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Analyte Incorporation and Ionization in Matrix-Assisted Laser Desorption/Ionization Visualized by pH Indicator Molecular Probes

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Despite the spreading applications of matrix-assisted laser desorption/ionization (MALDI), its fundamental understanding is still limited and under constant debate. This report focuses on the initial state of the analyte in the host matrix. pH indicator dyes serve as molecular probes since their color directly indicates their (de)protonation state. For a set of matrixes at their intrinsic pH, solution color was maintained, delivering clear proof for analyte incorporation in the solution charge state. Moreover, substantial solvent inclusion is determined by ^{1}H NMR spectroscopy. MALDI mass spectra show a clear correlation to the dye charge state. However, the dominant solution species are not observed exclusively in the mass spectra, pointing to a proton transfer or proton neutralization activity of the matrix.

Although matrix-assisted laser desorption/ionization (MALDI) mass spectrometry[1,2] has been used for over 10 years now and has become a powerful tool, especially in bioanalysis, the mechanisms involved in ion generation and desorption are still not fully understood. Many efforts have been made to improve both the practical aspects for successful applications[3−11] and the theoretical understanding of the involved mechanisms.[12−20] But whereas this lead to several new techniques concerning the instrumentation and the preparation protocols, there is still a lack of information about ion formation in MALDI. Nevertheless, despite many aspects that are still unknown, there are also some features of the MALDI process, i.e., analyte incorporation, energy absorption, and ion−molecule reactions in the ablated material plume, that are commonly accepted.[12,21−24] These key roles of the matrix are, however, inadequate and insufficient to explain some of the most striking features of the MALDI process such as the formation of essentially singly charged ions. Recently, a new approach was presented[20] in order to start a new discussion and to focus on essential aspects of the MALDI process, which are obvious from many years of practical experience but not accounted for by the early models. The key feature of the "lucky survivor"[20] model was to connect desorption and ionization by proposing cluster-ionization processes as a dominant ionization and charge-separation pathway. The initial formation of more highly charged cluster—and finally analyte ions—and their charge reduction by electron capture or proton transfer, or both, in the MALDI plume was proposed to account for the detection of essentially singly charged ions.

Within the model, some key starting considerations were clearly hypothetical. One aspect referred to statistical charging of clusters by excess or deficit of ions upon solid ablation, which clearly requires precharged analytes (and their counterions) to be incorporated into the host matrix crystals. The hypothesis was that the solution pH and thus the charge state of analyte molecules is roughly preserved in the crystals. Moreover, to facilitate charge separation, which is needed for the production of charged clusters

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and subsequent ion formation by desolvation, reduction of ion pair interaction by residual solvent was included in the concept. Within the primary ionization step, ionization by generation of charged clusters and photochemical ionization via matrix ions were both still considered to be important without any preference given for their relative contribution. The concept of preformed ions is well known in other desorption techniques such as secondary ion mass spectrometry (SIMS),[25−27] fast atom bombardment (FAB),[28−31] and laser desorption/ionization (LDI).[32,33] Consequently, the existence of cationized[34−36] as well as "preprotonated"[37] analytes in solution and in the matrix crystals was suggested for MALDI, too.

Nevertheless, to date there is no proof that the analytes are incorporated in MALDI matrixes in the same form, e.g., protonation state or solvation, as they exist in solution, although incorporation in the matrix crystals was shown earlier.[23,24,38−41] Moreover, MALDI-MS spectra are influenced by the solution conditions, such as the presence of buffers[42,43] or salts,[15] the choice of solvent,[44−46] and the acidity of analytes,[15,47] matrix,[15,48,49] and solution.[42] On one hand, it is common sense that acidic solutions are beneficial to enhance signal intensities in the positive ion mode. On the other hand, $[M + H]^+$ signals are generally also observed from less acidic solutions and $[M - H]^-$ ions are also easily generated from acidic preparations, e.g., in the case of peptides.

This work utilizes organic pH indicators as molecular probes to address the question of the existence of preformed ions in the host matrix crystals. By inspecting the solution color and that of the corresponding matrix crystals, the analyte's charge state within the crystals can be determined. Moreover, by using matrixes of different inherent pH, the correlation between the state of the analyte in the matrix and the MALDI signal was investigated in a titration-like experiment. In a further approach, matrix crystals were inspected by ^{1}H NMR spectroscopy to give evidence of solvent inclusion.

Several different dyes have been analyzed successfully with MALDI,[50−52] and dyes have been used to examine incorporation in matrixes before.[24,38] However, it has to be considered that LDI is possible without matrix for most of the dyes used for this study due to their strong absorption at the laser wavelength ($\lambda = 337$ nm) and that some of the tested compounds have matrix properties as well.[53−57] Moreover, incorporation of the dyes in the matrix crystals must be guaranteed prior to interpretation of the data. A suitable independent method to ensure incorporation and standard MALDI conditions is the measurement of the initial analyte velocities: MALDI-generated ions show high initial velocities, whereas LDI without matrix leads to slow ions.[58,59] One of the several different approaches to determine initial velocities of MALDI-generated ions[22,58−62] is based on the delayed extraction technique and was first proposed by Juhasz et al.[62] We used a slightly modified method[19] that was recently applied successfully to incorporation studies of MALDI matrixes.[41]

EXPERIMENTAL SECTION

Samples. Dyes and matrixes were obtained from Sigma-Aldrich (Deisenhofen, Germany), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany). Samples were used as supplied commercially without further purification. As standard solvent for sample solutions, a mixture of acetonitrile/water (1:1, v/v) was used.

