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Investigations of theoretical principles for MALDI-MS derived from solvent-free sample preparation Part I. Preorganization

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Abstract

The success of the recently developed solvent-free MALDI-MS method suggests that the common understanding of a successful MALDI process may not be justified. Analyte incorporation into a matrix crystal is apparently not necessary for the MALDI process and it further appears that crystallinity is obstructive. The analyte cytochrome *c* (Cyt *c*) and various matrices were employed in these studies. Light microscopy revealed microscopic defects on the surface of a single crystal of 2,5-dihydroxy benzoic acid (2,5-DHB) with incorporated Cyt *c*. MALDI analysis of this intact crystal required considerably higher laser power for obtaining mass spectra and showed much more fluctuation in the threshold laser power than was observed for the analysis of a fine powder of the same crystal. These results show that the required threshold laser power is in the order: intact crystal with perfect surface > intact crystal with defect surface > pulverized crystal. The smaller the remaining crystals are, the milder the MALDI process. Further investigations using the solvent-free MALDI method showed that the analysis of Cyt *c* is possible directly from the individual powders of Cyt *c* and 2,4-, 2,5- and 2,6-DHB, a result which was not obtained using the solvent-based MALDI method, and revealed, the stronger the absorption of a matrix at the applied laser wavelength, the milder is the MALDI process. Analyte incorporation into a matrix crystal apparently is not necessary, a concept that is strengthened by the successful characterization of Cyt *c* using incompatible matrices such as dithranol and anthracene. An optimized matrix-assistance in MALDI-MS is achieved by intimate contact between analyte and matrix, the smallest possible remaining crystallinity, and a sufficient absorption of the applied matrix at the laser wavelength.

Keywords: MALDI; Solvent-free sample preparation; Analyte incorporation; Threshold laser power; Fragmentation; Labile molecules; Decreased desorption threshold

1. Introduction

Since the development of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), investigations of its basic processes have been continuously performed parallel to application-oriented research efforts. The actual understanding of the processes is, however, still incomplete. This insufficiency is due to the complexity of the processes and instrumental limitations, e.g., to observe processes on a nanosecond time scale or in the nanoliter spatial measure. Only ions are directly accessible to the measurement, and ions form only a small proportion of the laser generated vapor phase species. The analyte is present only in small concentrations relative to the matrix, hence, investigations of the interaction of matrix and analyte are difficult, nevertheless there is a great interest in the influence of this interaction on the desorption behavior. A series of fundamental investigations show that different matrices incorporate macromolecules in their crystal lattices [1–10].

At the end of 1999, more than ten years after the introduction of MALDI-MS, the model conception was changed: The incorporation of the analyte into the crystal is helpful, but not generally necessary to produce large analyte ions [7]. This conclusion resulted from investigations of dihydroxy benzoic acid

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(DHB) isomers as matrices and Cyt *c* as analyte. The study also suggested that with UV-MALDI-TOF-MS, a direct proximity of the analyte to the matrix is sufficient, as would be found with an adsorption of the analyte to the matrix surface. It is assumed that a large surface-to-volume-ratio increases the chance that an analyte joins to an appropriate surface. This hypothesis relies in particular on investigations of the 2,6-DHB and Cyt *c* system, in which only the "thin layer" sample deposition protocol supplied good quality mass spectra. The "thin layer" protocol produces submicrometer-sized matrix:analyte crystals with a large surface-to-volume-ratio due to the fast evaporation of the solvent. No mass spectra of the Cyt *c* could be achieved from large prism disks, single crystals or from long needles, which were formed with the "dried droplet" sample deposition protocol. Other studies that are concerned with fundamental processes in MALDI but not particularly relevant here, have been reported; a few are cited [11–17].

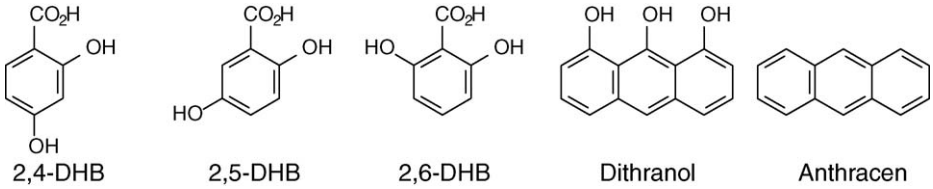
Recent studies on the implementation of the solvent-free MALDI method [14,15,17–29] give rise to renewed theoretical investigations of the MALDI process. In any solvent-based sample preparation, a separation of the analyte and matrix has to be prevented. Optimizing the conventional solvent-based MALDI sample preparation conditions can circumvent this intrinsic problem and make the MS analysis a great success; a few are cited [30–37]. In case of inhomogeneities, increasing the laser power during the measurement can enforce a sufficient desorption/ionization process, so that the analyte becomes accessible [14,25]. On the other hand, the solvent-free MALDI sample preparation method depends on the miscibility behavior in the solid state, which is caused by external forces. The method permits a true[1] matrix-assisted desorption/ionization process [14,17] with no dependency on the origin of the analyte [14,15,17–29], the molecular weight (polystyrene 100 kDa [14], bovine serum albumin 66 kDa [17]), the solubility [15,19,21,29] or the compatibility between the polarities of analyte and matrix [29]. The influence of the applied, external force is small, thus, a simple mortar and pestle treatment [14,18] generally ensures sufficient homogeneity of the analyte:matrix mixture. The degree of homogenization seems to exert the largest influence on the success of the mass spectrometric analysis. Surprisingly, solvent-free MALDI-TOF-MS often yields better results, particularly with synthetic polymers, and often requires less laser power for successful desorption/ionization of the analyte [14,29]. The pressed pellet MALDI powder mixture makes the desorption/ionization process more difficult, in comparison to the loosely applied powder [14], and is, based on an unusually low and restricted analyte:matrix ratio [9,18]. For example, the pellet method employing various analytes (e.g. mellitin 2.8 kDa, insulin 5.7 kDa, Cyt *c* 12.3 kDa, lysozyme 14.4 kDa, BSA 66 kDa) and matrices (3-HPA, ferulic acid, CHCA, 2,4-, 2,5-, 2,6-DHB) revealed no peptide or protein signals with the exception of employing 2,5-DHB for the analysis of mellitin and bovine insulin, however yielded only weak signals [9]. Extending the homogenization period (10 min) yielded small improvements in the spectra quality and made it possible to observe now also an ion signal for Cyt *c* [9]. The observation of the more difficult desorption/ionization [9] suggests that the surface condition of the MALDI sample strongly influences the analysis. Since the loosely applied powder, 100 kDa synthetic polymer [14] and 66 kDa protein [17], as well as the pressed pellet, 12.3 kDa protein, led to such different MALDI results, as in particularly distinguished with the accessible molecular weight range we extrapolated that an even more compact surface, as is found with crystal lattices, must make the desorption/ionization process even more difficult. This can also be observed with the solvent-based MALDI method (e.g., generally higher laser power is needed with larger crystals). Hence, we postulate that crystallinity is obstructive.

