changed significantly as fluence was increased above threshold, but that above some fluence these remained quite constant. The results were interpreted in terms of charge transfer kinetics, in an LTE-like framework.

The many discussions of charge transfer thermodynamics in MALDI, and its utility for predicting or interpreting spectra are also all based on an assumption, sometimes not precisely articulated, of LTE or similar. Most recently Kim and coworkers have proposed that spectra can be selected for their effective temperature (as reflected in matrix fragment ion ratios), and that the corresponding analyte ion intensities can be calibrated based on an LTE interpretation.[41]

On the other hand, positive vs negative analyte ion ratios have been shown to be far from LTE, for a series of analytes and matrixes.[4] Recent results from the Kim lab also seem to show trends opposite from the thermodynamic expectations.[27]

How can these results be reconciled with each other, and with a plume that is highly nonuniform both in space and time? There are at least two major contributing factors. First, the different layers of the sample acquire very different velocities, and expand at much different rates, so that it is extremely difficult to collect ions from them all in a vacuum or low pressure ToF instrument. (If ions are collected at atmospheric pressure or in a trap, they pass through a period of collisional cooling and equilibration, which leaves them in a state that has limited memory of the starting conditions). There is therefore a strong selection bias, probably almost always for upper layers, which are faster, have higher ion densities, and which reach collision free conditions sooner. The sampled ions therefore represent a relatively homogenous subpopulation of the total MALDI event.

Second, LTE becomes increasingly probable at higher temperatures and pressures, since these factors increase reaction rates. These are the conditions in the earliest part of the plume. As it expands, reaction rates drop due both to cooling and to decreased collision rates. Since both change exponentially, the LTE concentrations of the early plume may become "frozen" downstream. In other words, LTE is a transient phenomenon in the MALDI plume. It therefore seems more accurate to speak of a kinetically limited reaction quotient, and not an equilibrium constant, when discussing MALDI spectra.

None of this is a problem for either the rate equation or molecular dynamics models discussed above. They make no assumptions of LTE or any kinetically limited state. Rather the kinetics of all reactions are explicitly treated, and allowed to evolve without constraint. This was critical in showing that the models are fully consistent with the observations of positive/negative ion ratio,[4] which are probably among the most kinetically limited in MALDI. This is because there is generally a large difference in reaction free energy for the forward and reverse matrix-analyte charge transfer reactions. If, for example, the forward reaction for proton transfer is strongly favorable, as it often is, the reverse reaction has a large activation energy to overcome. Forward and backward reactions must balance for LTE, but the backward reaction is very slow, so the positive and negative polarities generally remain far away from LTE in MALDI.[4]

