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Matrix–analyte-interaction in MALDI-MS: Pellet and nano-electrospray preparations

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Abstract

The incorporation of analytes into matrix crystals and even more so its mechanistic aspects as a prerequisite for a successful MALDI-MS has been discussed controversially in the literature. Solventless sample preparation techniques can shed new light on this question. In order to investigate some crucial aspects of these preparation techniques, lyophylized peptides and proteins were ground or milled with the powder of two different matrices, 2,5-DHB as incorporating matrix and 2,6-DHB for which protein incorporation was definitely excluded in a prior study, and pressed into pellets. The dependence of the quality of the UV-MALDI-spectra on the mass (up to 12,360 Da) and the milling time in a ball mill is reported. For mellitin different initial axial ion velocities were found, when desorbed from 2,5-DHB-pellets as prepared and after wetting and re-drying. Velocities of 150 and 580 m s^{-1} for dry and wetted pellets are taken as representative for hard desorption from a surface and soft desorption of matrix-incorporated analytes, respectively. Proteins labeled with either fluorescein isothiocyanate (FITC) or Texas Red (TR) were nano-electrosprayed onto a bed of ferulic acid in a 'dry' or 'wet' mode. All 'dry' deposits exhibit strong fluorescence but do not yield MALDI-ion signals. All 'wet' deposits yield MALDI-signals of the proteins; the fluorescence of FITC is quenched in 'wet' deposits because of the low matrix pH.

Keywords: MALDI; MALDI-dry-preparation; MALDI-analyte-incorporation; Mass spectrometry; Fluorescence confocal laser scan microscopy

1. Introduction

The interaction between matrix and analyte molecules in matrix-assisted laser desorption/ionization (MALDI)-samples prior to and during the desorption/ionization event has been the subject of interest in the MALDI-community and the aim of investigations for many years [1–6]. So far, no general and conclusive model has evolved from these studies. In a number of publications, a 'dry' preparation technique has been reported mostly for the analysis of synthetic polymers [7–11]. This technique has obvious implications for the models put forth so far. In this technique matrix and analyte are mixed as dry powders and ground or milled to a fine grain size. In a recent paper, it has been shown that simple mixing suffices instead of the grinding or milling, if the HCCA-matrix powder was fine enough [12]. The resulting powder is either pressed into a pellet or applied directly to the sample support by dusting onto double side adhesive tape or pressing it down with a teflon covered spatula for analysis in a mass spectrometer [12–15].

Besides some practical advantages particularly for the analysis of non-soluble analytes, it also offers the opportunity to conduct some mechanistic experiments on the MALDI-process, which are described in this paper. This is a follow-up of several earlier publications [16–18], particularly the publication by Glückmann et al. [13]. The background is discussed extensively in the latter paper and shall not be repeated here in all detail. In short, it has been a paradigm in MALDI-MS for many years that incorporation of the analyte molecules into the matrix crystals of solid matrices is a prerequisite for a successful MALDI-

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analysis. This model was supported by early results proving that analyte molecules are, indeed, incorporated into single crystals of 2,5-DHB [1,20], succinic acid [4,5], sinapic acid [2], and alpha-cyano-4-hydroxy cinnamic acid (HCCA) [21]. Doubt was, however, cast on the generality of this assumption as a necessary requirement for MALDI by the finding that the position isomer 2,6-DHB does not incorporate analyte molecules but functions as a reasonable, though not optimal MALDI-matrix, if prepared as a microcrystalline sample [6,22]. It was concluded that a large specific matrix surface area is a prerequisite for a successful MALDI-mass analysis with non-incorporating matrices.

In the first part of this paper, investigations of pellet samples are reported. Pellets were prepared by grinding dry matrix and analyte material in a ball mill for different milling times (5 s–7 min) to find out whether: (a) decrease of the grain size resulting in an increase in specific surface area facilitates spectra acquisition and/or spectra quality, (b) whether a more intense milling of matrix and analyte material provides a better matrix isolation of analyte molecules and thereby a higher sensitivity, and (c) whether a higher mass range is accessible by longer milling times. Pellets were prepared under ambient atmosphere as well as under dry nitrogen to access a potential influence of the ambient humidity. The initial axial ion velocity of protein ions from pellets is investigated as a measure of the "softness" of the desorption process from pellet preparations [18].