To investigate the above hypothesizes, experiments are performed on Cyt *c* and DHB isomers. Our approach includes suitable mass spectrometric and light microscopic investigations of single crystals from Cyt *c* and 2,5-DHB. Comparative studies are acquired by means of solvent-based[2] and solvent-free[3] MALDI sample preparation methods. These investigations are model experiments mainly towards the obstructive nature of crystallinity for the MALDI process. Subsequently, direct solvent-free MALDI-MS experiments of the analyte and various matrices (2,4-, 2,5- and 2,6-DHB) are considered for the characterization of Cyt *c* primarily to investigate the non-necessity of analyte incorporation for matrix-assistance (Scheme 1). The results are correlated with the tendencies of analyte incorporation and the absorption at the applied laser wavelength of these matrices. Studies of the matrix effect for the characterization of Cyt *c* are also performed with incompatible matrices such as dithranol and anthracene.

[1] Classification of the used term was broadly defined as follows [9,10]: 'Matrix support effect' facilitates desorption/ionization of intact analyte molecules but appears to be limited in mass (<30 kDa). A 'true MALDI' is supposed to require analyte molecule incorporation and is functional for a larger mass range.

[2] *Definition of solvent-based MALDI-MS*: In solvent-based MALDI analysis there is an appropriate solvent employed to dissolve the matrix which is subsequently mixed either in the sample tube or on the MALDI plate with the dissolved sample to give the MALDI sample. Hence, homogenization and transfer of the MALDI sample preparation is based on a solvent or solvent mixture. A variety of different techniques were previously employed: dried droplet [30] and thin layer [32] are the most common. The ultimate step to deliver the MALDI sample on the MALDI plate is the evaporation of the solvent or solvent mixture; hence, the homogenization of the sample occurs on the MALDI plate. Examples may include solutions or suspensions.

[3] *Definition of solvent-free MALDI-MS*: In solvent-free MALDI analysis, at no point is solvent employed to mix analyte and matrix or transfer the MALDI sample on to the MALDI plate. Hence, homogenization and transfer of the MALDI sample is not based on solvent. A variety of external forces were previously employed for homogenization: (mini) ball mill [14,17,19], mortar and pestle [14,18], BB method [24], bead free homogenization [17], spraying techniques [25]. Approaches that were previously employed to transfer and attach the MALDI sample: pressed pellet preparation [18], loose powder [14,17,19] and more. The ultimate step to obtain the MALDI sample is a dry transfer to the MALDI plate. Examples may include powders, pressed pellets, intact single crystal, pulverized single crystals, or sprays. It is worth noting that the analysis of for example the intact crystal is a distorted case of solvent-free MALDI analysis as such that the "mixing" of the analyte and the matrix occurred over time, with the aid of solvent, during the growing process, however the dry sample has to be transferred to the MALDI plate.



Scheme 1. Applied matrices.

2. Experimental

2.1. Materials

Dihydroxy benzoic acid (DHB) isomers were purchased as follows: 2,4-DHB (Merck), 2,5-DHB (Sigma–Aldrich), and 2,6-DHB (Lancaster). The matrix compounds were purified by re-crystallization from aqueous solution by adding activated carbon. The purification was repeated (two to three times) until white crystals were obtained for all matrices. The obtained precipitate was then washed with cold ethanol and dried under vacuum for complete dryness. Cyt *c* (Fluka) was used without further purification. The structures of applied matrices are depicted in Scheme 1. Ethanol was purchased from Fisher Scientific and PEG from Lancaster. 1,8,9-Trihydroxyanthracene (dithranol) and sodium trifluoroacetate were obtained from Aldrich (Steinheim, Germany). Tetrahydrofuran (THF) was obtained from Fluka (Buchs, Switzerland).

2.2. Crystal growth

Crystallization was performed to obtain incorporation of Cyt *c* [5]. 2,5-DHB (50 g L^{-1}) solution in water:ethanol (9:1, v:v) was placed in a water bath at 36 °C for several hours to insure complete solvation of all crystals. The obtained solution was split in two parts: one part was mixed with the Cyt *c* solution, which had been pre-dissolved in water at 36 °C. The mixture, with a molar analyte:matrix ratio of 10^{-5} mol L^{-1}, was strained through a 0.2 μm syringe filter. Volumes of 5 mL were filled into small glass vials and covered with perforated plastic caps. The second part of the 2,5-DHB solution was directly strained through a 0.2 μm syringe filter; volumes of 5 mL were filled into small glass vials and covered with perforated plastic caps. All vials were stored for several weeks at room temperature in the dark.