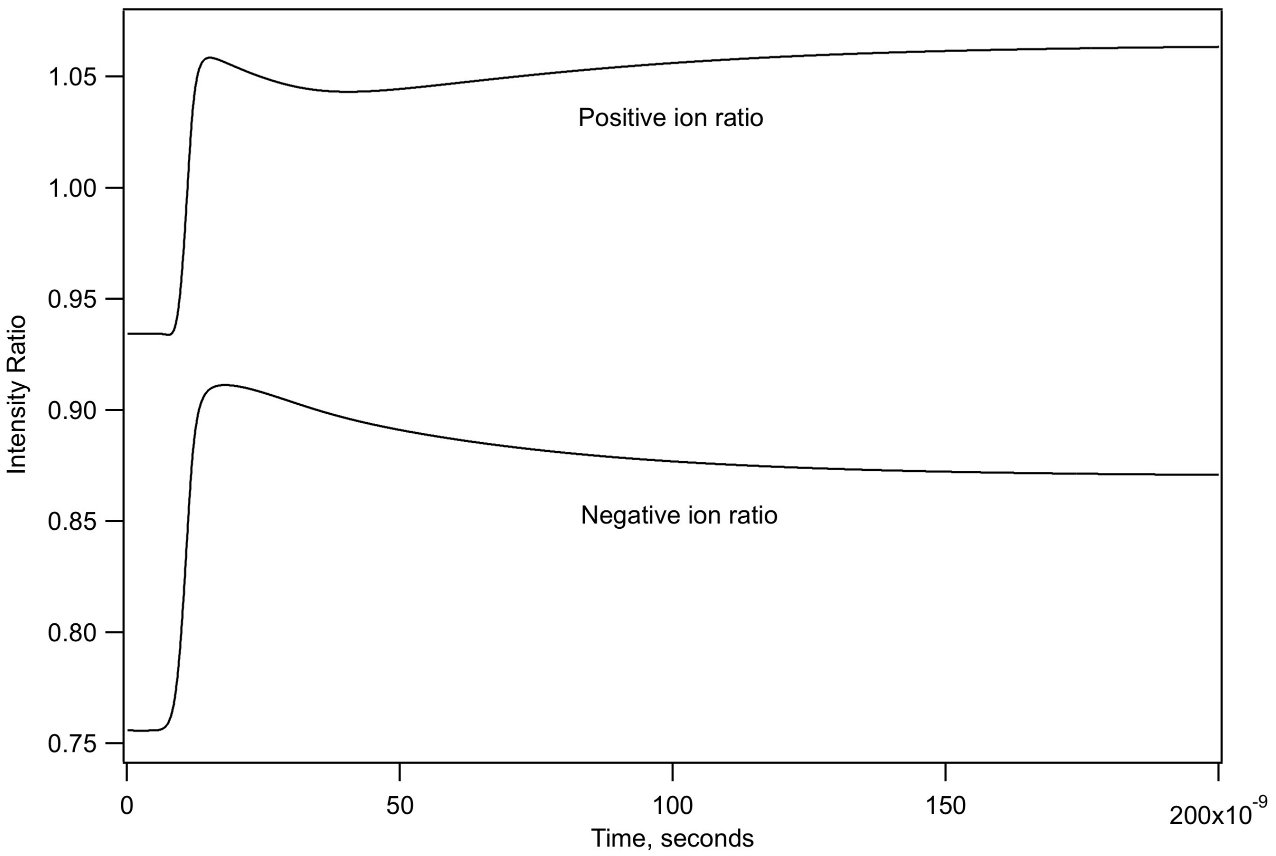

Figure 3. Rate equation calculations of MALDI ion intensity ratios for the analytes of Figure 2, versus time during the MALDI event. The upper curve is the ratio of positive analyte 1 to positive analyte 2 ions, the lower curve is the corresponding negative ion ratio. The matrix/analyte mole ratio in the sample was 0.005 for both analytes. Since the analyte 1 charge transfer reaction with matrix is 100 kJ/mol more favorable than that of analyte 2, a large positive ratio is expected, if no kinetic limitations exist. However, the ratio is modest, 1.05. The opposite should be true in negative mode, analyte 2 is 25 kJ/mol more favorable than analyte 1. Again the ratio deviates by less than 15% from 1. However, in neither case does the ratio correspond to the mole ratios in the sample. Note also the pronounced time dependence, which is a consequence of the multiple coupled formation, transfer and loss mechanisms.

Loss Mechanisms

While much discussion has focussed on the means by which charge might be separated in the MALDI event, far less effort has been devoted to understanding the fate of those ions. This is surprising since it is likely that loss mechanisms will as much determine the final outcome as formation. The degree of ionization expected at equilibrium in the cool downstream plume is extremely low, so the final stages of MALDI are a race between expansion and recombination.

If ions are widely dispersed in a sea of neutrals, many collisions will occur until two ions find each other and can neutralize. In this case, it is unimportant exactly how they neutralize, this step will be much faster than the diffusion time needed to come into contact. This is the Langevin model of recombination, that finds application in solid state physics.

In MALDI, however, calculated charge densities are in the range of several percent, at their peak. The approximation of diffusion limited rates is certainly no longer valid. Ions will be often in proximity, but will obviously not react instantly. A more refined and chemically reasonable model for recombination rates is needed.

However, in recent work,[12] it has been proposed that electron tunneling is the dominant charge transfer mechanism in MALDI recombination, even for ions which are not radicals. The reasoning is that electronic wavefunctions are far more diffuse than nuclear ones, so electrons will transfer and higher rates and longer intermolecular distances than protons or heavier ions. After neutralization, hydrogen (or other) atom transfer follows at a slower rate.

Tunneling rates have been measured for hundreds of donor-acceptor pairs, so the associated parameters are very well established. They fall into rather narrow ranges, so it is likely that MALDI tunneling is similar. Molecular dynamics simulations with these parameters gave very plausible time courses and recombination rates, consistent with those empirically determined for the rate equation model.[12]

One remarkable tunneling phenomenon is the so-called Marcus inverted region, where the charge transfer rate decreases with increasingly favorable reaction free energy.[42, 43] This is due to unfavorable vibrational wavefunction overlap. Evidence for this effect in MALDI may have recently been provided by the Kim group. They investigated a series of ionic liquids, which are believed to have significantly different binding energies.[27] The free energy of the recombination reaction is expected to be close to the binding energies. The measured ion yields were higher for the most strongly bound ionic liquids, rather than for the least strongly. This is the opposite of what would be expected due to simple reaction equilibria or in many models of chemical reaction rates. It is, however, consistent with these reactions being in the inverted tunneling regime.