In the second part of the paper, some details of the interaction of analyte molecules at the surface of the underlying matrix are investigated. For this purpose, proteins labeled with two different fluorochromes, one, pH-sensitive fluorescein isothiocyanate (FITC) and the other, pH-independent Texas Red (TR), were sprayed onto a bed of ferulic acid [13]. Spray conditions were chosen such that in one case the protein arrived at the matrix as dry particles and as still partly solvated in the other. These samples were then analyzed in a fluorescence microscope and a MALDI-TOF-MS.

2. Materials and methods

Matrices 2,5-dihydroxybenzoic acid (2,5-DHB, Sigma–Aldrich, Germany), 2,6-DHB (Fluka, Switzerland) were purified by re-crystallization before use. Ferulic acid (Sigma–Aldrich, Germany) was used as delivered. The peptides and proteins bradikinin (RPPGFSPFR triacetate salt), honeybee melittin (Mel), bovine insulin (Ins), horse heart cytochrome *c* (CC), and bovine serum albumin (BSA), as well as FITC-labeled insulin were purchased from Sigma–Aldrich (Germany). Texas Red-labeled avidin was supplied by Molecular Probes (The Netherlands). Mass spectrometric analysis ascertained that no free dye molecules were present in the labeled protein samples. All chemicals were used as delivered. Tetrahydrofuran (THF) and acetonitrile (ACN) were supplied by Merck (Germany). Doubly distilled water (H_2O_{bidest}) was used as a solvent.

Pellets were prepared by mixing dry matrix and analyte in a mass ratio of 250:1 and either grinding the mixture in a mortar for several (1–3 min) as described before [13] or in a ball mill (Perkin-Elmer, Rodgau-Jügesheim, Germany, volume 1.5 ml, diameter of balls approximately 3 mm) for milling times of 5 s, 30 s, 1 min, 3 min, or 7 min. The ground matrix–analyte powder was pressed into pellets at a pressure of 30–100 bar using a manual hydraulic press as used for KBr-pellets in IR-spectroscopy. The press had only a course pressure indicator. The size of the pellets was approximately 8 mm in diameter and 1–2 mm in thickness. For MS-analyses, the pellets were placed into wells in the stainless steel sample plates such that their surfaces was level with the sample plate in order minimize fringing field effects and affixed with double sided adhesive conducting tape (Plano, Germany). No special attempts were made to optimize the matrix/analyte ratio or the pressure for pressing the pellets for each combination of analyte and matrix, because this was not the goal of this study and prior experiments had shown that very little differences in performance and spectra quality were observed between about 100:1 and 1000:1 for the matrix/analyte ratio and the range of pressures available with the manual hydraulic press.

For preparations in a dry nitrogen atmosphere, pellets of 2,5-DHB and CC were prepared in a glove box containing the mortar, the hydraulic press the MALDI-target plates, and a small transportation box. The glove box was flushed with dry nitrogen (Westfalen Gas, Germany) for 10 min prior to sample preparation. The analyte/matrix mixture was ground manually for several minutes. Sample plates with the mounted pellets were stored in the transportation box and transferred immediately into the vacuum of the mass spectrometer. During transfer from the box into the vacuum lock of the mass spectrometer, the samples were exposed to the atmosphere for at most 0.5 min.

A second set of samples was prepared by nano-electrospray as described by Glückmann et al. [13]. First a microcrystalline matrix bed was prepared by dissolving ferulic acid in THF at a concentration of 20 g/l, ca. 5 μl of this solution was dripped onto the stainless steel sample plate. Fast evaporation of the solvent produced a thin layer of microcrystals on the target plate. The analyte molecules were deposited onto this matrix bed by nano-electrospray of 2–3 μl of analyte solution, sprayed from laboratory-pulled glass capillaries. The capillaries were mounted 2–3 mm above the surface of the matrix layer; the spray voltage was adjusted to maintain a current of 50–200 nA. Two different capillaries were used. For 'dry' deposits the orifice diameter was 1–2 μm. For these spray conditions, the solvent of the very small droplets fully evaporates before dry analyte particles reach the matrix surface. For 'wet' deposits capillaries with a 20–40 μm, diameter orifice were used. Under these spray conditions, droplets reaching the surface still contain a substantial amount of solvent which can then locally dissolve at least a superficial matrix layer, visible as an initially glossy surface. FITC-labeled insulin and TR-labeled avidin were used as analytes, dissolved to a concentration of 10^{-5} M in a mixture of H_2O_{bidest}/ACN (1/1, v/v). For 'dry' deposits, a deposition time of 5 h was chosen, for 'wet' deposits one of 20 min. For control purposes, the solutions were also 'wet' sprayed directly onto the neat stainless steel sample plates. After inspection by mass spectrometry and fluorescence microscopy, some of the 'dry' deposits were wetted with a small volume of solvent (1 μl H_2O_{bidest}/ACN, 1/1, v/v), and allowed to dry again. After drying again these samples were re-analyzed for fluorescence

