6.5 EVIDENCE OF POOR MALDI SAMPLE PREPARATION

As described above, successful MALDI sample preparation involves creating intimate contact between the matrix (which may consist of a few different molecules) and the analyte. The experiment requires each of the matrix roles to be completed to obtain a usable mass spectrum. To demonstrate the importance of the matrix roles, Figures 6.7 and 6.8 show two examples where a specific role of the matrix was not fulfilled.

Example 1: Figure 6.7 shows a problem with the role of desorber. Here a PEG 1000 sample is prepared for analysis using DHB (panel a) and the potassium salt of DHB (KDHB) in panel b as the matrix. In panel a, strong PEG peaks cationized with Na\(^+\) are observed (by adventitious Na\(^+\) from the matrix). While ample cation is present in the sample shown in panel b, the KDHB is ineffective at desorbing the PEG analyte from the MALDI sample when illuminated with the laser beam.

Example 2: Figure 6.8 shows a problem with the role of ionization agent. Here a PS 5050 sample is prepared for analysis using IAA as a matrix both without (panel a) and with (panel b) the addition of AgTFA as a cationization reagent. Note that as both the proton and the alkali ion affinity of PS is low, only Ag\(^+\) cationized peaks are observed in panel b. When no cationization agent is present in the sample, no sample related peaks are observed. Interestingly, in the case whose data are shown

![MALDI mass spectra](image)

Figure 6.7. MALDI mass spectra of PMMA 2900 obtained using (a) DHB and (b) KDHB as the matrix. The analyte peaks observed in panel a are cationized with adventitious sodium.
in panel a, the PS analyte is desorbed from the matrix into the gas-phase; the absence of an appropriate cationization agent allows the preparation of isolated neutral gas-phase molecules of high-molecular-mass analytes for analysis using other techniques.

The heterogeneous incorporation of analyte within the solid matrix preparation, which is characteristic of using the dried droplet method, often results in a wide range of analyte concentrations throughout the MALDI sample. In view of the M/A plots discussed above, it is expected that the analyte signal will therefore also be variable as the laser beam is rastered around the sample. Those positions that yield high analyte signal intensities have been termed “sweet spots.” In fact, the simple observation of “sweet spots” on the sample (i.e., implying highly variable ion signal intensities as a function of spatial position) is evidence of a poor sample preparation. Further evidence of a poor preparation is given by the observation of sample discrimination effects. However, in order to uncover these discrimination effects, the true composition of the sample must be known either by preparation or from an alternate analytical analysis. These discrimination effects fall into two categories, molar mass discrimination and structural discrimination.

For polymer samples that cover a broad mass range (i.e., they have a large polydispersity), molar mass discrimination is evidenced by a bias in the observed MALDI spectrum. In most cases, the bias is to low mass; that is, the low-mass oligomers are overrepresented in the observed mass spectrum. This type of discrimination is most likely due to sample preparation issues, in many cases the solubility of the oligomers varies significantly as a function of mass. This was demonstrated for low (<5000 Da) polyethylene terephthalate (PET) samples analyzed using THF (a poor solvent which dissolves only the lower-mass oligomers)
and a 70:30 (v/v) methylene chloride (CH₂Cl₂): hexafluorisopropanol (HFIP) azeotropic [58]. As described above regarding the importance of M/A ratio, it is also possible that different molecular mass species require different M/A ratios for analysis. Molar mass discrimination may also be due to ionization effects; higher molecular mass species are usually ionized more efficiently than lower molecular mass species. Overall, polymer samples with PD > 1.2 are found to exhibit molar mass discrimination. The Montaudo group [59, 60] has demonstrated that this problem may be overcome by preseparation of the polymer sample using gel permeation chromatography. MALDI analysis is then performed on each of the collected fractions, and the data are combined to give a full mass spectrum. It should not be forgotten, however, that TOFMS instruments have a severely restricted dynamic range (the high-speed digitizers used usually have an intensity axis resolution of 256). To accurately reproduce the entire molar mass distribution, the required dynamic range is generally much higher [61].