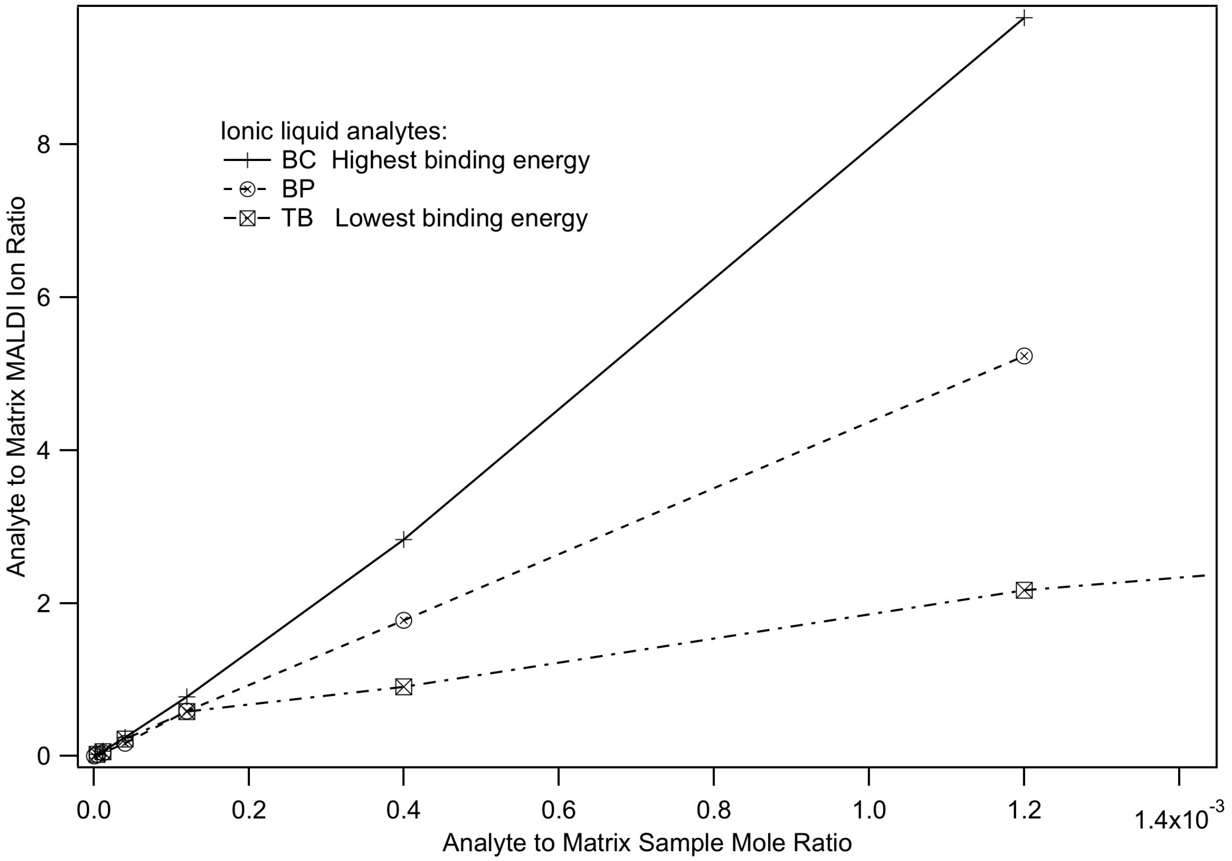

Figure 4. MALDI ion ratios for some ionic liquids, versus concentration in the sample before ablation, adapted from Ref. [27]. The liquids with the higher binding energies, and therefore higher ion-ion recombination energies, give larger signals, at all concentrations. This is contrary to the usual expectation that reactions with larger driving force are faster. On the other hand, it is consistent with a tunneling model for recombination reactions, in the Marcus inverted region. The analytes were BC: benzyltriphenylphosphonium chloride, BP: 1-butyl-

3-methylimidazolium hexafluorophosphate, and TB: trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate.

"Backside" Methods

It has long been known that the MALDI laser does not have to impinge on the sample from the exposed, free side of the sample.[44-47] If the substrate is transparent, the laser energy can be deposited on the "back" side of the sample. Even if the substrate is opaque, ions can be observed, though the mechanism(s) are unclear.

There has been a recent resurgence of interest in such methods, including some that do not use a laser.[48] It is therefore of interest to consider what modern models can contribute to understanding them. Molecular dynamics calculations were carried out for the backside geometry, using the otherwise standard primary/secondary model.