Sample Preparation (MALDI and LDI). For MALDI standard dried droplet preparations, 1 μL of matrix solution plus 1 μL of analyte solution (10^{-4}−10^{-3} M), both dissolved in acetonitrile/water (1:1, v/v), were mixed on the MALDI target and dried by a gentle flow of air. 3-Aminoquinoline (3-AQ) was dissolved in water (10 g/L) and 2-(2-aminoethylamino)-5-nitropyridine (ANP)

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in ethanol/water (1:1) (10 g/L); all other matrixes were dissolved in acetonitrile/water (20 g/L). The same analyte solutions without addition of matrix were used for LDI experiments. Usually neither acid nor base was added in order to maintain the intrinsic pH of the matrix solution, only if full protonation/deprotonation was required, was sodium hydroxide (0.01−0.5 mol/L) or trifluoroacetic acid (0.1%) added. To produce larger crystals, dye solutions (10^{-5}−10^{-3} M) saturated with matrix were stored either at room temperature (slow evaporation method) or in the refrigerator (4 °C), yielding analyte-doped colored crystals within ∼12 h. The procedures were similar to previously described methods,[23,24] but only water/acetonitrile were used as solvents. Matrix crystals of 2,5-dihydroxybenzoic acid (2,5-DHB) and sinapinic acid (SIN) were grown in the same way (at 4 °C) for NMR experiments; 20 μL of bovine insulin (10^{-5} M) was added to produce analyte-doped crystals.

Another protocol was used for the production of 4-hydroxy-α-cyanocinnamic acid (αCHCA) single crystals: a saturated solution of αCHCA (∼15 mg) was prepared in 400 μL of TFA (1%)/acetonitrile (1:1, v/v) in an Eppendorf tube. A 20-μL aliquot of a solution of the peptide or protein (5×10^{-5} M in water) was added to generate analyte-doped crystals. The solution was vortexed and then centrifuged for 15 min. The supernatant was filled into a new tube and placed in a water bath at 40−50 °C for ∼1 h. After this time, the heating was stopped and the water bath was covered, resulting in a slow cooling of the solution. After ∼6 h, the water was back to ambient temperature and the tube was removed from the bath. A small hole was drilled into the cap, the tube was let at ambient temperature, and some crystals showed up after one or two days.

The crystals were dried carefully, redissolved in deuterated methanol, and examined with ^{1}H NMR spectroscopy. For comparison, neat matrix as supplied commercially was examined in the same way. Some of the crystals were dried prior to spectroscopic examination by two different methods: crystals were either heated in an oven at 100 °C for 3 h or fixed on a MALDI target and stored under vacuum ($p \sim 10^{-7}$ Torr) for 3 h.

Determination of Acid−Base Properties. The intrinsic pH values of the used matrix solutions were measured to have an estimate of the pH during crystallization, taking into account that this pH may change slightly during evaporation of the solvent. The pK_A values were taken from the literature[63,64] or determined by titration: solutions of matrix (10^{-3} M in water) or dye (10^{-4} M in water), respectively, were titrated with sodium hydroxide (0.01−0.1 M) using a pH-sensitive electrode (Hanna Instruments, Woonsocket, RI). Hydrochloric acid was added prior to titration to ensure protonation when necessary, so that all compounds could be titrated with the same basic solution.

Instruments. MALDI-MS experiments were performed on a Voyager-DE PRO time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). The mass spectrometer is equipped with a nitrogen laser (Laser Science, Franklin, MA) emitting at λ = 337 nm. The spectra were mass calibrated with the GRAMS/386 (Galactic, Salem, NH) software. ^{1}H NMR experiments were performed on a Bruker AMX 250 (250 MHz) instrument. Matrix crystals were dissolved in deuterated methanol, and 80 scans were summarized to obtain a sufficient signal-to-noise ratio. The amount of acetonitrile in the crystals was calculated by integration of all ^{1}H signal intensities of acetonitrile and comparison with the integrated aromatic and olefinic ^{1}H signal intensities of the matrix within the same spectrum, taking the number of hydrogen atoms per molecule into account.

RESULTS AND DISCUSSION

Protonation State of Matrix-Incorporated Dyes. The first step was to check whether the protonation state indicated by the dye color changed upon crystallization of the matrix/analyte mixture. Several dyes with different color change ranges and acid−base properties were selected to cover a wide range of pH values (see Figure 1 and Table 1). Crystal Violet (CV) was used as a reference, because it showed no color change in the examined pH range. Solutions of the dyes were mixed with five different matrixes (see Table 2) without addition of acids or buffers, and the color of the solution after mixing was documented. Generally, the color did not change notably after drying. This indicates that incorporation into the matrix crystals does not change the (de)-protonation state of the dyes to a significant extent (see Figure 2). The intensity differences observed are most likely due to local variations of concentration and thickness within the crystalline matrix−analyte layers, and in no case was a significant color change found after crystallization. In a second experiment, matrix and dye solutions were mixed and larger crystals were grown by slow evaporation of the solvent (several days). Again, the crystals showed the same color as the solution before (although not in all cases equally distributed in the crystals), and the color was maintained even after washing with cold water and their partial redissolution (see Figure 3).

These results agree with recent incorporation experiments using confocal laser scanning microscopy (CLSM): Horneffer et al.[65] reported that fluorescence quenching of fluorescein isothiocyanate (FITC)-labeled proteins occurred within crystals of acidic matrixes, whereas only a moderate pH influence on the fluorescence of Texas Red-labeled proteins was observed. This effect was attributed to the protonation of FITC in FITC-labeled proteins, and it is well known that only the anions existing at pH >5 show fluorescence,[66] whereas Texas Red cannot be protonated.

Moreover, the preservation of the dye color throughout the experiments indicates that analyte incorporation is independent of the analyte net charge. For the selected dyes and the pH range inspected, dyes may be protonated (CV in all cases), neutral, or (singly or doubly) negatively charged. Moreover, the used pH indicators are mostly zwitterionic, depending on analyte and pH, but again the net charge is not zero in all cases. Since counterions also have to be included, this raises the question of the final state of precharged analytes in the host crystal, i.e., if they are incorporated as ion pairs or if they are at least partially separated by residual solvent.