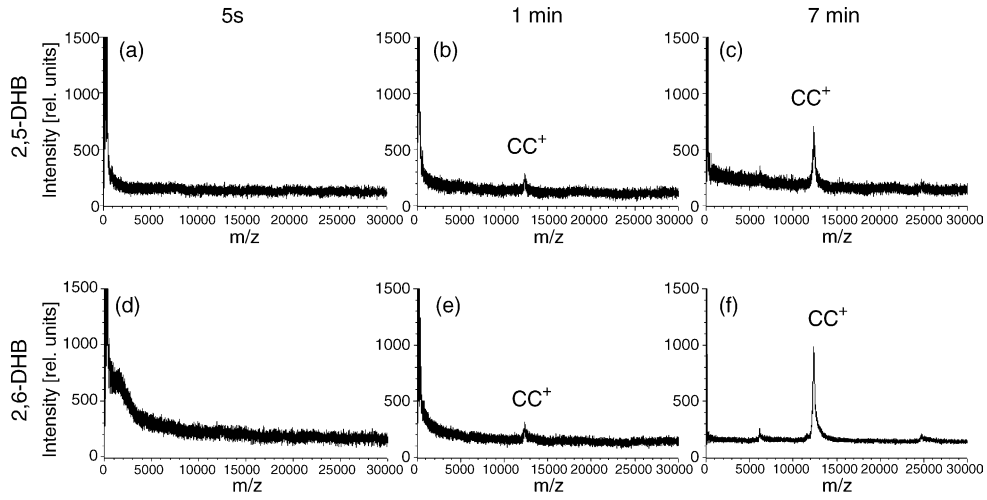

Fig. 1. UV-MALDI-mass spectra of matrix–analyte pellets after mixing the materials in a ball mill. Wavelength 337 nm; reflectron TOF; each spectrum sum of 20 single spectra. Spectra after different milling times are shown for 2,5-DHB–cytochrome *c* (CC^+): (a) 5 s, (b) 1 min, (c) 7 min and 2,6-DHB–cytochrome *c*: (d): 5 s, (e) 1 min, (f) 7 min. Signals of protonated and cationized species not resolved. The spectra (a–c) and (d–e), respectively, were recorded at the same laser fluence. Before milling the (purified by re-crystallization) matrix crystals were prismatic in shape with a 3–5 μm square base and a length between ca. 30 and 100 μm. Increasing milling time led to a fraction of prismatic structures of 10–30 μm length and a fraction of agglomerated crystalline particles in the <1–3 μm size range exhibiting a sponge-like structure. At 1 min milling time, the volume fraction of small particles was about 50%, at 7 min milling time this fraction was ca. 90%.

and mass signals. A modified VISION 2000 (Thermo-Finnigan, Germany) MALDI-mass analyzer was used for the mass spectrometry of pellets from mortar and ball mill preparations as well as the nano-electrosprayed samples. The reflectron TOF modes was used for the pellets with a total acceleration voltage of 12.5 kV and a postacceleration of 2.5 kV for peptides and 20 kV for proteins, respectively. Sprayed samples were analyzed in the delayed extraction linear TOF-mode with an extraction voltage of 2.0 kV and an extraction delay of 700 ns, a full acceleration voltage of 20 kV and 2.5 kV postacceleration.

The initial velocity v_0 of analyte ions from pellets prepared by manual grinding was measured with a Voyager DE PRO MALDI-TOF-Mass Spectrometer (Applied Biosystems, Framingham, MA, USA) operated in the delayed extraction linear mode. v_0 was determined from the slope of the total flight time plotted against the ion extraction delay as described in detail elsewhere [16,18]. In a control experiment, pellets were wetted with H_2O_{bidest}/ACN, 1/1 (v/v) and dried again before determination of the initial velocity.