In addition to molar mass discrimination, structural discrimination may also be observed in MALDI samples. Structural discrimination can result from differences in the solubility of different components in the solid state (e.g., due to the presence of hydrophilic versus hydrophobic end groups), or from differences in ionization of the components of the mixture. This is particularly important in sample mixtures containing different end groups, as an important result of the analysis may be the quantitative determination of the quantity of each end group present in the sample. “Simple” polymer blends can be some of the hardest samples to analyze via MALDI. It may be possible to prepare the sample using one matrix, cationization agent, or set of additives that will produce signal from one component of the mixture, and use a different sample preparation to view the second component (remember the PEG/PTMEG blend example given above). While this may be useful when looking for small changes in low-concentration contaminants that affect the sample properties, it can be an analytical nightmare for determining the composition of even the major components of true unknowns. Without extensive sample knowledge, the intensity of a particular polymer analyte series in the observed mass spectrum cannot be used directly for quantitative analysis.

### 6.6 A DETAILED EXAMPLE

To illustrate the value of understanding the underlying principles of the matrix roles and the key issues involved in developing new sample preparation methods for polymer MALDI, we provide the following example of a new sample preparation method that was developed in a few minutes for a novel polymer material [18]. In collaboration with Prof. Li Jia of Lehigh University, we developed a MALDI sample preparation method to characterize the chemical structures of novel materials resulting from the copolymerization of N-allylaziridines and carbon monoxide [62]. One example of this type of material is the copolymerization of N-ethylaziridine with CO. The resulting polymer was soluble in water and water/methanol mixtures, and partially soluble in methanol. This solubility most closely matches that of poly(ethynylformamide) PEF 1500 in Figure 6.3. Using Figure 6.3, PEF 1500 is in
the most hydrophilic group, which suggests that either thiourea (TU) or DHB would be the best first choice for a matrix. Using a sample preparation of methanol, DHB, and dried droplet deposition, we acquired the mass spectrum shown in Figure 6.9. The mass spectrum shows the sodium cationized repeat units and end groups expected for this polymer. This is an example of the analysis of a new polymeric material that to our knowledge had never been analyzed previously by MALDI. In a matter of a few minutes, a sample preparation method was developed using the principles described above.

6.7 CONCLUSION

Sample preparation is the key to a successful MALDI experiment. Even before work at the bench begins, a large number of decisions must be made, the goal of which is to produce a homogeneous solid sample solution. One of the key concepts is the incorporation of the analyte into the chosen matrix solid. Heterogeneous incorporation results in the observation of large spot-to-spot variability in the analyte signal, which generally leads experimenters to search for the “sweet spots” on the sample surface. Invoking the concepts of solubility or miscibility of the analyte in the solid matrix enables an organized means of searching for the proper matrix to use for a given analyte. It also is seen to explain many of the practices found in the literature (the use of matrix additives, the application of heat, vacuum, and fast-drying solvents, etc.) suggested as a means of empirically producing better MALDI samples. In fact, anything that affects the rate of precipitation will have an effect on the distribution of analyte, cationization agent, and other impurities in the solid sample.
As described in this chapter, the chosen “matrix” must fulfill a number of distinct roles (dispersant, desorber, ionization agent) in the MALDI analysis. In our experience, dispersion, desorption, and ionization are separate processes that can generally be optimized independently. Most importantly, each of these roles must be fulfilled in order to result in a successful MALDI experiment. While many researchers search for a single molecule that may be a good matrix for a given class of analytes, it is possible that a mixture of chemical compounds may prove more effective if each component of the mixture is selected to optimize its role in the MALDI process.