Inclusion of Solvent in Matrix Crystals. Several observations point to the fact that analytes might be solvated in matrix crystals. For example, all dyes show their distinct color only in solution, whereas the neat and dry dye has a different, in most cases not

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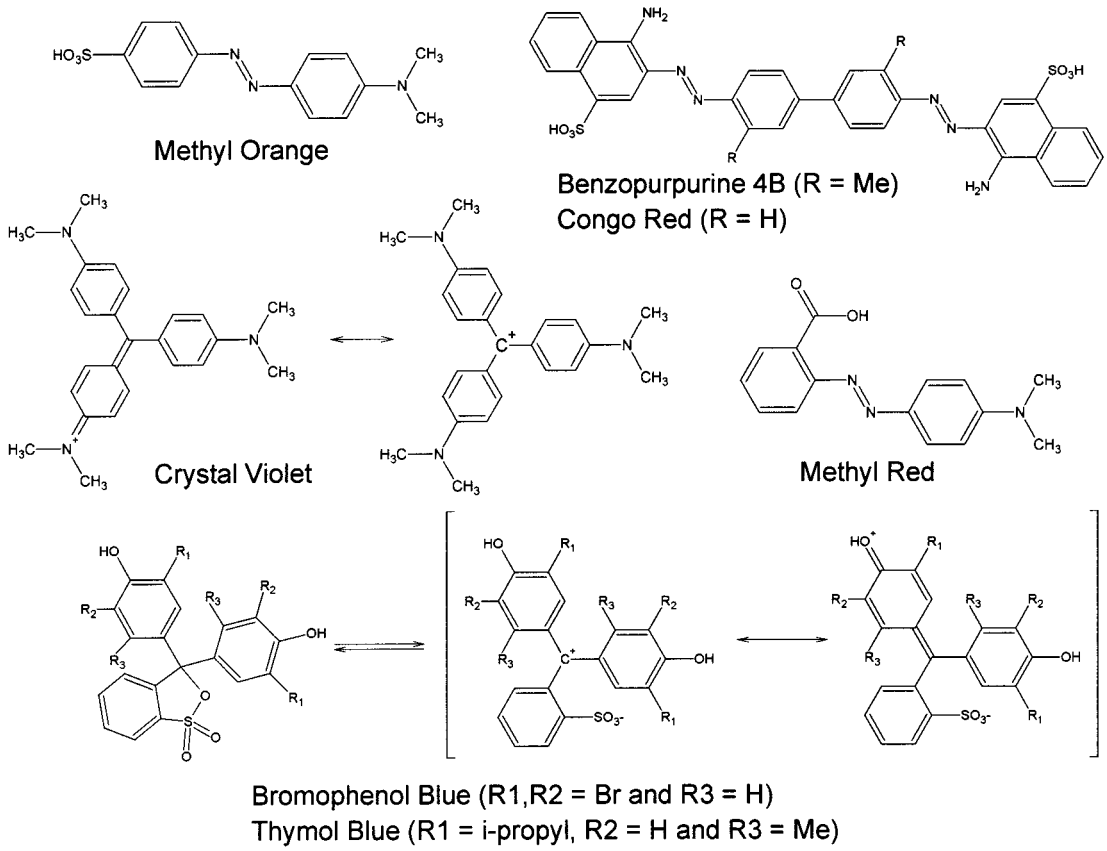

Figure 1. Structures of the investigated pH indicators.

Table 1. Acid−Base Properties of the Investigated Dyes

dye	abbrev	transition pH[a]	transition colors[a]	pK_A values[a]	EP[a] ($c = 10^{-3}$ mol/L)
Crystal Violet	CV	0.0−1.8	yellow → violet	nd[b]	nd[b]
Benzopurpurine 4B	BE	2.3−4.4	violet → light red	∼4[c]	7.5[c]
Congo Red	CO	3.0−5.0	blue → red	5.4[c]	8.2[c]
Bromophenol Blue	BP	3.0−4.6	orange → blue	4.1	7.6
Methyl Orange	MO	3.1−4.4	red → yellow	4.2	7.6
Methyl Red	MR	4.4−6.2	red → yellow	2.5	6.1
			(only 1 transition interval visible)	9.6	10.3
Thymol Blue	TB	1.2−2.8	red → yellow	1.7	5.4
		8.0−9.2	yellow → blue	9.2	10.1

[a] transition pH and colors as given by the suppliers; pK_A values taken from 63 and 64 except for BE and CO, which were determined by titration of 10^{-4} mol of the dye with 0.01 M NaOH; equivalence points (EP) were calculated from pK_A values. [b] nd, not detected (pK_A values and equivalence points below pH 1, e.g., sulfonic acids, were generally not detected). [c] After neutralization of two acidic groups per molecule; resolution of two distinct protonation states was not possible by titration.

very well-defined color. Additionally, a bleaching of the color occurred within the matrix−analyte preparations after a certain time, depending on the matrix used. This aging process was very slow for big crystals (e.g., 2,5-DHB, ATT), whereas it was fast for fine crystalline preparations (e.g., SIN). The larger 2,5-DHB crystals at the rim of the preparation—in contrast to the inner, polycrystalline part—did not lose the color of the dye over a period of several days. These aging processes might therefore be attributed to the loss of solvent. It is well known that samples can show decreasing analyte ion response when they are kept for a longer time in vacuo, sometimes preventing analyte detection after a certain storage time.

To verify the inclusion of solvent, matrix crystals were grown in a mixture of water/acetonitrile (1:1, v/v), dried, redissolved in deuterated methanol, and examined with ^{1}H NMR spectroscopy (see Experimental Section). The amount of acetonitrile was calculated from the relative signal intensities. To rule out absorption at the surface of the crystals and to examine possible loss of solvent after crystallization, the crystals were dried by two different methods: crystals were either heated in an oven at 100 °C for 3 h or fixed on a MALDI target and stored under vacuum for 3 h. The values of all single measurements are summarized in Table 3 and show clearly that acetonitrile is incorporated in the crystals in a range of 0.3% up to 3.7% (n/n dye/matrix) and that the amount is significantly higher compared to the reference, the matrix as obtained from the supplier. Even drying by heating or applying vacuum does not remove all solvent from the crystals. Although different amounts of solvent were determined for different