A Leica TCS (Germany) was used for the fluorescence microscopy to image the fluorescence of 'dry' and 'wet' deposits of fluorescence-labeled analyte molecules on ferulic acid matrix layers. The instrument was equipped with an Ar–Kr laser, and a 10×-objective with a numerical aperture of 0.3. For FITC-insulin 488 nm was used as excitation wavelength, while the emission was detected in the wavelength range of 515–545 nm. TR was excited with a wavelength of 568 nm, and the emission was detected at wavelengths above 590 nm.

3. Results

The incorporation of analytes into matrix crystals and even more so its mechanistic aspects as a prerequisite for a successful MALDI-MS of biopolymers particularly of higher mass proteins, e.g., from pellet preparations has been discussed controversially in the literature [19]. In order to investigate the analyte–matrix interaction and specifically the importance of analyte incorporation into the matrix for the MALDI-MS of bioorganic compounds, pellets were prepared with two different matrices, 2,5-DHB as incorporating matrix and 2,6-DHB for which protein incorporation was definitely excluded in a prior study [6,13]. In addition the influence of the milling time on spectra quality was studied. Mass spectra were obtained for several different peptides/proteins with masses up to cytochrome *c*, with a mass of 12,360 Da. The quality of the spectra was evaluated with respect to mass resolution, signal intensity, signal-to-noise ratio, and reproducibility from shot-to-shot and spot-to-spot. Mass spectra of cytochrome *c* (CC), obtained after different milling times are shown in Fig. 1 for the two matrices. The results are summarized in Table 1. The relatively low mass resolution of less than 50 and the symmetric peak shape are indicative of a substantial metastable ion fragmentation. For all spectra of a given matrix, the laser fluence was chosen at about 20% above the threshold fluence for the desorption of peptides or insulin from pellets prepared by the same procedure. For the samples ground for only 5 s, no analyte signal could be obtained even for substantially higher fluences.

Within the usual variability of MALDI-spectra, no significant differences are observed for the two matrices. With increasing mass of the analyte molecule spectral quality of the signals degrades for both matrices; in particular, for larger masses, the spectra quality never matches that of optimal standard wet preparations. It can be concluded from the data shown in Table 1 and Fig. 1, that a longer milling times clearly improves the sensitivity as well as the quality of spectra and extends the accessible mass range up to at least 12 kDa. Compared to standard MALDI-preparations the spot-to-spot reproducibility is greatly improved for pellets.

It could be argued that the ambient humidity could induce an at least superficial analyte incorporation during the grind-

Table 1
Quality of MALDI-mass spectra obtained for different milling times of matrix and analyte material in the ball mill

Matrix	Analyte	Milling time in the ball mill				
		7 min	3 min	1 min	30 s	5 s
2,5-DHB	Bradykinin 1060.25 Da	+++++	++++	+++	++	+
	Insulin 5733.6 Da	+++	++	+	+/−	−
	Cytochrom *c* 12359.0 Da	+	+	+/−	−	−
	BSA 66430 Da	−	−	−	−	−
2,6-DHB	Bradykinin	+++++	+++	++	+	+/−
	Insulin	++	+	+	+/−	+/−
	Cytochrom *c*	++	+	+/−	+/−	−
	BSA	−	−	−	−	−

(+++++) Very good; (++++) good; (+++) medium; (++) weak; (+) very weak; (+/−) barely detectable; (−) no ion signal.

ing/milling process. To exclude this possibility, pellets of 2,5-DHB with CC were prepared by grinding the mixture for several minutes by hand and pellerizing at a pressure of 30 bar in a dry nitrogen atmosphere. The pellets were then quickly transferred into the vacuum of the mass spectrometer for analysis. Fig. 2 shows the resulting mass spectrum. A significant CC-signal is obtained even from such water free pellets.