The 2,5-DHB:Cyt *c* solution formed reddish colored crystals whereas the 2,5-DHB solution gave colorless crystals. The crystals were removed from the mother liquor and washed carefully with small quantities of ice-cold distilled water to remove residual protein, which might have adsorbed to the surface of the crystal. The crystals were dried briefly and then introduced to the source of the MALDI mass spectrometer.

2.3. Light microscopy

Zeiss Photomikroskop III was employed.

2.4. Mass spectrometry

Analysis of single crystals. The single crystals were mounted onto a stainless steel MALDI sample holder with double-sided adhesive tape.

Solvent-free sample preparation. The procedure is described in detail in a previous report [14]. Briefly, the analyte and matrix were simply mixed in the appropriate molar ratio and shaken by a ball mill for homogenization. The powder was crushed on the target as a very fine powder to produce a very thin coverage of the analyte:matrix mixture.

Solvent-based standard sample preparation ("*dried droplet*"). 2,5-DHB was dissolved in water:ethanol (9:1, v:v).

MALDI-TOF instrument. MALDI-TOF mass spectra were recorded using a Bruker Reflex II^{TM} MALDI-TOF mass spectrometer (Bremen, Germany) equipped with a N_2-laser (λ = 337 nm) operating at a pulse rate of 3 Hz. The ions were accelerated with pulsed ion extraction (PIE^{TM} design from Bruker) by a voltage of 20 kV. The analyzer was operated in reflection mode and the ions were detected using a microchannel plate detector.

Calibration. Calibration was carried out before each measurement. PEG was dissolved in THF together with dithranol and sodium salt (1:500:10). A multi point calibration of the isotopically resolved PEG-oligomers was carried out.

3. Results and discussion

3.1. Characterization of the crystals

3.1.1. Crystal growth and light microscopic investigations

The re-crystallization of the matrix 2,5-DHB and the growth of the crystals to incorporate Cyt *c* as an analyte into 2,5-DHB matrix were performed using previously described procedures [7]. The light microscopic studies of the crystal from 2,5-DHB and Cyt *c* indicated a plate-like appearance (Fig. 1(IA)). In the enlargement of the plate-like crystal (Fig. 1(II)), changes in the crystal surface were found. Some crystals show pronounced stair-like formations, A, close together. These surface-increased rough areas are called "defect surfaces". In contrast, areas were also found with a very smooth, intact crystal surface B (Fig. 1(II)) and will be called "perfect surfaces". Light microscopic investigations of the crystal of the pure 2,5-DHB matrix (Fig. 1(IB)) showed a different crystal growth comparing it with the crystal of the 2,5-DHB matrix with incorporated Cyt *c* (Fig. 1(IA) and (II)). However, this pure DHB crystal revealed

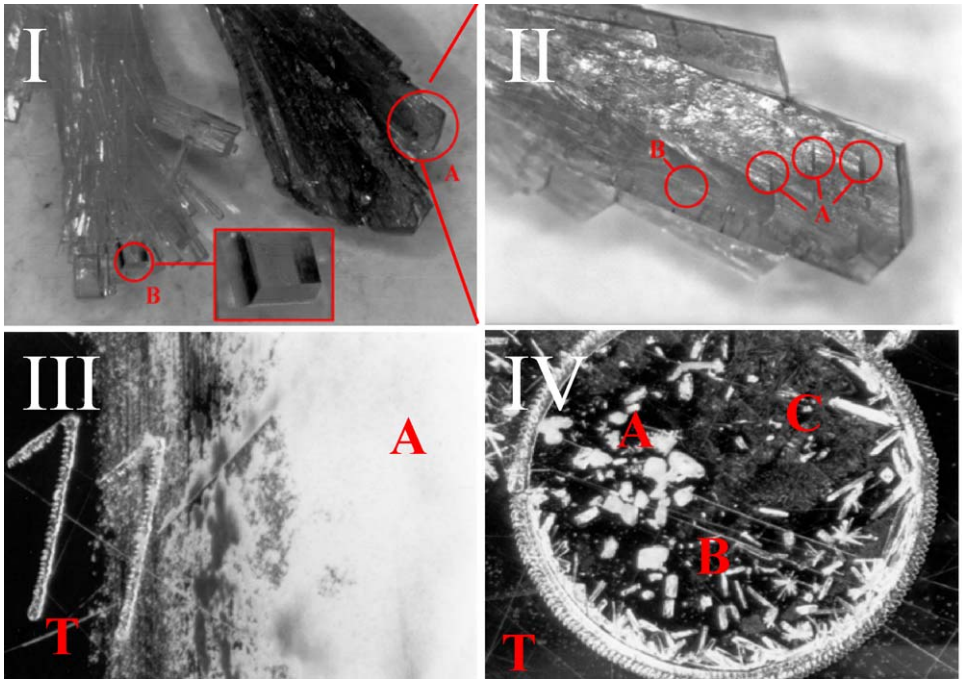


Fig. 1. Light microscopy images: (I) comparison of single crystals—(A) 2,5-DHB matrix incorporated with Cyt *c* (for more details see (II)) and (B) pure 2,5-DHB matrix; (II) 2,5-DHB matrix incorporated with Cyt *c* and its surface appearance—(A) stair-like formation ("defect surface") and (B) very smooth, intact areas ("perfect surfaces"); (III) pulverized single crystal of 2,5-DHB matrix incorporated with Cyt *c*; loosely applied on MALDI sample holder (generally referred to as "solvent-free"); (IV) typical MALDI sample appearance of 2,5-DHB and Cyt *c* after solvent-based sample preparation ("dried droplet")—(A) large, prism-like disk, (B) needle-like crystals and (C) very finely distributed, submicrometer-sized crystals. [T = MALDI target].

again perfect and defect surfaces. It can be summarized, that microscopic defects and areas of heterogeneity on the crystal surface of the single crystals are visible from 2,5-DHB with and without incorporated Cyt *c*.

