Solvent-free MALDI-MS for the Analysis of Biological Samples via a Mini-Ball Mill Approach

Sarah Trimpin and Max L. Deinzer

Department of Chemistry, Oregon State University, Corvallis, Oregon, USA

An efficient, low sample load mini-ball mill (MBM) sample preparation procedure was developed for solvent-free MALDI analysis of peptides and proteins. Picomole sample amounts can be handled conveniently, with 30 s grinding times being sufficient. Matrix purity and molar analyte/matrix ratios are not as critical as with methods employing solvent. Ammonium salt is employed for protonation of the peptide and suppression of sodiation. This strategy allows for peptide mapping and other biochemical manipulations to be performed prior to MBM sample preparation and mass analysis. The analysis of bovine serum albumin (66 kDa) yielded good results, indicating that higher molecular weight proteins are accessible. A semi-solvent-free strategy by the MBM sample preparation method is also described. (J Am Soc Mass Spectrom 2005, 16, 542–547) © 2005 American Society for Mass Spectrometry

Methods that avoid the use of solvent during preparation of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) samples for analyzing polyamide, <5 kDa [1], and poly(ethylene glycol), 6 kDa [2], have been reported. A method involving mechanical mixing of sample and matrix by ball mill, followed by transfer of the resulting powder to the MALDI-target, and direct MALDI-MS measurement have been thoroughly investigated [3–7]. The procedure is generally referred to as “solvent-free MALDI-MS”. It was shown that this MALDI method permits a true matrix-assistance [5]. No limits on the molecular weight (MW) of the analyte [5], the solubility [3, 6, 7] or the compatibility between the polarities of analyte and matrix [7] have been reported. The ball mill procedure [5, 7] exhibited excellent homogeneity and better efficiency as a result of automatic pulverization and mixing. Three different approaches for avoiding solvent in the sample preparation seem to vary in methodology only slightly, but differences in the resulting data can be significant. Solvent-free MALDI-MS is believed to be the most promising approach [8, 9].

Surprisingly, solvent-free MALDI-MS often yields better spectra than solvent-based MALDI-MS, particularly for synthetic polymers, and it typically requires less laser power for successful analysis of the analyte [5, 7]. Advantages are that the analyte segregation phe-

nomena are overcome, the method is facile and reduces the time-consuming optimization of the three-component matrix/solvent/analyte system, fewer sample/matrix combinations need to be considered, fewer problems arising from trace impurities from binary solvents are produced, it is more universal since insoluble analytes are accessible, and the reliability is greater since suppression effects are reduced [6, 7, 10]. The potential for quantitation, high throughput, and automation of MALDI-MS is also enhanced [7]. Only a few reports [4, 5, 10–13] describe solvent-free MALDI-MS for the analysis of proteins and peptides. In this study, we developed a universal mini-ball mill (MBM) method to prepare biological target samples for solvent-free MALDI-MS.

Experimental

Materials

α -Cyanohydroxycinnamic acid (HCCA), 2,5-dihydroxybenzoic acid (DHB), dithranol, diammonium citrate, platelet factor 1, cytochrome *c* 2, and bovine serum albumin (BSA) 3, were obtained from Aldrich (St. Louis, MO) and used without further purification.

Instrumentation

MALDI was performed on an ABI4700-TOF/TOF mass spectrometer with an accelerating voltage of 20 kV. Spectra were acquired in the reflector mode using external calibration standards, except analytes 2 and 3,

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Address reprint requests to Dr. M. L. Deinzer, Department of Chemistry, Oregon State University, ALS 1047, 153 Gilbert Hall, Corvallis, OR 97331, USA. E-mail: max.deinzer@oregonstate.edu

which were measured in the linear mode. Experimental conditions (e.g., laser power) were kept constant to insure relevant comparisons within specific sets of experiments (e.g., grinding time E-7A to E-7E).

Solvent-Free MALDI-MS Based on the Mini-Ball Mill (MBM) Method

A general protocol was developed by adapting existing grinding procedures [5, 8] in order to downscale the method for biologically-relevant analyte amounts (Table 1). The sample/matrix-ratio was varied, as were other experimental conditions which are depicted by numbers E-1 to E-10 (Table 1). Mixing of the analyte and matrix powders was performed using a mini-bead-beater (Biospec Products), glass or zirconia/silica beads (0.5 mm, 1.0 mm sizes: Biospec Products, Bartlesville, OK), and plastic tubes (0.2 mL PCR-tubes; Molecular Bioproducts, San Diego, CA). More detailed information to the novel MBM procedure is available in the Supplementary Material section. The remaining sample mixture can be stored in the tube if desired.