In a recent publication, Karas et al. [18] have shown a correlation between the initial axial velocity v_0 of analyte ions and the softness of the desorption: the slower the velocity, the harder the desorption. Given the fact that spectral quality, obtainable from pellet preparations is significantly lower than that of standard 'wet' MALDI-preparations of the same proteins, which moreover degrades significantly with increasing mass, it was of interest to determine the initial velocity of the analyte ions. Mellitin in 2,5-DHB was chosen as analyte for these measurements, because its protonated and cationized ions could still be fully resolved in the spectra. Pellets were measured dry as prepared and after additional wetting. The results are listed in Table 2. The velocities of $154 \pm 97\,\mathrm{m\,s^{-1}}$ for the protonated ions and $118 \pm 65\,\mathrm{m\,s^{-1}}$ for sodiated ions obtained for dry pellets are significantly lower than the corresponding values of 580 and $446\,\mathrm{m\,s^{-1}}$ after wetting.

In the former publication [13], it was reported that analytes, nano-electrosprayed onto a ferulic acid matrix bed such that the particles arrived dry at the matrix surface do not yield any mass signals under MALDI-conditions. In contrast, 'wet' deposits yielded good protein signals. To better understand this observation, the state of the analyte on the matrix surface, for 'dry' and 'wet' deposits was inspected by fluorescence imaging with a Leica TCS microscope. In addition, 'dry' deposits were wetted by a small solvent volume (1 μl H_2O_{bidest}/ACN, 1/1, v/v) after a first analysis and re-measured for fluorescence and mass signals. These experiments take advantage of the different pH-dependence of the fluorescence yield of fluorescein isothiocyanate and Texas Red. FITC loses its fluorescence at low pH (yield at pH 2–3 is 1% of that at pH 10 [24,25]), while the fluorescence of TR has only a very weak dependence on pH.

In a first set of experiments, FITC-labeled insulin was 'wet' sprayed either directly onto the clean stainless steel MALDI-target or onto the matrix bed. The nano-electrospray preparation on the clean metal surface generates a thin protein layer with an almost homogeneous coverage as documented by the fluorescence image shown in Fig. 3a; no hints of protein clotting are observed. No mass spectra were obtained from such samples because of a lack of matrix.

When FITC-insulin was 'dry' sprayed onto the ferulic acid matrix bed, a strong fluorescence is observed from isolated islands of several micrometer in diameter (Fig. 3b). The homogeneously distributed fluorescence of FITC-insulin on the neat target surface at the bottom edge of the picture (arrow) proves that the clustering of the protein on the ferulic acid bed must result from lateral diffusion of the only loosely bound insulin, rather than be caused by the spray conditions. After taking the fluorescence image, the samples were transferred to a mass spectrometer for analysis. In agreement with the result reported in

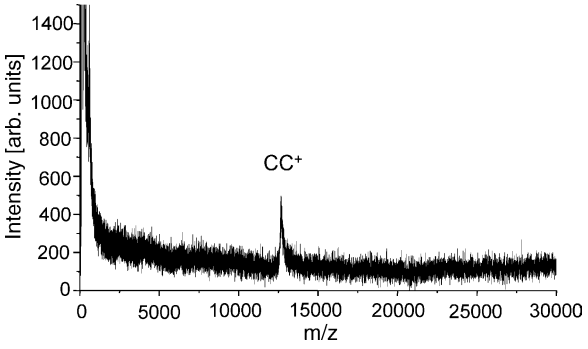

Fig. 2. UV-MALDI-mass spectrum of a 2,5-DHB–cytochrome *c* (CC^+) pellet after mixing for several minutes of grinding in a mortar and pressing of the materials, all in a dry nitrogen atmosphere. Wavelength: 337 nm; reflectron TOF. Sum of 20 single shot spectra.

Table 2
Axial initial velocity v_0 of Melittin ions out of a 2,5-DHB-pellet preparation after intense grinding of both components in a mortar and pressing the material into pellets

Ion	Pellet	v_0 (m/s)
$(Melittin + H)^+$	Dry surface	154 ± 97
$(Melittin + Na)^+$	Dry surface	118 ± 65
$(Melittin + H)^+$	Wetted surface	580 ± 11
$(Melittin + Na)^+$	Wetted surface	446

Pellets were investigated either dried or after wetting their surface with 1 μl water:acetonitrile containing 0.1% TFA (1:1).