3.2. MALDI-TOF-MS of the intact and pulverized crystals

The threshold laser power leading to the desorption/ionization of the Cyt *c* analyte from the 2,5-DHB:Cyt *c* crystal and from the pulverized crystal was compared. The pulverized crystals were obtained by ball mill treatment of the crystallized 2,5-DHB with Cyt *c*. The loose powder was applied to the MALDI sample holder (Fig. 1(III)). The threshold laser power for the 2,5-DHB:Cyt *c* crystal which results in desorption/ionization of Cyt *c* was determined. Subsequently, 10 × 20 laser shots were summed at the threshold laser power. The resulting mass spectrum of 200 laser shots from randomly chosen spots was saved as an experiment number. The MS investigations of the solvent-free prepared pulverized crystal were handled identical. With the video camera of the MALDI mass spectrometer, the sample was displayed on the control monitor with approximately 100-fold enlargement. Therefore, the macroscopic inhomogeneities and surface conditions were visualized directly during the MALDI-TOF-MS measurement.

In Fig. 2, the experiment number is plotted against a relative measure of the laser power (=100% − laser attenuation). Data acquisition of the MALDI-TOF mass spectra of the crystal from 2,5-DHB and incorporated Cyt *c* showed large fluctuations (Fig. 2(a)) in the threshold laser power that needs to be applied for successful desorption/ionization of the analyte. Thus, the distribution of the relative laser power was situated between 25 and 43 with an averaged threshold laser power of 30.1. Similar MALDI-TOF-MS investigations of the pulverized crystal from 2,5-DHB and incorporated Cyt *c* showed a substantially smaller distribution (Fig. 2(b)) and a clearly lower threshold laser power of 25.4, averaged from 24 to 27 in the relative laser power. The lower and less fluctuating laser power required for the powder sample can be explained by the increased overall surface of the powder leading to less crystal lattice energy that must be broken in order to transfer the matrix and analyte molecules from the

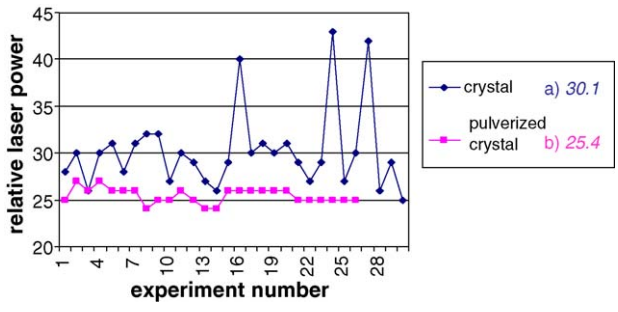


Fig. 2. A plot of experiment number against the average relative threshold laser power (in parentheses) needed to be applied to characterize Cyt *c*—(a) in blue diamonds: MALDI-TOF-MS of intact single crystal of 2,5-DHB matrix incorporated with Cyt *c* (30.1); (b) in pink squares: MALDI-TOF-MS of the pulverized single crystal of 2,5-DHB matrix incorporated with Cyt *c* (25.4) applied loosely to the sample holder ("solvent-free"). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

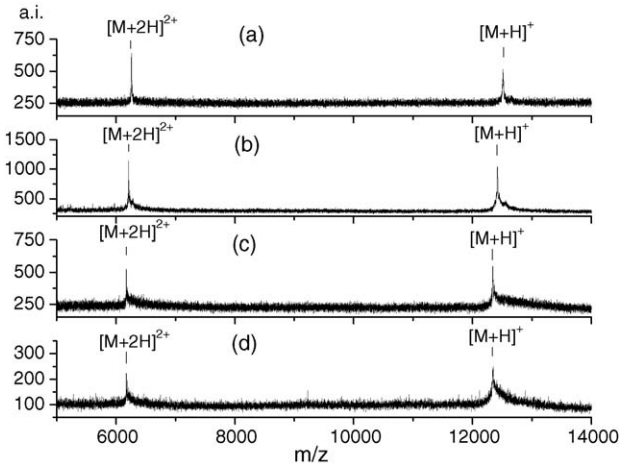


Fig. 3. MALDI-TOF mass spectra of Cyt *c* in dependence of the sample preparation and its corresponding threshold laser power (in parentheses): (a) perfect crystal (50); (b) defect crystal (30); (c) pulverized crystal (25); (d) solvent-based sample preparation (30). The mass shift observed (a, b) is expected because of the change in surface position caused by the thickness of the crystals.

crystal lattice into the gas phase. Smaller fluctuations in the laser power required for the pulverized crystal can be explained by the higher uniformity of the sample.

Thus, these results suggest that by maximization of areas with defect surfaces the desorption/ionization process is facilitated, which directly corresponds to the hypothesis that the incorporation of analyte into a matrix crystal is obstructive. More detailed investigations of the crystal of 2,5-DHB and incorporated Cyt *c* show that the experiments with relative laser power >35 (Fig. 2(a)) strictly correlate with the smooth, perfect crystal surfaces B (Fig. 1(II)). The experiments with minimum relative laser power (<30) occurred with defect surface A (Fig. 1(II)). It has been experimentally observed [8,13,14] that less laser power imparts less superfluous energy into the analyte:matrix system so that the MALDI process is improved by the more gentle conditions and that, in consequence, the fragmentation tendency of the analyte may be lowered. A greater number of defect surfaces in a crystal lattice facilitates the MALDI process.

Further studies show a correlation between the crystal surface and the quality of the MS results (Fig. 3). Fig. 3(a) shows a mass spectrum obtained from the perfect surface with a relative laser power of 50, and Fig. 3(b) of the defect surface with a relative laser power of 30. Hence, considerably less laser power had to be applied in the case of the defect surface and led to about twice the signal intensity for the protonated parent ion. The perfect surfaces B (Fig. 1(II)) did not supply reproducible results requiring a laser power of 80 in one of the measurements. Light microscopic investigations of the crystal of 2,5-DHB and incorporated Cyt *c* after MS investigations revealed that after intensive laser irradiation of the crystal, the surface is sometimes altered which might be due to a melting process.