As development of the MALDI technique continues, we have seen it grow from a qualitative (“what is there?”) to a quantitative (“how much is there?”) analysis tool. The next development, which is generally occurring now, is to answer the question “how well do we know how much is there?” This can be done by statistically analyzing replicate experiments, the judicious addition of internal standards, and the application of standard additions techniques and factorial design experiments. Careful control of the sample preparation, coupled with the use of techniques other than MALDI to study the prepared MALDI samples, leads to better understanding of the MALDI process, which ultimately leads to greater success in the analysis of real-world samples.

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CHAPTER 7

SOLVENT-FREE MALDI SAMPLE PREPARATION

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7.1 INTRODUCTION AND SCOPE

Analytical techniques ideally aim for a maximum degree of unbiased analytical information. Mass spectrometry (MS) should be capable of characterizing any synthetic polymer qualitatively and quantitatively. In reality, of course, this is not the case; however, simplification can certainly make techniques more efficient and often more reliable [1]. Such is the case with solvent-free matrix-assisted laser desorption/ionization mass spectrometry (MALDI) MS which continues to gain in analytical importance [2]. The major breakthrough of the solvent-free MALDI sample preparation method came with the first successful analysis of an insoluble poly(9,9-diphenyl-2,7-fluorene) (polyfluorene) (Figure 7.1) [3]. Recent developmental improvements to the original solvent-free MALDI method were made through industrial and governmental collaborations [4, 5, 6] or directly by industrial laboratories [7], and indicates a general interest in improving the MALDI method for analyzing synthetic polymers. The ability to simultaneously and precisely prepare over 100 synthetic polymer samples by a solvent-free on-target homogenization/transfer MALDI method [4] offers the powerful opportunity for decongestion of solubility-restricted and even insoluble complex mixtures by coupling to powerful solvent-based separation utilizing liquid chromatography (LC) (see Chapter 11) [6] and/or solvent-free gas phase separation utilizing ion mobility spectrometry (IMS)-MS [8, 9]. These developments provide a likely optimistic and productive future for the field of MALDI-MS.

This chapter will focus on different MALDI concepts, both past and present, all of which are currently valid approaches. It is left to the reader to employ the approach most appropriate to the individual needs and/or applications. The challenges associated with MALDI-MS will be addressed, including segregation due to crystallization effects (e.g., polydimethylsiloxane (PDMS)), unusual solvent-driven problems in highly compatible systems (e.g., polystyrene (PS)/1,8,9-anthracenetiol (dithranol)/tetrahydrofuran (THF)), desorption/ionization preferences in mixture
Figure 7.1. MALDI mass spectrum of the insoluble fraction of polyfluorene after Soxhlet extraction in toluene for 5 days; the sample was prepared with dithranol as matrix and BM for homogenization: (a) full scale, (b) inset of the three most intense signals, the signals at -69 Da to each of the expected oligomer ions were characterized by detailed post-source decay (PSD) studies to be fragment ions where one phenyl side chain is cleaved. This fragmentation also occurred already as in-source decay (ISD) when more laser power was employed, which was than additionally to the PSD fragment ion (-69 Da) present in the mass spectra as ISD fragment ion (-77 Da). Mass spectra were recorded using a Baiker Reflex II™ MALDI-TOF mass spectrometer (Bremen, Germany) equipped with a N$_2$-laser.

analysis (e.g., poly(para-phenylene vinylene) (PPV) versus poly(ethylene glycole) (PEG)), as well as high-throughput issues in an industrial setting. Qualitative and quantitative improvements of analytical results for these examples will be demonstrated from the implementation of the nontraditional solvent-free MALDI method for the analysis of synthetic polymers.