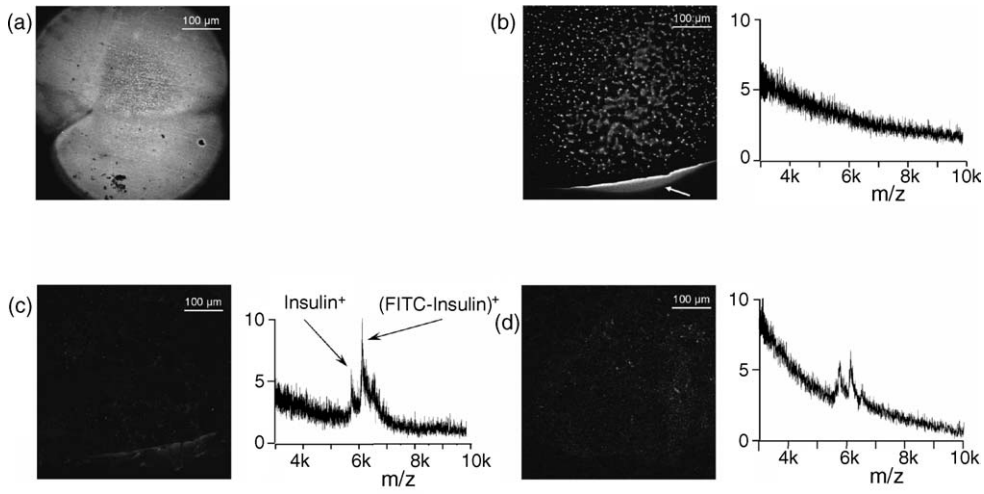

Fig. 3. Fluorescence images and mass spectra of insulin labeled with fluorescein isothiocyanate (FITC). (a) FITC-fluorescence after nano-electrospraying of FITC-insulin onto the plain stainless steel target. (b) 'Dry' deposit of FITC-insulin onto a ferulic acid layer. Fluorescence of FITC is detected, while no MALDI-signals of FITC-insulin is obtained. (c) Same sample as in (b) after wetting with 1 μl of solvent and subsequent drying. Fluorescence is quenched, but MALDI-signals of FITC-insulin are obtained. (d) 'Wet' deposit of FITC-insulin onto a ferulic acid layer. Fluorescence of FITC is quenched upon interaction with the acidic matrix. MALDI-signals of a distribution of FITC-insulin are obtained.

the earlier publication [13], no MALDI-mass signals of FITC-insulin could be obtained from such 'dry' deposits (Fig. 3b). After wetting the sample with a small amount of solvent (about 1 μl H_2O_{bidest}/ACN, 1/1, v/v), the fluorescence signal disappears and a distribution of FITC-labeled insulin signals appear in the mass spectrum (Fig. 3c). Identical results are obtained from samples 'wet' sprayed onto the ferulic acid matrix bed (Fig. 3d). These results clearly show that FITC-fluorescence and protein mass signals are mutually exclusive under the chosen conditions.

In a set of control experiments, the FITC-labeled insulin was then replaced by TR-labeled avidin. The experiments with TR-avidin were conducted identically to those with FITC-insulin. Again, a homogeneous distribution of fluorescence and no hints of protein clotting are observed for a 'dry' spray onto the neat stainless steel target (Fig. 4a). The clear visibility of the target scratches proves that the protein indeed forms a thin homogeneous (on the microscope resolution level) layer, rather than a thick preparation of clustered protein. 'Dry' spraying of TR-avidin onto the ferulic acid bed resulted in an intense fluorescence of distinct islands of 2–20 μm in diameter (Fig. 4b) as was observed for the FITC-insulin. As in the case of FITC-insulin, no MALDI-mass signals of TR-avidin could be obtained from such 'dry' deposits (Fig. 4b). Adding 1 μl of solvent (H_2O_{bidest}/ACN, 1/1, v/v) to the sample resulted in the fluorescence image shown in Fig. 4c. A significant TR-fluorescence is still detectable, not much of a surprise, keeping in mind that TR is much less pH-dependent than FITC. The fluorescence image of Fig. 4c also reveals that the matrix as well as the protein clusters get fully re-