Table 2. Acid−Base Properties of the Used Matrixes

matrix	abbrev	pK_A values[a]	EP[a] (c = 0.1 mol/L)	intrinsic pH[a] (c = 0.1 mol/L) exptl	intrinsic pH[a] (c = 0.1 mol/L) calcd from pK_A values
2,5-dihydroxybenzoic acid	2,5-DHB	3.3	6.8	2.0	2.1
sinapinic acid (4-hydroxy-3,5-dimethoxycinnamic acid)	SIN	5.6	7.0	3.5	3.3
		∼9.5	∼10−11		
6-aza-2-thiothymine	ATT	6.6	9.0	4.0	3.8
3-aminoquinoline	3-AQ	5.1 (pK_B 8.9)	8.8	8.0	9.1
2-(2-aminoethylamino)-5-nitropyridine	ANP	2.2 (pK_B 12.8)	5.2		
		nd[b]	nd[b]	9.5	

[a] pK_A values were determined by titration of 10^{-3} mol of the matrix with 0.1 M NaOH; intrinsic pH and EP were calculated from pK_A values; intrinsic pH was confirmed experimental with a pH-sensitive electrode (3-AQ 10 g/L in water; ANP 10 g/L in ethanol/water (1:1); all other matrixes c = 20 g/L in acetonitrile/water (1:1)). [b] Resolution of the secondary and tertiary protonation states was not possible by titration.

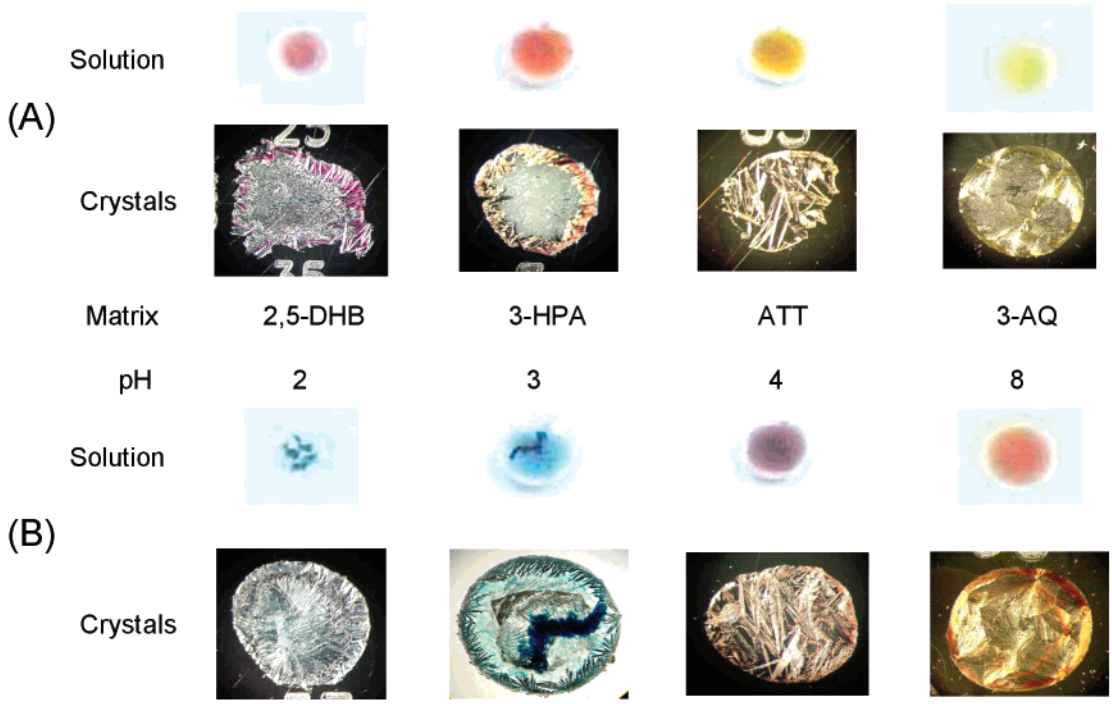

Figure 2. Color comparison of pH indicators in solution and in dried droplet preparations using matrixes with different intrinsic pH: (A) Methyl Orange and (B) Congo Red.

matrixes, there was no significant difference between nondoped crystals and crystals doped with bovine insulin using the same matrix. The highest abundance of acetonitrile as well as the most significant decrease of the solvent content upon drying was found for SIN. On the other hand, 2,5-DHB showed the highest difference between the grown crystals and the reference, whereas the loss of solvent upon drying was not as pronounced in general. These results are in good agreement with the faster aging process of SIN compared to 2,5-DHB. Unfortunately, it was not possible to verify the existence of water in the crystals by ^{1}H NMR spectroscopy because of the fast H−D exchange of the acidic protons of water. Nevertheless, it is not likely that out of a mixture of solvents only organic solvents are incorporated and water is excluded. The relative permittivities are ∼80 for water and 37.5 for acetonitrile, respectively (20 °C),[63] high enough to enable at least partial separation of ions within the crystals, which will facilitate charge separation during desorption.

A recent investigation showed that in the best case a very weak $[M + H]^+$ signal is detected if analyte (e.g., proteins) and matrix are physically mixed and deposited in a solvent-free way.[41] This observation might well be attributed to the missing solvent, because close vicinity between analyte and matrix was clearly confirmed by the experiments. Indeed, the normal MALDI spectrum was restored by addition of solvent, redissolution, and crystallization upon drying. These experiments point again to the importance of residual solvent and thus charge separation by ion solvation for a successful MALDI analysis.