As seen in the next figure, the most highly excited and ionized material, at the back, is strongly confined for a long time. While the thick layer of cold material at the surface would certainly break up over larger lateral dimensions than simulated, it clearly hinders ion release considerably. This has the consequence that secondary reactions take place in an environment that is considerably different that in a frontside MALDI event. The material remains hotter and denser for a longer time. LTE may be better approached, but recombination losses will also be greater. The final result after longer simulations remains to be seen.

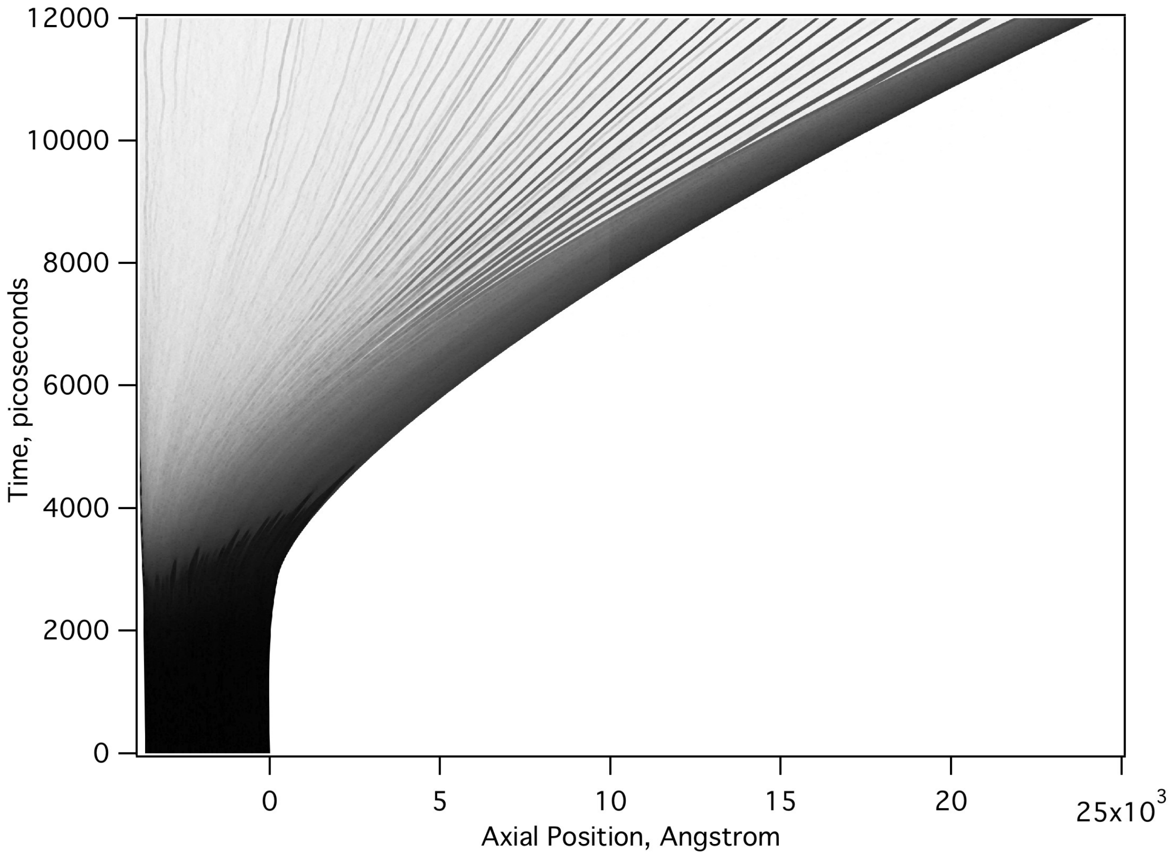

Figure 5. Molecular dynamics simulation of a backside MALDI ablation event, showing time-dependent density. The laser is incident from the left. The most energized, highly charged and thoroughly vaporized material is trapped for a significant time behind cold, thick layers. This can be compared to the very rapid escape of top-layer ions and the decreased time for secondary reactions in Fig. 1a.

Conclusions

This brief discussion has attempted to touch on both old and new topics and results in MALDI mechanistic studies. A few primary ionization models were compared, no single model is expected to be valid in all cases. Subsequent secondary reactions of matrix and analyte ions are widely accepted, but there remain areas of discussion regarding reaction rates, approach to LTE, inhomogeneity, and plume sampling. In contrast to formation mechanisms, loss processes are only starting to be examined.

Key concepts that have been very profitable in the MALDI discussion, and which will continue to be so are: primary vs secondary mechanisms, coupling of ablation and ionization, and the plume as a reaction vessel.

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