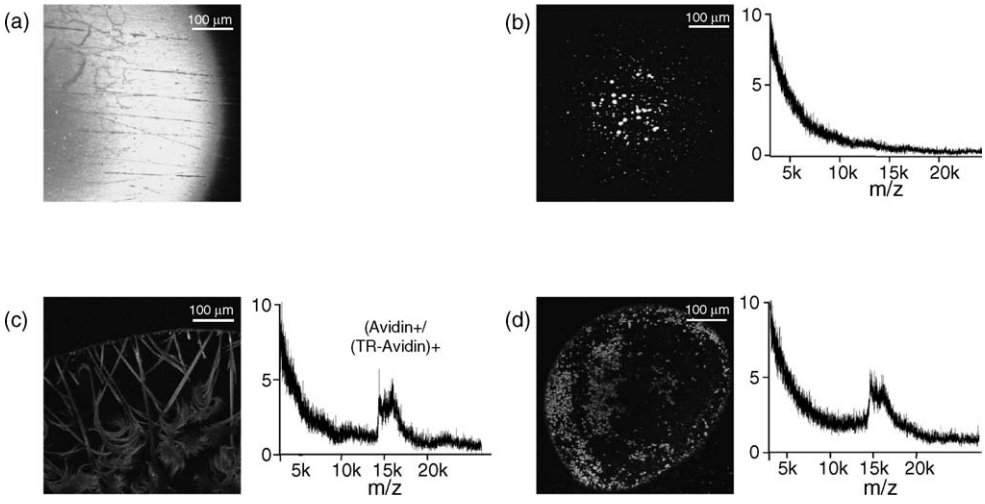

Fig. 4. Fluorescence images and mass spectra of Texas Red (TR)-labeled avidin. (a) Fluorescence of TR after nano-electrospraying of TR-avidin onto the plain stainless steel target. (b) 'dry' deposit of TR-avidin onto ferulic acid layer. Fluorescence of TR is detected, while no MALDI-signals of TR-avidin are obtained. (c) Same sample as in (b), after wetting with 1 μl of solvent and subsequent drying. Fluorescence as well as MALDI-signals of TR-avidin are obtained. (d) 'Wet' deposit of TR-avidin onto a ferulic acid layer. Fluorescence as well as MALDI-signals of TR-avidin are obtained.

dissolved and subsequently re-crystallize into the typical pattern known for dried droplet preparations of this matrix. Accordingly, mass signals of avidin with a distribution of TR-labels were obtained from such samples (Fig. 4c). 'Wet' deposits of TR-avidin onto ferulic acid layers also resulted in fluorescence as well as protein mass signals as expected (Fig. 4d). Here, the morphology of the sample, as documented in the fluorescence image, is different from that of Fig. 4c, because crystallization is a local event, whereas wetting of a dry deposit results in an entire droplet which crystallizes as a whole.

4. Discussion

The goal of the experiments described in this paper was to shed some light on the mechanisms of MALDI-MS particularly of biopolymers. No attempt was made to optimize practical analytical procedures in particular not for the analysis of synthetic polymers for which the 'dry' preparation method was originally developed and for which it is mostly used. The results obtained from pellet preparations can be understood in the light of previous experiences, particularly with 2,6-DHB which was conclusively shown to not incorporate proteins. For this matrix spectra of reasonable quality could only be obtained from microcrystalline preparations [6,22] and it was assumed then that if analytes are restricted to crystal surfaces, a high specific surface is needed for MALDI. There is all reason to assume that grinding/milling and pellet pressing will also restrict the protein to the grain surfaces, certainly for 2,6-DHB, but also for 2,5-DHB. The increased milling time decreases the average grain size and thereby increase the specific surface. An estimate of the accessible matrix crystal surface based on SEM micrographs shows an increase of roughly a factor of 10 between the non-milled matrix and the matrix after 1 min of milling and at least a factor of 50 after 7 min of milling. In this study, the upper mass limit for which spectra of reasonable quality could be obtained from pellets was that of cytochrome *c* at 12,360 Da, but under a somewhat modified sample preparation protocol signals even of bovine serum albumin have been obtained, albeit of inferior quality [15]. Metastable ion fragmentation is the main reason for an upper mass limit and the deterioration of the spectra quality with increasing mass. A similar upper mass limit for biopolymers and a concurrent deterioration of spectra quality has also been observed for standard microcrystalline 2,6-DHB preparations; this is obviously a general limitation for surface localization of analytes in comparison to preparations with an incorporation of the analyte into the matrix.