However, recently a solvent-free preparation protocol was presented[67,68] yielding typical MALDI mass spectra for a selection of neutral polymers, and also for insulin, but indeed not (yet) for

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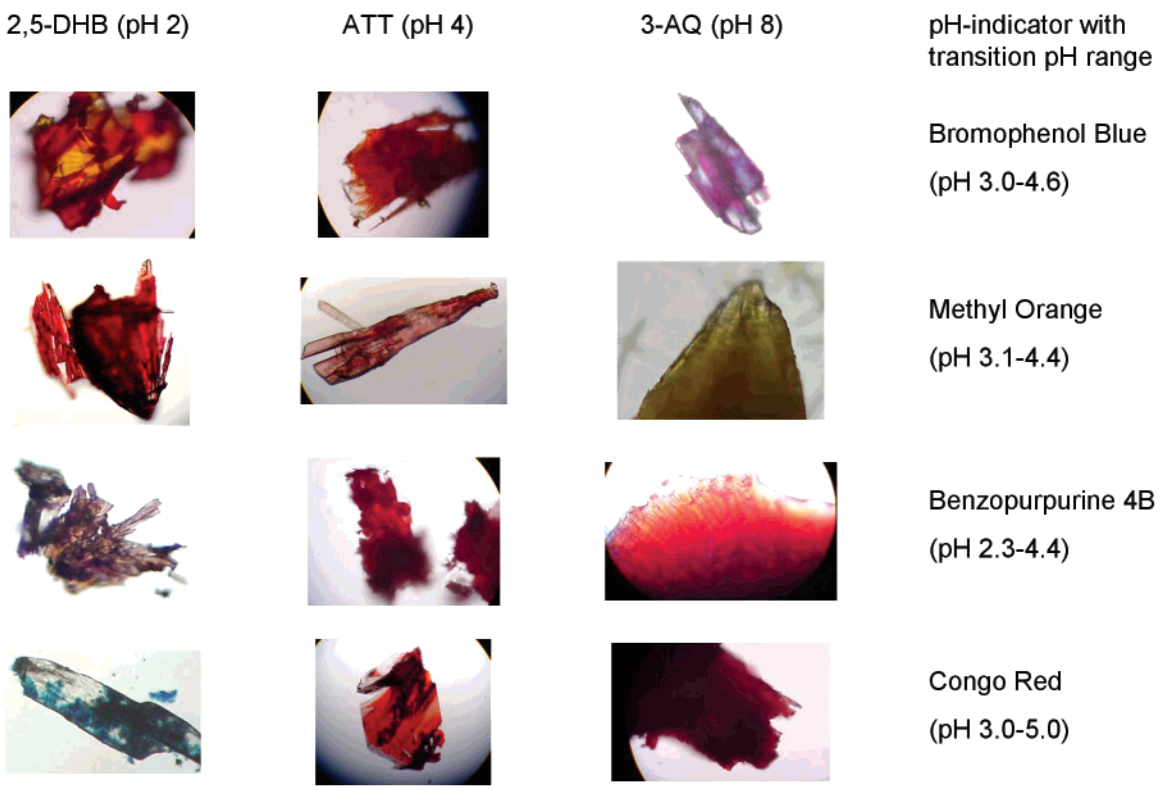

Figure 3. Color comparison of pH indicators incorporated in matrix crystals with different intrinsic pH.

Table 3. Amount of Acetonitrile (% *n*/*n*) in Matrix Crystals Determined by ^{1}H NMR Spectroscopy (Each Value Represents One Single Measurement)[a]

matrix	solid matrix as obtained from the supplier	grown crystals (acetonitrile/water (1:1))	after drying (oven, 100 °C, 3 h)	after drying (vacuum MALDI, ~10^{-7} Torr, 3 h)
2,5-DHB	<0.01	0.43/0.88	0.34/0.85	0.33/0.36
2,5-DHB + insulin	<0.01	0.77	0.80	0.84
SIN	0.62	2.54/3.73	2.13/2.71	1.76/1.36
SIN + insulin	0.62	3.37	1.61	1.57
αCHCA	0.22	0.64	0.59	
αCHCA + insulin	0.22	0.65		
αCHCA + Texas Red-labeled protein	0.22	0.48		

[a] The solvent amount was calculated as follows: ^{1}H-signal intensities of acetonitrile (CH_3 group) were compared with the aromatic and olefinic ^{1}H-signals of the matrix, taking into account the number of H-atoms.

larger proteins. Provided the interference of water (e.g., from air moisture) within the elaborate grinding and loading procedure can be definitely excluded, an important role of the residual solvent as discussed above would indeed be questionable.

Correlation of MALDI Spectra with Solution Acid−Base Properties. In a further set of experiments, a possible correlation between the signals observed in the MALDI spectra and the pH of the solution was interrogated. The relevant acid−base properties (pK_A values, equivalence points (EP), and intrinsic pH of the used matrixes) are given in Tables 1 and 2. MALDI-MS spectra were recorded for all combinations of the dyes and matrixes as a function of the intrinsic matrix solution pH.

Considering the acid−base equilibrium, it is clear that even though the color is changing around the pH of the pK_A of the dye (depending also on concentrations and absorption coefficients of the involved species), there is a broad transition range where both species coexist; only at and above the equivalence point is a strong excess of the product species given. However, if the protonated molecule exists in the solution at least as a minor component (which is true in the vicinity of the pK_A value), it can easily be detected by mass spectrometry. Therefore, only a correlation between the equivalence points of the dyes and the detection of the respective species is to be expected. Clearly incorporation for all equilibrium dye species was indicated by the color, and the MALDI-generated ions are also strongly influenced by the incorporated species. It should be noted that, although pH indicators have been investigated for a long time now, there are still some uncertainties about their structures and the relative contributions of the different forms in solution, e.g., concerning the equilibrium of zwitterionic, quinoid, or sultone structures of the triphenylmethane dyes.[69−73] Moreover, p*K* values are generally higher in acetonitrile than in water,[70,71] but only a small influence (visible by the solution color) was found using up to 50%

Table 4. Relative Signal Intensities of Protonated Dye Molecules $[M + H]^+$ Obtained with Different Matrixes in the Positive Ion Mode ($c = 10^{-3}$ M)[a] (for Comparison, the Color of the Dye within the Matrix Preparation Is Given)

dye	2,5-DHB (pH 2.0)	SIN (pH 3.5)	ATT (pH 4.0)	3-AQ (pH 8.0)	ANP[b] (pH 9.5)
Crystal Violet	+++ violet	+++ violet	+++ violet	+++ violet	++ green
Benzopurpurine 4B	+ nearly no color	+ light red	+ red	- red	- brown
Congo Red	+ blue	+ violet	+ red	- light red	- light red
Bromophenol Blue	+ light orange	+ orange	+ orange	− blue	− blue-green
Methyl Orange	++ red	+ red	+ yellow	− yellow	− yellow
Methyl Red	++ red	++ red	++ red	++ orange	+ yellow
Thymol Blue	++ red	++ orange	++ yellow	++ yellow	+ green

[a] Key: +++, very high signal intensity (higher than matrix ions, maximum 10% cationized analyte signals observed); ++, high signal intensity (similar to matrix ions, maximum 30% cationized analyte signals observed); +, mediate signal intensity (lower than matrix signals, but still similar or higher than cationized analyte signals); −, no $[M + H]^+$ signal or spectrum dominated by cationized analyte signals ($[M + H]^+$ signal intensity maximum 20% of both matrix and cationized analyte signals). [b] Preparations show a mixed color due to the yellow color of ANP.

acetonitrile; these effects are thus disregarded here for simplification.