Results and Discussion

Bead Sizes and Materials

Under identical experimental conditions, but with different bead material or size (E-1, more spectra in Supplementary Material section.), very similar high quality MS (Figure 1b and c) and MS/MS spectra (Figure 1e) of 1 were acquired using the MBM method. Minor differences were found; the spectra obtained when employing silica beads showed slightly increased $[M + Na]^+$ signals as observed previously [10] using a solvent-free method. After four months, the premixed samples were reinvestigated by MALDI-MS. Again, no differences were observed in the quality of the MS and MS/MS spectra. The effect of mixing analyte and matrix in the absence of beads was also examined (E-1G). The MALDI analysis of this sample yielded a spectrum with equal quality to the ones in which beads were included in the grinding step. In the spectra, the signal intensities for the parent ion are slightly higher in the case of the “no beads” (Figure 1a) experiment than that with the “larger glass beads (1 mm)” (Figure 1c). However, the signal intensity with the “smaller glass beads (0.5 mm)” (Figure 1b) was significantly higher than the other two. The tendency for protonation and sodiation were about the same in all three cases (Figure 1a–c). The MS/MS results were very similar in the “no beads” (Figure 1d) and “smaller glass beads” (Figure 1e) experiments. The effect of the grinding procedure has already been described and again it was found to be minimal [5]. Laser desorption-MS (E-1H) did not yield a spectrum of 1, thus indicating that a true matrix-assistance must be operative in homogenized but unground samples (E-1G). This showed us that the beads, and therefore the grinding, are not necessarily mandatory to achieve

homogeneity sufficient for a MALDI effect. Any method that brings about analyte/matrix homogeneity seems to be sufficient. The results of this particular experiment are rationalized on the basis that the powders of analyte and matrix were sufficiently fine to achieve adequate homogenization for matrix-assistance without the grinding step. In a previous contribution, micron-size grains prepared with the ball mill treatment were found to be sufficient to obtain matrix-assisted spectra [5]. In this contribution, it is shown that even bigger analytes and matrix grain sizes successfully yield matrix-assisted spectra. We postulate that the grain sizes, thus, reduced crystallinity [11] as well as the intimate contact between analyte and matrix [11], are the ultimate determinant for the MALDI effect. Hence, the result of the bead-free mixing should not be generalized, but interpreted in terms of a sufficient MALDI effect having been achieved in this particular case, and presumably in similar cases of sufficiently fine powders. Thus, beads should be used in all cases where the powders are coarser, which is most often the case. Nevertheless, the previous uncertainty [5] of possible effects caused by mechanical mixing by the ball mill, such as changes in morphology of analyte and matrix that might aid matrix-assistance, can most likely be excluded by this result. Overall, the novel MBM approach is effective and allowed us to apply lower sample amounts than described in the literature utilizing solvent-free MALDI-MS.

Matrices, Evaporation Procedures, Salts

It is well-known, though the underlying reasons are not understood, that the matrix has a large impact on the quality of the spectra. Often, matrices even of sufficient purity do not work well and need to be recrystallized before they can be employed as a good matrix in solvent-based MALDI-MS. It has been described in the literature that the choice of matrix is less restrictive with solvent-free MALDI-MS [7], and that even disfavored analyte/matrix combinations can be applied. We investigated (E-2) several different HCCA samples that worked to varying degrees in solvent-based MALDI-MS. Hence, the HCCA matrix batch considered being of good quality produced reproducible spectra whereas the HCCA matrices considered being of mediocre or of poor quality again gave reproducible results, but poor quality spectra. Interestingly, the “best” matrix was 12 years old, was yellow in color, and had a purity of only 97% but it had the smallest grain size, which appears to be the determining factor for obtaining high quality reproducible spectra. The MBM approach did not show any difference in the quality of the spectra obtained of 1 regardless of the HCCA quality described above. Hence, it is concluded that solvent-free MALDI-MS is less easily influenced by matrix batches that might perform poorly in solvent-based experiments. The reason for the solvent-free approach being less sensi-

Table 1. Experimental conditions for solvent-less MALDI analysis of analytes (1) peptide platelet factor; (2) Cytochrome c; (3) BSA using varied amounts of matrices, HCCA, DHB, dithranol, and diammonium citrate addend under different grinding times (0.5–10 min.) and drying procedure