Expectedly, a pellet preparation in dry nitrogen atmosphere did not principally change the situation, strongly supporting the notion that even for 2,5-DHB grinding/milling under ambient humidity does not result in a significant protein incorporation into the matrix grains. 2,6-DHB is not water-soluble to begin with.

The initial velocity measurements of melittin in 2,5-DHB-pellets perfectly fit this picture. The value of 580 m s^{-1} for the wetted pellets is in good agreement with values reported by Glückmann and Karas for protonated insulin ions desorbed from 2,5-DHB standard (wet) dried droplet preparations [16]. It strongly suggests that the protein got incorporated into the matrix upon re-drying. The strong differences of the initial velocity for the dry versus the wetted pellets as well as all the other observations for pellets as discussed above render the assumption that high local pressures during grinding/milling lead to melting and protein incorporation highly unlikely. The values of 154 and 118 m s^{-1} for dry pellets are close to values reported for small neutral oligosaccharides [18] and lower by ca. a factor of two than initial velocities obtained for insulin from 2,6-DHB microcrystalline preparation. These low velocities together with the observed stronger metastable ion fragmentation confirm the notion that low initial velocities are associated with a loss of softness in the ion formation. Whether this correlation reflects differences in the desorption of the neutral analytes as a result of their location inside or at the surface of the matrix [16] or a different ionization process, e.g., in the gas phase as is assumed for the cationization of neutral oligosaccharides [23] or a combination of both is not clear at this point in time.

While the pellet experiments clearly demonstrate that mass signals of proteins can be obtained even if not incorporated into the matrix, they do not provide information about the state of the protein molecules at the surface and their interaction with the underlying matrix. Such information can, however, be obtained from the nano-electrospray samples. When the protein arrives at the surface as a dry particle, it gets physisorbed by a weak (van der Waals) interaction. As shown by the corresponding fluorescence images (Figs. 3b and 4b), a sizable lateral diffusion commences in these cases leading to an agglomeration of protein in well-defined islands, at least in case of the chosen matrix ferulic acid. In addition, the strong FITC-fluorescence signals from these islands (Fig. 3b) proves that there is no chemical interaction between the protein and the matrix bed. The fact that there is also no protein mass signal from such samples could be caused by either the lack of isolation of single protein molecules from each other, one of the properties commonly assigned to functional matrices, or by the lack of matrix support for the desorption/ionization. The loss of the FITC-fluorescence after wetting clearly proves a chemical interaction between the FITC-labeled protein and the matrix making the FITC aware of the low matrix pH of ca. 2.0. This is not too surprising because the fluorescence image of a Texas Red-labeled avidin in Fig. 4c exhibits a full dissolution and re-crystallization of the matrix with the accompanying protein incorporation. However, a complete resolution of the sample may not even be necessary to regain the protein mass signal of dry deposits. Anecdotal observations suggest that leaving 'dry' sprayed samples exposed to the ambient humidity for some time, may result in protein mass signals from such samples. In this case, a full protein incorporation into the matrix crystal is rather unlikely, and it is most probably, more appropriate to describe such samples by chemisorption of proteins on matrix surfaces, a situation which would also agree with the situation most probable for pellet preparations. In this most likely scenario, the main function of the matrix would indeed be a support of the desorption/ionization process rather than a simple isolation of the analytes and absorption of the laser energy. Such a chemical interaction of analyte molecules at the surface of matrix crystals has been suggested before for the analysis of

non-covalently bound complexes by the first shot phenomenon [26,27].

5. Conclusion

The experiments and their interpretation are a clear demonstration that 'dry' matrix preparations such as the milling and pellerizing can generate ions of rather large labile analytes such as proteins, however, with a somewhat compromised spectra quality. Such preparations are not restricted to the analysis of synthetic polymers, they were originally developed for. The experiments have also given strong support to the assumption that even for pellet and other "surface" preparations, the function of the matrix is similar to that for standard preparations. Whether this should still be called MALDI-MS or be separated out as a different method seems to be mostly a question of nomenclature. There obviously is a continuous transition from the very hard direct laser desorption (LDI) without any matrix all the way to the softest traditional MALDI-MS with wet preparations of incorporating matrices. As long as the matrix has a key function in the process besides absorption of the laser energy, it is, most probably, reasonable to call it MALDI.

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