7.2 BACKGROUND AND MOTIVATION

MALDI-MS has been extensively applied to the characterization of soluble macro-molecules [1, 10, 11] and has even been used to detect intact soluble molecules with masses higher than 1,000,000Da [12, 13]. The potential of MALDI for the characterization of synthetic polymers was first demonstrated by Tanaka et al. [10]. The principles of MALDI-MS based on the traditional solvent-based method are described in Chapter 6. One of the central aspects of MALDI analysis is the preparation of a
“good” MALDI sample. It is not always as trivial as applying a matrix material and an analyte to the surface of the MALDI plate since it is the special analyte-matrix preorganization which makes the MALDI analysis successful. Many variables influence the integrity of a “good” MALDI sample and may include the concentration of the matrix and analyte, choice of solvent [14], matrix, cationization reagent, sample history, contaminants, and compatible solubilities of matrix and analyte solutions. Hence, in MALDI-MS, sample preparation is one of the key factors that greatly influences the success and the quality of the analytical result. Because there are too many contributing parameters in solvent-based sample preparation for MALDI-MS, there was a need for a more simplistic method that is easily applicable and has fewer critical parameters. One critical parameter is the solvent. There are three crucial aspects to “solvent.” First, the chosen solvent must solubilize the entire sample, which may not be an easy task due to varying differences in solubility throughout different parts of the sample. For example, strong adhesion, aggregation, and low solubility of some of the constituents may exist. Subsequently, the analysis of these molecules fails and leads to the erroneous conclusion that they were not present in the sample. The second crucial aspect to the “solvent” is that the solvent system used must initially homogenize a sample with matrix in the liquid MALDI phase. Lastly, the mixture on the MALDI plate must subsequently yield a locally homogenous sample and matrix composition upon evaporation of the solvent, which is ultimately important in achieving reproducibility. Any of these issues can introduce serious limitations to the success and reliability of the analytical results. However, the third aspect is the most complex and often responds poorly in optimization trials. To understand the generality of this problem (often referred to as segregation), one has to keep in mind that (re)-crystallization is commonly used as a purification step in synthetic chemistry. Any crystallization procedure involves separation phenomena of two or more compounds in differing degrees. Intrinsically, any solvent-based MALDI sample preparation suffers a lack of homogeneity caused by solvent evaporation during the crystallization step. For these various reasons, dry sample preparation strategies were introduced [15, 16, 17, IB] and established [19] as nontraditional methods for MALDI-MS for the analysis of synthetic polymers. The strength of the solvent-free approach is its capacity to yield reproducible analytical results for some insoluble and poorly soluble polymers (Figure 7.1) [3].

7.3 EXPERIMENTAL CONCEPTS

7.3.1 Original Concepts

In the year 2000, several laboratories independently published work that focused on the MALDI sample preparation of soluble [16] and solubility-limited synthetic polymers [15], insoluble large polyaromatic hydrocarbons (PAHs) [17], and fragmentation labile peptides [18] prepared in the absence of solvent. Solvent-free methods generally consist of two steps: “dry” homogenization, usually carried out by mechanical mixing of sample, matrix, and salt, and transfer of the resulting powder mixture to the MALDI plate. In solvent-free MALDI-MS, at no point is
solvent employed to homogenize analyte, matrix, and salt or in the transfer to the MALDI plate. In the case of a dissolved sample, the sample is dried prior to solvent-free preparation. Hence, homogenization and transfer of the sample in these approaches is not based on the solvent and its properties. The organic or aqueous medium the polymer was originally dissolved in is entirely irrelevant. The MALDI analysis is therefore simplified because fewer combinations and issues of compatibility or solubility have to be considered and explored.