Similar investigations for the pulverized crystal of 2,5-DHB and incorporated Cyt *c* showed superior results regarding the MS reproducibility. Solvent-free mass spectra could be obtained by applying a constant relative laser power of 25 (Fig. 3(c)) and repeating 10 sets of 20 laser shots. This was repeated six times at exactly the same spot. The signal intensities and signal width of the analyte (Fig. 3(c)) were almost identical for each measurement and corresponded closely with the mass spectrum obtained from the perfect surface (Fig. 3(a)) with regard to signal intensity and signal-to-noise-ratio when relative laser power of 50 was applied. High uniformity and homogeneity in the depth profile of the MALDI sample and also showed that laser irradiation does not initiate alteration of the sample. Light microscopic investigations determined complete depletion of the analyte:matrix sample mixture after intensive MALDI laser irradiation of the pulverized crystal. It can be concluded that the laser irradiation of the powder mixture of the pulverized crystal of 2,5-DHB and incorporated Cyt *c* leads primarily to the desorption/ionization of matrix and analyte.

Comparative investigations of the pure crystal of the 2,5-DHB matrix showed that the averaged threshold laser power, which leads to desorption/ionization of the matrix, was 19.4 and those of the pulverized crystal 16.2. After intensive laser irradiation of the crystal, surface alterations were found to be identical to the 2,5-DHB:Cyt *c* crystal. These surface alterations again were probably due to melting processes. This was not observed in case of the powder of the pulverized 2,5-DHB crystal. These results indicate that pulverizing the crystal, which directly corresponds to an increase of the surface-to-volume-ratio or maximization of defect surfaces of a crystal, facilitates the desorption/ionization during the MALDI process and reduces surface alterations of the sample.

Overall, the desorption/ionization of the Cyt *c* analyte from the 2,5-DHB matrix is favored in the pulverized over the intact crystal as seen by higher reproducibility and lower threshold laser power. Therefore, incorporation of the analyte into the matrix crystal is of disadvantage for the MALDI process since the crystal lattice energy must be surmounted first in order to enable the desorption/ionization process. It is worth noting that the determined relative laser power of the single crystal is a value rather underestimated, since perfectly planar areas require substantially higher laser power yet are less often observed within this particular crystal.

3.2.1. Investigations in dependence of the MALDI sample preparation

3.2.1.1. Solvent-based MALDI-TOF-MS applying "dried droplet" sample deposition protocol. There is little practical relevance of single crystal growth for MALDI-TOF-MS. For this reason the solvent-based "dried droplet" sample deposition protocol was evaluated similarly to the single crystal investigations described above. The light microscopic investigations of the solvent-based "dried droplet" sample deposition protocol (Fig. 1(IV)) display areas on the MALDI target holder, which apart from areas with very finely distributed, submicrometer-sized crystals (C), indicate large, prism-like disk (A) and needle-like (B) crystals.

The evaluation of the threshold laser power was performed similarly to the MALDI-TOF-MS investigations of the single crystal of 2,5-DHB and incorporated Cyt *c* and its pulverized crystal analog. The mean of the laser power of these solvent-

based experiments resulted in a value of 32.2 with a range from 30 to 39. In Fig. 3(d) the mass spectrum is shown for the relative laser power 30. More detailed investigations of the solvent-based sample preparation showed that there was a close correlation between the need to apply high laser power and large, prism disks crystals (A). This correlation between macroscopic appearance and the MALDI-TOF-MS result was again possible by visualization with the assistance of the video camera installed on the MALDI device. The experiments in which the lowest laser power needed to be applied were found with submicrometer-sized crystals (C). Hence, submicrometer-sized crystals, correlating to an increased number of defect surfaces and decrease in crystal lattice energy, facilitate and improve the MALDI process, shown by the lower threshold laser power (Fig. 2(b)) that has to be applied for successful desorption/ionization of the analyte.

The investigations regarding the reproducibility of the "dried droplet" sample deposition protocol showed that the areas with the large crystals (A) did not produce reproducible results, whereas the submicrometer-sized crystals resulted in increased reproducibility (C). The light microscopic investigations did not show surface alterations in the large crystals (A), while the submicrometer-sized crystals were to a large extent removed (C) after laser irradiation. The MS results for solvent-based MALDI sample preparation supports the hypothesis that due to the lattice energy in the crystal, which has to be surmounted for successful desorption/ionization of the analyte, higher laser power relative to the solvent-free method must be applied. The solvent-based "dried droplet" MALDI sample deposition protocol produced results that resemble, due to the observed considerably higher threshold laser power and the poorer reproducibility, the results of the single crystal rather than those of the pulverized single crystal.

3.2.1.2. Direct solvent-free MALDI-TOF-MS applying 2,5-DHB. The solvent-free sample preparation of Cyt *c* and 2,5-DHB directly combined and mixed as powders (molar ratio analyte:matrix of 1:10,000) enabled the production of mass spectra of Cyt *c*. The direct mixture produced by means of ball mill treatment of a commonly used matrix and analyte ratio enables sufficient homogenization and ensures an effective MALDI process. Therefore, there is no mandatory requirement to embed the analyte into the matrix crystal lattice. The ball mill used in this work supplies particle sizes of approximately micrometers according to the suppliers' information [38]. The order of magnitude of a micrometer is, however, some powers of ten larger than molecular dimensions (order of magnitude 10^{-10} m). A homogeneous mixture of analyte and matrix on the molecular level will not be achieved with solvent-free MALDI-MS. Hence, the original model that the matrix effect is based on mixed crystal formation between matrix and analyte is not supported. However, at least a partial homogenization on the molecular level appears to be sufficient for a successful measurement. This suggests that in solvent-based MALDI samples, the homogeneities achieved in the solid state are inferior to what was anticipated so far for a successful MALDI process. The process of any crystallization of two or more components shows separation phenomena of differing degrees. Therefore, the matrix and analyte molecules must be protected from separation in the crystallization step in the conventional solvent-based MALDI sample preparation. Certainly, minimization of separation or maximization of homogeneity can be introduced by optimization of the solvent-based sample preparation [7,30–37]. Thus, similar polarities of the analyte and matrix molecules lead inevitably to reduction of separation during the crystallization based on our past experiences in solvent-based approaches. The crystallization can be directly accelerated and, hence, the isolation of the components can be suppressed to a large extent, by using more volatile solvents. Thus, the effects that come along with the phase transition from the dissolved to the solid state are minimized. Nevertheless, the conventional method using the dissolved state is simple and fast and, in most cases, leads to sufficient homogeneity. However, inhomogeneity is reflected by different crystals, in size and appearance, within a single MALDI target "spot". When less homogeneity is achieved [14], time-intensive searching for "hot spots" on the MALDI target "spot" will be required or application of higher laser power may suffice to acquire a mass spectrum. However, the latter can significantly lower the quality of the MS result.