Depending on the matrix and thus pH and on the dye, the following cases can be differentiated (see Table 4; dye structures are depicted in Figure 1): (1) CV exists as a positive ion (MH^+) in all cases; (2a) MR is predominantly protonated up to pH 2.5 and (2b) predominantly neutral up to pH 9.6 (zwitterionic (+1/−1) as well as uncharged), with contribution of the deprotonated anionic form at pH >6.1; (3a) MO is predominantly zwitterionic (+1/−1) up to pH 4.2 and (3b) deprotonated above pH 7.6; (4a) BE and CO are zwitterionic neutrals (2+/2−) in the case of acidic matrixes and (4b) doubly deprotonated in basic matrixes; (5a) BP exists predominantly as zwitterionic anion (+1/−2) up to pH 4.1 (besides a neutral sultone form in strong acidic solutions) and is (5b) doubly deprotonated above pH 7.6; (6a) TB exists predominantly as zwitterionic anion (+1/−2) in the examined pH range (with contribution of a neutral zwitterionic form in acidic solutions and of a doubly deprotonated form at pH >5.4).

Protonated dye (analyte) ions are only detected with high signal intensities in the case of "pre"protonated (1, 2a) and (at least partially) zwitterionic species (2b, 3a−6a), but never for fully deprotonated anionic compounds (3b−5b). In the latter case, cationized ion species dominate the mass spectra and the $[M + H]^+$ analyte signal was either very weak or even completely absent (see Figure 4). These findings are summarized in Table 4. Although this pH effect was clearly visible, no direct correlation with the analyte's net charge was found since $[M + H]^+$ is observed from positive (1, 2a), neutral (3a−4a), and negative (5a−6a) analytes. However, a gradual intensity shift from protonated to sodiated species was clearly visible upon switching to more basic matrixes within this "titration-like" experiment. Moreover, $[M + H]^+$ signal suppression was only found in the case of incorporated anions and might be related to a missing zwitterionic form. Deprotonated azo dyes (MO, MR, BE, CO) are definitely no zwitterions, but the strong bathochromic shift in the absorption bands of the sulfonphthalein (BP, TB) dianions (causing a change of the complementary color from yellow to blue) points to enhanced electron delocalization compared to the monoanionic form, suggesting a mainly nonzwitterionic form for these compounds, too.

However, partial proton neutralization for MALDI is observed for deprotonated anionic species and can be verified by the inspection of the direct LDI mass spectra of the dyes: dyes were mixed with 0.01 M NaOH to ensure deprotonation and dried for direct laser desorption. In this case, no singly sodiated ions were observed from completely deprotonated anionic dyes (3b−5b). Instead, the deprotonated doubly sodiated molecule $[M - H + 2Na]^+$ was the most abundant peak, and no molecular ion signal was found for BE and CO at all (see Figure 5). This shows clearly that these dyes are completely deprotonated in the solid state after addition of NaOH and signals in the positive ion mode are only observable by attachment of at least two singly-charged cations. However, MR and TB were not completely deprotonated, visible by the occurrence of $[M + H]^+$ with low abundance; thus, small $[M + Na]^+$ signals were also detected.

The same basic solutions were then mixed with a matrix in a standard dried droplet preparation. Only the basic matrixes 3-AQ and ANP could be used for these experiments, because deprotonation of acidic matrixes leads to the loss of the matrix function. Addition of the basic matrix ANP did not change the overall picture found in the LDI case. However, using 3-AQ as a matrix lead to a significant difference: singly sodiated analytes were detected for all dyes incorporated as completely deprotonated anions (3b−5b) with high intensities (see Figure 5). Additionally, even BE and CO were detected in their singly and doubly sodiated forms besides the expected doubly deprotonated molecule charged by three sodium ions $[M - 2H + 3Na]^+$, which was not possible

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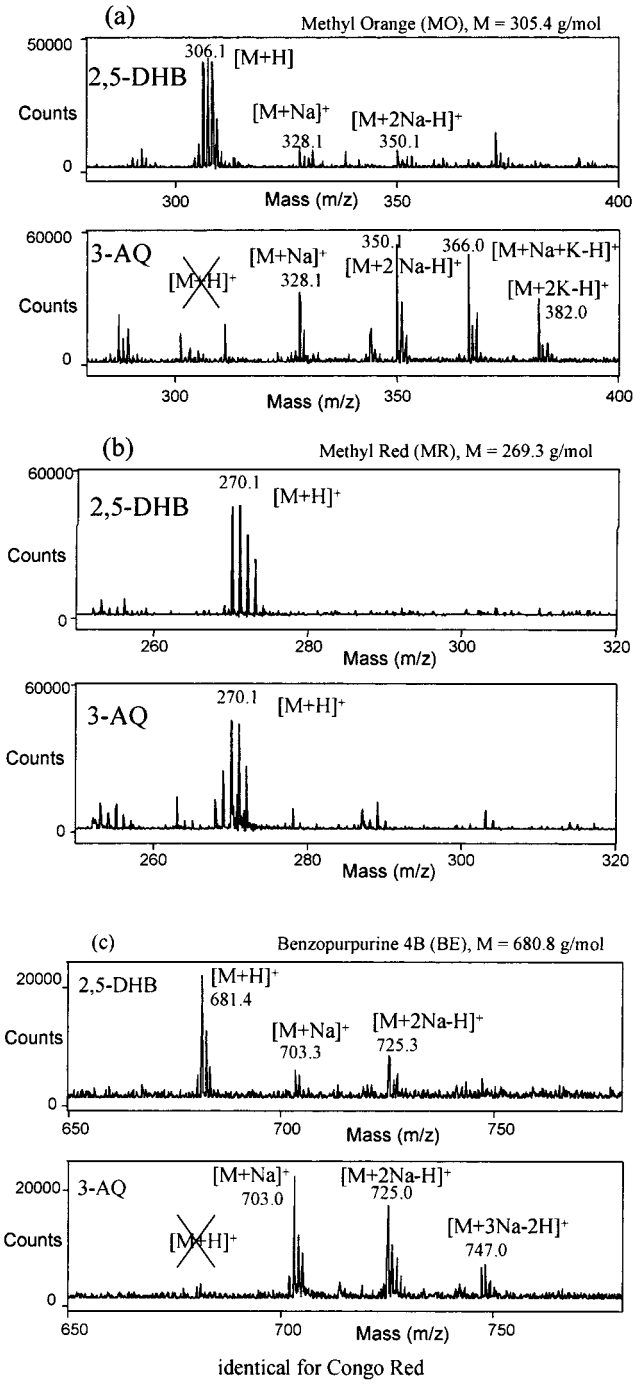