Two general methods were originally employed for homogenization of polymeric material, MALDI matrix, and salt: grinding by mortar and pestle (about 5 min) [15, 19] and shaking by ball-mill (BM) (about 5-10 min) [3, 19, 20], the latter having the advantage in that the process is accomplished automatically. Both methods were applicable over a broad homogenization time period; however, it is not known what is the minimum or maximum period needed to yield satisfactory results. In its infancy, ball-milling was carried out in a metal vessel (tungsten carbide (WC)), into which the matrix and sample is added to be ground, along with a suitably sized ball that is preferably made of the same material as the vessel. Over time, it was shown that the vessel or ball material has no significant impact on performance, thus disposable aluminum vessels were custom-built, avoiding cleaning issues and troublesome carryover. Grinding takes place as a result of the interaction between the ball, sample particles, and grinding vessel wall. The technique works equally well on soft, medium-hard, and even extremely hard, oily, brittle, and fibrous materials. In fact, viscous material (PS, 100 kDa) exerted one of the greatest challenges yet, but was overcome by cooling the vessel during homogenization, which made the polymer more brittle and easier to mix [19]. The grinding ball is free to move around during the shaking process, which gives the technique the ability to produce much finer particles than other methods provide. Generally speaking, smaller balls and longer grinding times yield smaller particles.

Originally, transfer of the dry MALDI sample to the MALDI plate used either a loose powder or a pressed pellet method. In the case of the loose power method, the homogenized MALDI sample is transferred with a spatula [3, 4, 5, 7, 19, 20] or, as later shown, the wooden end of a Q-tip [4], with the latter avoiding undesired scratching of the plate. The prepared sample is gently pressed directly to the MALDI plate [3, 4, 7, 19, 20] or to a conducting carbon disc affixed to the MALDI plate [16] to produce a thin film. In the other approach, a pressed pellet (similar to the potassium bromide (KBr) pellets for infrared analysis) is prepared and attached to the MALDI plate with double-sided adhesive tape [15, 19], which has the intrinsic disadvantage of being more involved and time-consuming in preparation.

The important similarities and differences in the MALDI analytical results are that the different homogenization methods do not produce significantly different analytical results, at least for low-mass polymers; however, the transfer methods of the sample to the plate appear to be important [19]. The loose powder approach, in contrast to the pellet variant, allows the use of a large excess of matrix [3, 19], and permits true matrix-assistance, as evident by its capacity for high-molecular-weight analysis [19], which has not been observed in other solvent-free approaches as reported for example in Reference 21. Further advantages of the loose powder compared to the pellet method include improved sensitivity, higher-mass resolution, and
Figure 7.2. Solvent-free MALDI mass spectrum of PS in comparison with the solvent-based MALDI mass spectrum. With increasing matrix dilution (A 1:50, B 1:500, and C 1:5000) the signal-to-noise-ratio appears to be less affected using (II) the solvent-free method than (I) the solvent-based MALDI method. Mass spectra were recorded using a Bruker Reflex II™ MALDI-TOF mass spectrometer equipped with a N\textsubscript{2}-laser.

less restrictive molar analyte: matrix ratios \[19\]. Hence, only the loose powder method achieves results that are at least comparable to that of the traditional solvent-based MALDI method (Figure 7.2; low-molecular-weight PS). Overall, the original approaches for avoiding solvent in sample preparation seem to vary in methodology only slightly, but the differences in the resulting data are significant. Solvent-free loose powder MALDI \[19\], irrespective of the employed homogenization procedure, appears to be the most promising sample preparation method \[4, 7, 22, 23, 24\] to improve the reliability and the potential of MALDI analysis. For this reason, this method was evaluated systematically by comparison with solvent-based MALDI-MS \[19\]. Numerous experiments established solvent-free analysis as a complementary MALDI method, frequently providing valuable additional information and improving analytical results. Generally, the solvent-free method allows for more homogeneous analyte: matrix mixtures, as well as higher shot-to-shot and sample-to-sample reproducibility \[3, 4, 5, 6, 7, 15, 17, 19, 25, 26, 27\]. As a result, less laser power has to be applied, which yields milder MALDI conditions, as evidenced by reduced analyte fragmentation and lessened background signals, and provides better resolution of the analyte signals \[3, 5, 18, 19\].
7.3.2 Concept of Vortexing