Exp.	Analyte; amount	Matrix; amount	Salt ¹ amount	Grinding details: bead material and size; grinding time	Drying details
E-1	1; 50 μ g	HCCA; 13 mg	–	Glass: E-1A) 1 mm; E-1B) 0.5 mm; E-1C) ² ; 5 min.	–
E-1H	1; 1 μ L ⁴			Silica: E-1D) 1 mm; E-1E) 0.5 mm; E-1F) ³ ; 5 min.	–
E-2	1; 50 μ g	HCCA; 13 mg; E-2A); E-2B); E-2C) ⁵	–	E-1G) No beads; 5 min.	directly on plate
E-3	1; 10 μ L ⁴	HCCA; 13 mg	13 mg E-4A) to E-1A; E-4B) to E-1B; E-4C) to E-1C; E-4D) to E-1F	2; 5 min.	–
E-4	–	–	–	E-3A) ⁶ ; E-3B) ⁷ ; 5 min. 2; 5 min.	E-3A) ⁶ E-3B) ⁷
E-5	1 ⁴ ; E-5A) 10 μ L; E-5B) 1 μ L; E-5C) 0.1 μ L 1 ⁸ ; E-5D) 10 μ L; E-5E) 1 μ L; E-5F) 0.1 μ L 1 ⁴ ; E-5G) 10 μ L; E-5H) 1 μ L; E-5I) 0.1 μ L 1 ⁸ ; E-5J) 10 μ L; E-5K) 1 μ L; E-5L) 0.1 μ L 1 ⁴ ; E-6A) 10 μ L; E-6B) 1 μ L; E-6C) 0.5 μ L; E-6D) 1 μ L; E-6E) 0.1 μ L; 1 ⁹ E-6F) 10 μ L 1; 0.5 μ L ⁴	HCCA; 13 mg HCCA; 1.3 mg HCCA; 13 mg HCCA; 6.5 mg HCCA; 6.5 mg	–	2; 5 min.	6
E-6	–	–	–	2; 5 min.	10
E-7	–	–	–	2; E-7A) 0.5 min.; E-7B) + 0.5 min. of E-6A; E-7C) + 1 min. of E-6B; E-7D) + 3 min. of E-6C; E-7E) + 5 min. of E-6D. 2; 5 min. 2; 5 min. 2; 5 min. 2; 5 min.	6
E-8	2 ¹¹ ; E-8A) 10 μ L; E-8B) 1 μ L; E-8C) 0.1 μ L 2 ¹¹ ; E-8G) 10 μ L; E-8H) 1 μ L; E-8I) 0.1 μ L 2; 100 μ g	HCCA; 13 mg HCCA; 1.3 mg HCCA; E-9A) 13 mg; E-9C) 1.3 mg	–	–	6
E-9	–	–	E-9B) 13 mg to E-9A; E-9D) 1.3 mg to E-9C.	–	6
	2; 100 μ g 2; 100 μ g	HCCA; E-9E) 1.3 mg	–	2; 5 min. 2; 5 min.	6
E-10	3; 100 μ g	E-9F) DHB; E-9G) Dithranol; 13 mg HCCA; 1.3 mg	E-10A) none; E-10B) 1.3 mg to E-10A	2; 5 min. 2; 5 min.	6

¹ dl ammonium citrate;² glass bead mixture of 1 and 0.5 mm.³ silica bead mixture of 1 and 0.5 mm.⁴ 2 μ g μ L⁻¹.⁵ various matrix qualities.⁶ aliquot diluted by 100 μ L MilliQ, shock frozen (liquid N₂), dried (speed vac) (A,B,C).⁷ directly dried (speed vac).⁸ 2 ng μ L⁻¹.⁹ 20 ng μ L⁻¹.¹⁰ analyte solution directly added to the matrix, then ground.¹¹ 9.4 μ g μ L⁻¹ of 2, trypsin, 37°C over night.¹² diluted in 100 μ L 1% TFA than shock frozen as ⁶.

Figure 1. MBM MALDI analysis of **1** (m/z 1573); (i) (E-1A,B,C): MS spectra (a) no beads; (b) small glass beads; (c) larger glass beads; MS/MS spectra (d) no beads, (e) smaller glass beads.; (ii) 1.3 mg HCCA, 2 μ g of **1** (E-5H): (f) MS; (g) MS/MS spectrum.

tive to matrix quality can only be speculated at this point. However, it might be attributable to the grain being of a size that permits good contact between sample and matrix that is more easily obtained through external shaking and grinding than when solvent is employed. The fact that matrix quality seems to be unimportant with the dry procedure is a clear advantage over solvent-based protocols.