Figure 4. Comparison of UV-MALDI spectra of Methyl Orange (a), Methyl Red (b), and Benzopurpurine 4B (c) obtained with two different matrixes (2,5-dihydroxybenzoic acid and 3-aminoquinoline).

without matrix. The observation of the singly sodiated species is only possible by assuming proton-transfer neutralization of the anions during the desorption process. Even though it cannot be read from the mass spectra whether protonation takes place at the sulfonic acids or at a basic site in the molecule, the latter case, i.e., a zwitterionic structure with attached cation(s), appears more probable. Furthermore, the absence of ammonia and the differences using either 3-AQ or ANP as matrix point to the fact that analytes are protonated by matrix molecules and make it unlikely that the proton is transferred from counterions.

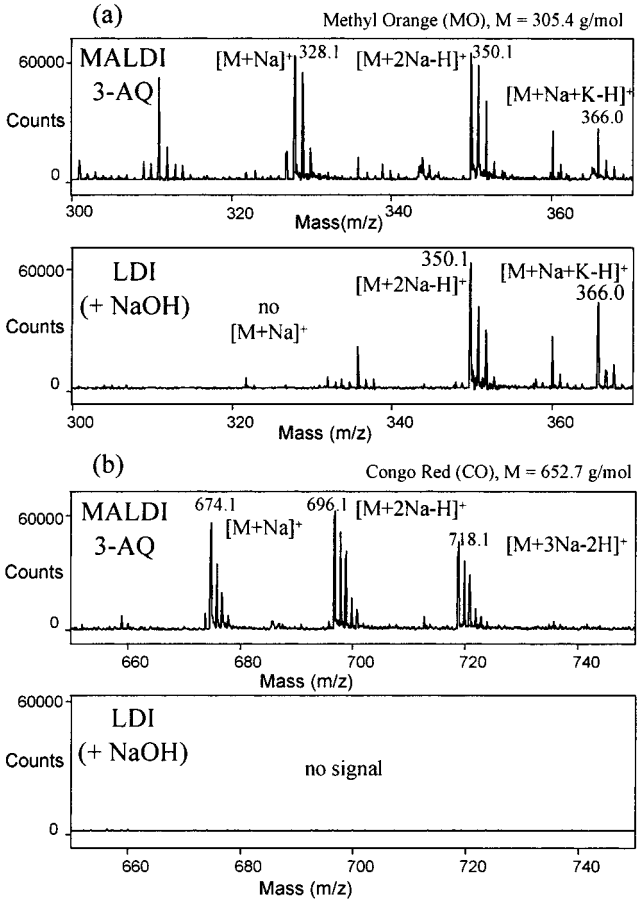

Figure 5. Comparison of spectra obtained with 3-aminoquinoline as matrix and without matrix after addition of 0.01 M NaOH for the analytes Methyl Orange (a) and Congo Red (b).

However, a complete proton neutralization replacing all cations leading to a protonated molecule was not evident using 3-AQ or ANP as matrix. Only MR showed an increased $[M + H]^+$ signal compared to the negligible signal intensity in the LDI case. Unlike all other dyes, MR has a carboxylic acid function instead of a sulfonic acid. Nevertheless, protonation of sulfonates must be possible at least with the acidic matrixes, otherwise no singly protonated analyte should appear in the spectra of the dyes incorporated as zwitterions.

Hence, a clear correlation between acid−base properties, namely, the EP, and the observation/vanishing of protonated dyes in the MALDI spectra as well as strong hints for proton neutralization reactions in the MALDI plume were found. Although $[M + H]^+$ signals were detected from incorporated molecules with different overall charge, proton transfer seems to be prohibited in the case of fully deprotonated anionic dyes, depending on the nature of both analyte and matrix. Although the reason for the nearly complete lack of $[M + H]^+$ signals is not clear, there are two reasonable explanations: first, a strong attachment of Na^+ counterions might prevent ion separation in the matrix crystals, which leads to incorporation and desorption of close-contact ion pairs. The attachment of sodium to the analyte within the crystal is supported by the high initial velocity (similar to protonated analytes) of sodiated species (see Table 5 and next chapter). Second, the observations might be attributed to different acidities/basicities of both matrixes and functional groups within the dyes.