3.2.1.3. Direct solvent-free MALDI-TOF-MS applying various DHB Isomers as matrices. The direct solvent-free MALDI sample preparation was evaluated for the characterization of Cyt *c*, in order to enable a unique statement about the matrix effect. Different experiments were performed, which include molar analyte:matrix conditions and analyte:matrix combinations. It can be assumed that due to the grinding effect, which leads to homogenization, analyte:matrix combinations may be successfully applied for MALDI-TOF-MS analysis via the solvent-free approach, which are only accessible with difficulties or inaccessible due to their different physiochemical characteristics in solvent-based methods.[4] Following this argument, solvent-free MALDI-MS analyses of different DHB matrix isomers were selected that, ideally, show sufficient absorption at the laser wavelength of 337 nm but failure in solvent-based UV-MALDI-TOF-MS analysis. The mass spectra of the 2,4-, 2,5- and 2,6-DHB isomers exhibited, with optimized deposition protocols and suitable laser wavelengths, generally better qualities than those of the 3,4- and 3,5-DHB matrix isomers [7,39]. Therefore, in this contribution model investigations were performed with the first three DHB matrix isomers only. The order of decreas-

[4] With "dried droplet" sample deposition protocol the analyte and the matrix are mixed first in the dissolved state and then put on the MALDI sample holder. By evaporation of the solvent, the crystallization takes place according to the mixture components and their physiochemical characteristics. In "thin layer" sample deposition protocol first the matrix, dissolved in acetone, is put on the MALDI sample holder and the solvent is evaporated. The aqueous analyte solution is applied thereafter on the crystallized matrix layer on the MALDI sample holder. Depending upon solubility of the matrix in water (2,6-DHB < 2,4-DHB < 2,5-DHB), the thin matrix layer undesirably partially redissolves [7]. The contact of both the analyte and matrix molecules takes place with sufficient miscibility only at the boundary of the two layers. "Thin layer" sample deposition protocol is therefore an advanced "dried droplet" sample deposition protocol, in order to minimize inhomogeneities between analyte and matrix in the solid state.

ing absorption at 337 nm in the UV-spectrum in the solid state, is 2,5-DHB > 2,6-DHB > 2,4-DHB [7]. In previous contribution [7], employing the solvent-based "dried droplet" sample deposition protocol, only the 2,5-DHB matrix supplied a MALDI-TOF mass spectra (337 nm). No mass spectra, or only some with poor quality, could be produced at this laser wavelength for 2,4-DHB with both solvent-based sample deposition protocols. The 2,6-DHB resulted in mass spectra via solvent-based MALDI-TOF-MS only after "thin layer" sample deposition protocol from an acetone solution. This success correlates with the theoretical expectations that this DHB isomer should work the best for "thin layer" based on its low solubility in water compared with other DHB isomers. For the DHB isomers the following Cyt *c* molar analyte:matrix incorporation conditions in the crystal were determined [7]—2,5-DHB: quantitative incorporation of $1{:}10^4$; 2,4-DHB: semi-quantitative incorporation of about $1{:}10^5$; 2,6-DHB: slightly to none incorporation ($<1{:}10^5$). MALDI-TOF mass spectra of Cyt *c* from these crystals were achieved, logically, only for the 2,4-DHB and 2,5-DHB with good quality, whereby the 2,6-DHB crystals produced no mass spectra. In this previous investigation [7], solubility, incorporation and segregation are in close relationship and dictate a successful MALDI analysis.