Analytes of biologically-relevant samples were prepared by different evaporation procedures (Table 1) of the solvent prior to MBM treatment (E-3). The solvent-free MALDI-MS and MS/MS spectra obtained using the MBM approach gave high quality results in all cases. The most intense signal was $[M + H]^+$; the $[M + Na]^+$ signal is noticeable but not very intense. In fact, the $[M + Na]^+$ signal (spectra in Supplementary Material) was much less intense in E-3 than it was in E-1, in which no solvent at all was employed. This observation is peculiar since the only real difference is that in E-3, analyte **1** was dissolved and dried prior to analysis. There is no explanation for the reduced availability of the sodiated adduct that reflects itself in the signal ratio with the protonated species in the spectrum. Overall, under the conditions and amounts applied, the impact of the drying procedure appears not to be particularly relevant, but it is presumed to become increasingly important with lower sample amounts. This is in comparison with solvent-based methods where complete dryness is

avoided in order to prevent sample loss to the walls of the tube.

In solvent-based methods, higher amounts of metal salts sometimes lower the quality of the spectra. Molecules forming $[M + Na]^+$ during ionization may complicate the interpretation of the spectrum. This is of particular interest since $[M + Na]^+$ signals were observed in some of the aforementioned experiments. Adding salts to synthetic polymers utilizing solvent-based or solvent-free MALDI-MS is often required for metal adduction of the analyte, so the molecule of interest can be detected by MS. It was of interest to determine (E-4, spectrum in Supplementary Material) if a positive effect can be introduced when a potential proton-donating salt, such as ammonium, is added to the sample, that is if the $[M + H]^+$ signal is detected rather than the $[M + Na]^+$ signal. In each case, $[M + Na]^+$ was suppressed in favor of $[M + H]^+$. However, the overall intensity of $[M + H]^+$ and the $[M + Na]^+$ signals decreased compared with samples with no added ammonium salt. MALDI-MS/MS spectra were conveniently obtained, too; the selected $[M + H]^+$ was observed with less intensity than the $[M + Na]^+$ signal, however, the latter did not contribute a high amount of fragment ions and therefore did not interfere with the data interpretation. Overall, it can be concluded that suppression of the undesired $[M + Na]^+$ signal can be introduced by adding ammonium salt to the sample

mixture as seen with solvent-based methods. Fine tuning of appropriate sample/matrix/salt conditions might allow for optimizing the result.

Sample Amounts, Semi-Solvent-Free, Grinding Times

These investigations were prompted by the recent report of Hanton and Parees [8], that showed equivalent results can be obtained by vortexing (grinding) synthetic polymers, matrices, and metal salts with metal balls in glass containers. The amounts used (about 1 mg analyte, 100 mg matrix) are far above that typically available for analysis of biological samples. The minimum sample amount for MBM that could be conveniently analyzed was investigated herein. Different amounts of **1** were used (E-5A to F, spectra in supplemental); it was found that 200 ng (127 pmol) could be dried in a PCR-tube to which matrix is added and processed with MBM. With 12.7 pmol, the signal intensity of **1** was poor in the MS spectrum and a MS/MS spectrum could not be acquired, but by reducing the matrix amount by a factor of ten (E-5G to L; more spectra in supplemental), MS/MS spectra of 20 ng (12.7 pmol) of **1** could be obtained. It was observed that for any given analyte amount the MS spectra showed strong $[M + H]^+$ signal intensities but also $[M + Na]^+$ signals (Figure 1f). Furthermore, the MS/MS spectra generally gave stronger absolute intensities of the parent ion, in the form of $[M + H]^+$ and $[M + Na]^+$ as well as the fragment ions (Figure 1g), which are similar to the results observed in the E-4 experiment. Due to the small mass difference (22 Da) between $[M + H]^+$ and $[M + Na]^+$, the unresolved sodiated parent ion was thought to contribute to a certain degree to the fragmentation in the MS/MS experiments. Hence, it was of interest to investigate the possible fragment ions arising only from the sodiated parent ion. Fragment ion contributions were not observed from the sodiated species. This result can be rationalized on the basis that metal adduction to peptides stabilizes the analyte, thereby reducing the fragmentation [4, 12]. Overall, the results from these experiments demonstrate that there is a large range of analyte/matrix ratios that is accessible by this method. They also confirm the putative intrinsic limitations of MALDI investigations that there is no direct correlation between analyte concentration and signal intensity. The low-pmol sample amount range is adequate for a more general use of solvent-free MALDI for the analysis of peptides.