Table 5. Initial Velocities of Dyes Obtained with Different Matrixes and without Matrix Compared to Initial Velocities of Other Analytes and Matrix Ions

dye	ion	LDI	2,5-DHB	3-AQ	ANP
Methyl Red	$M^{\bullet+}$	253 ± 16	595 ± 69	284 ± 42	
	$[M + H]^+$	267 ± 35	594 ± 76	288 ± 62	564 ± 30
	$[M + Na]^+$	245 ± 30	592 ± 14		
Methyl Orange	$[M + H]^+$	271 ± 26	595 ± 69		
	$[M + Na]^+$	253 ± 12	598 ± 17		
	$[M + 2Na - H]^+$	240 ± 13	650 ± 37		
Thymol Blue	$[M + H]^+$	244 ± 44	673 ± 49	290 ± 28	669 ± 84
	$[M + Na]^+$		602 ± 16		
	$[M + 2Na - H]^+$	234 ± 22	621 ± 08		
Crystal Violet	$[M + H]^+$	242 ± 39	596 ± 94	319 ± 116	442 ± 41

matrix/analyte	initial velocity (m/s)
3-aminoquinoline $[M + H]^+$	262 ± 37
2-(2-aminoethylamino)-5-nitropyridine $[M + H]^+$	491 ± 95
2,5-dihydroxybenzoic acid $[M + H]^+$	572 ± 17
proteins/peptides $[M + H]^+$ with DHB as matrix[19]	530−600

The acidity of protonated carbonic acid matrixes (formed upon laser irradiation) is considerably higher compared to protonated basic matrixes (existing already in solution). The latter species might not be able to protonate very weak bases (e.g., sulfonates), although proton transfer to more basic sites was clearly shown. Moreover, the second explanation is supported by the dominance of nonsodiated species in the negative ion mode, where in all cases except MR $[M - H]^-$ was detected as the most abundant peak.

Are the Experimental Conditions Comparable to Standard MALDI Applications? All dyes absorb laser light at the wavelength used for the MALDI experiments ($\lambda = 337$ nm). Therefore, it has to be proven that the observed spectral characteristics are indeed the result of a MALDI event and not induced by direct LDI. To rule this out, dye solutions were mixed with 0.1% trifluoroacetic acid to ensure protonation and were allowed to dry directly on the MALDI target without addition of a matrix. The results were then compared to spectra of the same acidic dye solution obtained with 2,5-DHB as matrix within a standard dried droplet preparation. In most cases, the spectra with and without matrix were considerably different: either the LDI spectra showed no $[M + H]^+$ signal of the intact molecule at all (e.g., BP, CO or BE) or high intensities of $M^{\bullet+}$ ions were detected without matrix, whereas (multiple) addition of H-atoms was found with 2,5-DHB besides the normal $[M + H]^+$ signal (MO and MR; the existence of H-radicals in the plume is well known[74]).

The differences of the spectra are obvious and point to the fact that we have "true MALDI conditions" for these dyes. However, additional experiments were necessary, because only protonated molecules of CV and TB were detected with and without matrix, preventing a clear distinction between LDI and MALDI for these compounds. The measurement of the initial velocities is an appropriate tool to distinguish between MALDI and direct LDI and was described before.[19] The initial velocities of protonated and sodiated dyes with different MALDI matrixes (2,5-DHB, 3-AQ, ANP) and without matrix were determined. The results are shown and compared to the initial velocities of other analytes and some matrix ions in Table 5. In the case of 2,5-DHB and ANP, the values obtained are significantly higher than in the LDI case for all compounds, as found before for other analytes.[19] Although determination of absolute values for initial velocities of desorbed ions is difficult (see refs 19, 22, and 62), the relative comparison of initial velocities within one experimental setup is indicative. These results prove that the observed dye signals stem indeed from incorporated analyte molecules and thus reflect a typical MALDI situation.

Reflections on MALDI of Biomolecules. The color of the pH indicators is a significant indication that the solution pH and thus the analyte's charge state is maintained in the host matrix crystals. This also explains the quenching of fluorescence for FITC-labeled proteins as reported in ref 65. Moreover, included solvent points to analyte inclusion in a (partially) solvated state; the existence of small inclusions in 2,5-DHB single crystals was reported recently and the possibility of "wet" inclusions was discussed earlier.[75] Solvent inclusion might also rationalize the fact that MALDI is a "soft" ionization technique: laser energy is only absorbed by the matrix and analytes might be "shielded" by the liquid or cooled by solvent evaporation.

It is emphasized that, even though typical bioanalytes are incorporated in a "precharged" state,[36] the term "preformed ion"[25−33] is avoided here, since most analytes carry several (different) charged groups (leading to different net charges) and are thus significantly different from the singly charged species detected in MALDI-MS. Moreover, electroneutrality has to be guaranteed by inclusion of counterions, but the solvated state of the analyte will, however, prohibit (complete) charge association and thus close ion pair formation upon incorporation. This justifies applying a simple cluster-ionization model[20] which postulates the formation of charged clusters by excess or deficit of counterions upon solid disintegration by the ablation process. In this approach, the primary "ionization" event is neither a gas-phase protonation

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mechanism (suggested by several authors[15,49,76−80]) nor the desorption of "preformed ions", but the generation of charged clusters via separation of the included precharged analytes followed by their subsequent "desolvation" (by evaporation of matrix) to the ionic species, including proton-transfer neutralization and proton-transfer chemical ionization processes.

Referring to the typical bioanalytes, peptides and proteins form the easy case. Typically they are investigated using acidic matrixes or acidified solutions. This results in the inclusion of polyprotonated species with matrix or additive acid anions as counterions, facilitating proton neutralization. The deficit or excess of one anion in a matrix−analyte cluster fully suffices to account for the observation of singly protonated or deprotonated species. This situation changes for zwitterionic species such as nucleic acids (at acidic pH) and peptides or other analytes having phosphate or sulfonic acid functional groups. In this case, the strong association of, for example, Na^+, will result in (poly)cationized species; therefore, preparation protocols have been developed to fully exchange metal cations toward NH_4^+.

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CONCLUSIONS

Within a matrix analyte preparation, the analyte's charge state is about the same as in solution. Most analytes carry at least one basic or acidic group, leading to incorporation of ions in the host matrix crystals. Residual solvent makes incorporation and partial separation of analyte ions from their counterions possible. As primary ionization step, clusters including precharged ions are desorbed during the MALDI process. The charge state of these ions determines the secondary reactions possible within the selvedge region, e.g., proton-transfer reactions with the matrix. As peptides and proteins are incorporated as (multiply) protonated molecular ions in the typically acidified solid matrixes, their MALDI performance in the positive ion mode is superior to other analytes such as neutral carbohydrates or polymers.

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