The solvent-free MALDI analysis of the re-crystallized matrices with a molar analyte:matrix ratio of 1:1000 allowed, in contrast to the solvent-based sample preparation, the characterization of Cyt *c* with all three DHB isomers (Fig. 4). The 2,5-DHB matrix enabled the production of high quality mass spectra of Cyt *c* using a relative laser power of 35 (Fig. 4(a)). With the 2,6-DHB matrix, mass spectra (Fig. 4(b)) could be obtained with a slightly increased relative laser power of 40 but the signal of Cyt *c* was relatively broad caused by extensive sodiation. The mass spectra obtained using the 2,4-DHB matrix (Fig. 4(c)), likewise supplied relatively broad signals of the Cyt *c*, however, only when a substantially higher relative laser power of 55 was applied. Surprisingly, an increase of the relative laser power to 60 clearly improved the quality of the mass spectrum. Fragmentation did not occur despite very high laser irradiation. The increase of the relative laser power that has to be applied for the different DHB isomers is attributed to the decreasing absorptions of the matrix isomers at 337 nm laser wavelength. The width of the analyte signal can be explained with the increased laser power and, additionally, with a Cyt *c*–matrix adduct formation. The latter is in conformity with other investigations, where the 2,5-DHB showed the smallest tendency for matrix adduct formation [7]. With these investigations, it is uniquely shown that the solvent-free MALDI sample preparation with 2,6-DHB as a matrix supplies good MALDI-TOF mass spectra even though this isomer, of the evaluated isomers, has the smallest tendency to incorporate Cyt *c* in single crystals. Therefore, solvent-free MALDI-TOF-MS provides sufficient contact between analyte and matrix that is not achieved in solvent-based methods or by the solvent-free pressed pellet method [9]. The failure in the solvent-based MALDI-TOF-MS analysis can be rationalized by the fact that the physiochemical interaction of the 2,6-DHB matrix and the Cyt *c* analyte molecule is disturbed upon the evaporation of the solvent during the conventional sample deposition protocol which is in agreement with literature described observations [7]. Hence, separation between analyte and matrix is initiated and the contact between analyte and matrix is minimized in such a way that the MS analysis fails. Nevertheless, sufficient homogenization of the less compatible molecules of 2,6-DHB and Cyt *c* can be achieved by homogenization in the solid state applying outside forces such as a ball mill treatment. Thus, limitations caused by segregation are eliminated in solvent-free MALDI-MS. However, modifications of the morphology on the molecular level (e.g., ball milling aids solid-state solubility so that the analyte dissolves to some extent into matrix surface layers) cannot be completely excluded due to mechanical handling with the sample preparation presented in this work. However, it was shown recently that an analyte was accessible via a solvent-free mini-ball mill MALDI approach [17] where the analyte and matrix powders were homogenized without any beads; hence, grinding did not take place. A further assumption regarding the theoretical aspect of the solvent-free MALDI sample preparation method is that an adsorption model between analyte and matrix can be considered, which is independent of crystallinity. The successful, solvent-free MALDI-TOF-MS analysis of Cyt *c* using different DHB matrix isomers is consistent with the statement that a close contact between analyte and matrix is a decisive factor in the MALDI process. It was demonstrated that mainly the absorption coefficient of the matrix determines the necessary laser threshold to yield analyte ions. An incorporation of an analyte into a matrix crystal is clearly not necessary for a successful MALDI process.

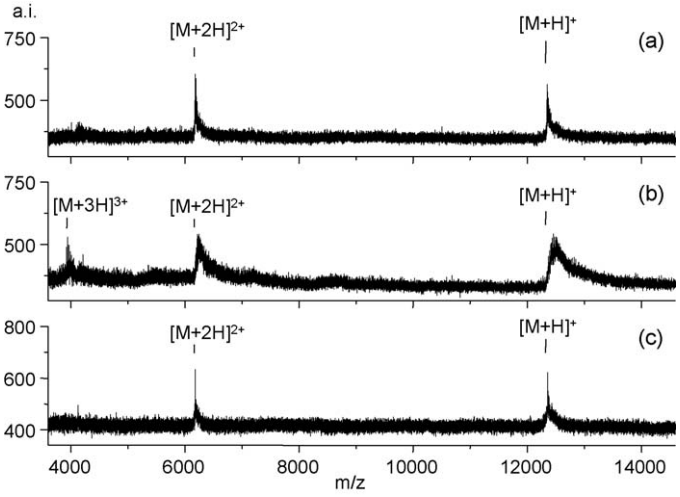


Fig. 4. Solvent-free MALDI-TOF mass spectra of Cyt *c* employing various DHB matrix isomers with a constant molar analyte:matrix ratio of 1:1000 and its corresponding threshold laser power (in parentheses): (a) 2,5-DHB (35); (b) 2,6-DHB (40); (c) 2,4-DHB (55).

3.2.1.4. Application of solvent-free MALDI-TOF-MS to incompatible analyte, matrix combinations. Dithranol (Scheme 1) is a matrix, which is preferentially used in the analysis of synthetic polymers or organic macromolecules and shows good solubility in organic solvents (e.g., THF) and is therefore solvent-

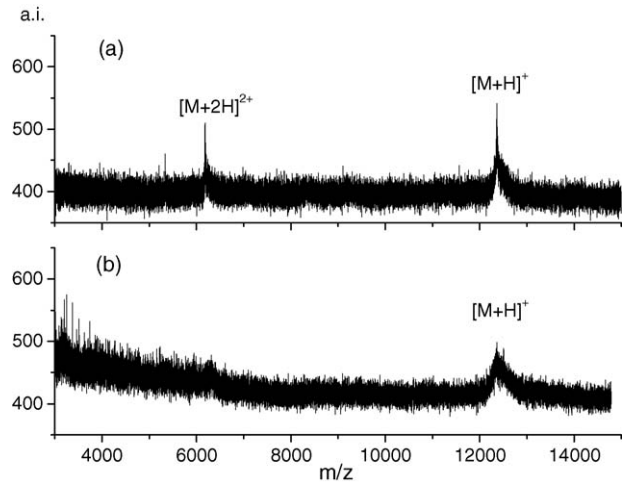


Fig. 5. Solvent-free MALDI-TOF mass spectra of Cyt *c* employing incompatible matrices with a constant molar analyte:matrix ratio of 1:500 and its corresponding threshold laser power (in parentheses)*:* (a) dithranol (40); (b) anthracene (45).

incompatible with Cyt *c*. The dithranol matrix is less polar than the DHB matrix. The solvent-free MALDI-TOF mass spectrum of Cyt *c* using a dithranol matrix (Fig. 5(a)), in the matrix-common analyte:matrix ratio of 1:500, was obtained with a relative laser power of 40 with good quality. There is an even larger difference in polarities between Cyt *c* analyte and anthracene (Scheme 1) matrix. Anthracene is a very non-polar matrix, which was previously applied for the characterization of non-polar polymers such as poly(butadiene), poly(isoprene) and polystyrene [40]. It improves the reproducibility of the measurement of non-polar compounds in particular. Anthracene is solvent- and polarity-incompatible with Cyt *c*. In Fig. 5(b), the solvent-free MALDI-TOF mass spectrum is shown, obtained by applying anthracene in an analyte:matrix ratio of 1:500 with a relative laser power of 45. A mass spectrum with very poor quality was obtained, in particularly shown by a poor signal-to-noise-ratio and a wide analyte signal. Because the analyte and matrix molecules are completely incompatible due to their polarities, it can be assumed that they mix exclusively by the external forces given by the ball mill treatment. The initial success of obtaining a mass spectrum shows that the absorption of the matrix represents a decisive factor of the matrix effect. Due to the poor quality of the mass spectrum, it must be assumed that a compatible polarity between matrix and analyte improves the MALDI process.