A semi-solvent-free method was also investigated; various different ratios of analyte/matrix/solvent were considered (E-6). In this approach, the dissolved analyte was added directly to the matrix and the MBM protocol applied for homogenization. When the matrix/solvent ratio was such that all solvent was absorbed by the matrix, good MS results were obtained for **1**. This approach could,

in principle, minimize sample loss and/or sample preparation time, since one sample preparation step is avoided.

The effects of different grinding periods were investigated. To maintain identical conditions within this experiment, the same sample was reground after a few grains were removed for MALDI analysis at each time point (E-7, spectra in supplemental). Assuming sufficient homogeneity was acquired after 30 s and that removal of a few grains of sample did not affect the molar ratios, this approach seemed best to insure the same conditions when used for comparing samples of interest. Poor quality spectra resulted after 10 min of grinding, while intense $[M + Na]^+$ and low $[M + H]^+$ signals resulted after 30 s of grinding. After 1 or 2 min good results were obtained, and after 5 min the best results were obtained and unequivocal characterization of **1** was possible. It is clear that exchange between H^+ and Na^+ takes place with increased grinding time favoring the production of $[M + Na]^+$. Hence, the appropriate grinding period appears to be between 1 and 5 min; a time frame that would allow two separate samples to be processed by the MBM method. This is reasonable and is competitive with solvent-based procedures if washing and desalting steps are taken into account.

Peptide Mapping Investigations

Wang and Fitzgerald [10] showed the advantage of significantly reducing the signal suppression effects in MALDI for multicomponent systems by applying their solvent-free method on purified model peptide mixtures. In these investigations (E-8, spectra in supplemental), the application to biologically-relevant procedures, such as enzymatic digests prior to solvent-free MBM treatment, is presented for the first time. The MBM approach allowed detection of the tryptic peptides of **2** in the low-pmol range [E-8I], and excellent MS and MS/MS signals, from the m/z 964.6 selected ion, were obtained in the medium- to high-pmol range [E-8G,H].

Molecular Weight Range

The solvent-free method was used to analyze high MW polymers giving at least equal quality mass spectra up to 100 kDa [5]. The solvent-free mortar and pestle approach showed major limitations in the high MW range of proteins as it proved to be unsuccessful at characterizing BSA [10]. However, in other work the ball mill approach was used successfully to analyze BSA [12]. In principle, the MBM method should yield the same results as the ball mill approach. The focus of this contribution is the evaluation of the MBM method for the analysis of biomacromolecules. Cytochrome *c*, **2**, previously investigated by solvent-free MALDI-MS [11, 13], is a small protein and thus, useful for optimization experiments (E-9, spectra in supplemental). The signal intensity is greater and less broad without the addi-

tion of a proton-donating salt. Lowering the matrix amount by one tenth (E-9C) improved the quality of the spectra. Employing TFA in the drying process doubled the intensity of the signal for **2** (E-9D), however the signal width also increased. DHB and dithranol (E-9F, G) gave spectra of **2**, but the signal was broad in both cases; therefore, the addition of ammonium salt decreased the quality of the spectrum.

In E-10 (spectra in Supplementary Material) we determined a m/z 66,746 Da (E-10A) for **3** (the theoretical MW 66,431 Da) using the MBM method. The signal was broad but the addition of proton-donating salt slightly narrowed the signal width giving m/z 66,442 Da (E-10B). It is conceivable that even larger proteins can be analyzed with this method.

Conclusions

A solvent-free MALDI-MS method was developed that meets important criteria for the universal analysis of biological samples. In some respects, the method appears to be an improvement over standard solvent-based MALDI procedures for analyzing biological samples. The results obtained from adding ammonium salt were a mixed blessing, since although the spectra of the peptide and protein digests were improved because of the reduction of sodiated species, adduct formation, which we speculate to be multiple matrix adductions to the analyte, appeared to result in broadened signals of a lower MW protein, i.e., cytochrome *c*. However, with this procedure the characterization of higher MW proteins, i.e., BSA was improved slightly with respect to signal width and the accuracy of the mass measurement. The application of MBM should improve the data quality in the analysis of proteins and peptides when the use of solvents creates difficulties as, e.g., hydrophobic and solubility-restricted peptides or proteins and transmembrane proteins, and where suppression effects [10] are a problem. The solvent-free, and to some extent semi-solvent-free, MBM MALDI approaches hold great promise for the analysis of biological reactions that involve peptide mapping and/or H/D-ratio measurements from exchange experiments,

where proton back exchange leads to insufficient retention of label to insure accurate measurements.

Supplementary Material

Details to MBM sample preparation procedure, Figures: MS and MS/MS data of E-1, 5, 7, 9, 10 are available with the electronic version of this article.

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