In comparison to solvent-free MALDI results of the Cyt *c* analysis obtained using analyte–uncommon dithranol as matrix with the analyte–common 2,5-DHB matrix (Fig. 6), an increased relative laser power, smaller signal intensity, a lower S/N, but smaller signal width was found. Signal widening using 2,5-DHB matrix (Fig. 6(I)) is mainly caused by metal-pseudo-molecular-ion-formation with sodium ions through varying degrees of exchange with protons as also described with solvent-based MALDI-MS for this analyte:matrix system [1]. Different matrices introduce different degrees of sodiation of Cyt *c* in solvent-

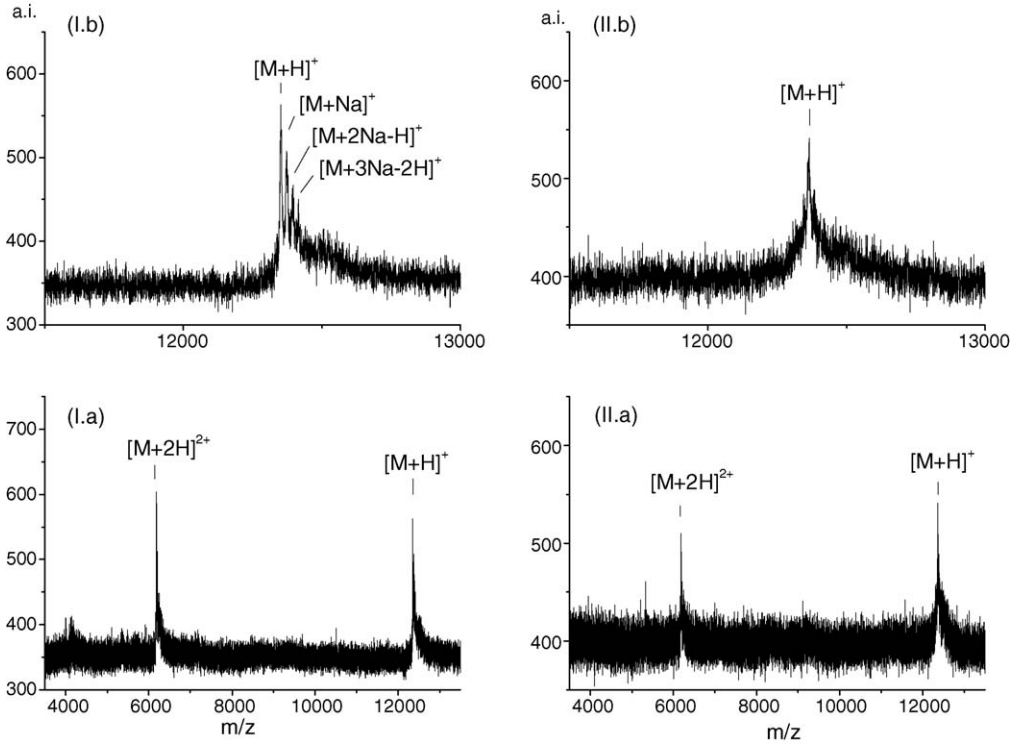


Fig. 6. A comparison of solvent-free MALDI-TOF mass spectra (a) and respective inset spectra (b) of Cyt *c* and dependence of the employed matrix and its corresponding threshold laser power (in parentheses)*:* (I) 2,5-DHB in a molar analyte:matrix ratio of 1:1000 (35); (II) dithranol in a molar analyte:matrix ratio of 1:500 (40).

free MALDI-MS; sodiation of peptides and proteins is described in previous work [17,22]. The "tailing" (Fig. 6(II)) of the Cyt *c* signal to lower masses using dithranol matrix is attributed to fragmentation by increased laser power.

4. Conclusion

An attempt to sketch a uniform model of the MALDI processes is not the focus of these investigations. Rather this investigation joins a multiplicity of detailed investigations seeking a deeper understanding of the physiochemical processes which are the basis for the desorption process in the MALDI method and lead to their unusual characteristics. The results presented in this work support the notion that a successful MALDI-TOF-MS analysis with a wavelength of 337 nm requires a close contact between analyte and matrix molecules [7,8] in general. The new realizations do not contradict the postulated adsorption model [7] between analyte and matrix. In these investigations, the process of the analyte ion production is favored by a sufficient absorption of the matrix at the applied laser wavelength of 337 nm and a maximized crystal-free homogenization between matrix and analyte in the solid state. The latter can significantly lower the threshold laser power of the ion formation, which makes the MALDI process milder. The conclusion for all these experimental results is that by increasing the surface-to-volume-ratio, the number of defect surfaces of the crystal is maximized. Therefore, the lattice energy of the crystal is minimized, whereby the release of the molecules from the lattice federation into the gas phase is facilitated. In particular, comparisons of the MALDI-TOF-MS investigations of the single crystal and pulverized single crystal of 2,5-DHB and incorporated Cyt *c*, showed explicitly that the crystallinity and laser power correlate. The smaller the remaining crystallinity, the smaller the laser power that needs to be applied for the MALDI analysis of the analyte. Contrary to Horneffer et al. [7] we must conclude with these results that the incorporation of analyte in matrix crystals is not helpful for a MALDI-TOF-MS analysis but obstructive, since it is crystallinity that makes the underlying process energetically more difficult.

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