A wider application of the solvent-free MALDI method for polymer analysis was delayed due to practical aspects of the initial methods. These may include characteristics such as being time-intensive, which is mainly due to intensive cleaning of the equipment to avoid carryover, particularly considering the mortar and pestle application, and availability, specifically in the case of the BM method. An efficient procedure was required to increase the meaningful impact of solvent-free MALDI-MS for the analysis of synthetic polymers. Modifications were made to the original solvent-free loose powder MALDI method which resulted in a simple method that utilizes a common laboratory vortex instrument, a pair of BBs (the type used in air rifles; Zn-plated, 4.5 mm), and common glass vials [7]. This refined solvent-free MALDI method has been found to be efficient, requiring a minimum amount of polymeric sample and preparation time (0.5-1 min). Disposable glass vials and metal balls are used, thereby avoiding carryover and cleaning procedures. Mixing of polymer sample and matrix powder is performed with a vortexer followed by direct measurement of the analyte: matrix: salt powder loosely applied to the MALDI target [7]. In these studies it has been found that this solvent-free method is applicable to liquid, soft, and waxy polymers as demonstrated in the example shown for low-molecular-weight PEG. We are not aware of the vortex method being applied to higher-molecular-weight polymer analysis (>10,000Da). The BM [19] and mini-ball-mill (MBM) methods [28] were successfully employed for high-molecular-weight analyses. This suggests that the quality of grinding gains in importance with increasing molecular weight of the polymer.

The vortex/BB technique made the workflow of the solvent-free and solvent-based MALDI methods comparable. If a sample is received in a MALDI-appropriate solution (in a vaporizable solvent), solvent-based MALDI can be used, and if the sample comes as a solid, solvent-free MALDI is preferred. However, solvent-free MALDI should be considered at all times since the analytical results of simple synthetic polymer standards generally improves with the implementation of the solvent-free method due to its lower laser threshold which provides less matrix background, better signal-to-noise ratio, less fragmentation, and fewer suppression effects, and overcomes artifacts related to the use of solvents [5, 19, 29]. Most importantly, if one deals with a problematic compound requiring a great deal of sample preparation and optimization, the solvent-free method, due to its simplicity in sample preparation by omission of the intractable solvent and possible oxidation/degradation of the analyte, clearly is the preferred method of choice.

Solvent-based MALDI analysis uses less material than solvent-free approaches (<0.1 mg of analyte). However, when optimization trials have to be performed, which are mainly related to solvent-based MALDI, then solvent-based methods may require more sample and be more time-intensive than the solvent-free MALDI method. In general, the sample amount requirement of ca. 0.1 mg employing solvent-free MALDI analysis is not a significant limitation because most polymeric samples are typically available in sufficient quantities. Biologically relevant amounts of analyte at the femtomole level [26] was recently achieved by utilizing the solvent-free MBM method by making use of smaller vials and beads [28],
Sufficient sample amounts may not be available for all synthetic polymers, as is the case, for example, with fractionated samples in gel-permeation chromatography (GPC) or LC-MALDI analysis. Additionally, high numbers of fractionated sample are generally produced in LC-MALDI which calls for the development of preparing multiple samples solvent-free simultaneously. Solvent-free sample preparation methods can even be simplified and the analytical utility increased beyond the vortex/BB method as is seen with the multi-sample on-target homogenization/transfer method [4] capable of effectively coupling LC to MALDI [6].

7.3.3 Concept of Multi-Sample Solvent-Free On-Target Homogenization/Transfer

Solvent-free MALDI-MS had been shown to simplify the sample preparation by removing the influence of solvent, which provides in cases where the solvent is problematic, considerable time efficiency. For samples in which the solvent is not problematic, the solvent-free method may be less efficient. The two major limitations to any of the previous solvent-free MALDI methods is that only one sample can be prepared at a time and the transfer of the sample from the homogenization vessel to the MALDI-plate is more time-demanding than when solvent-based MALDI methods are employed. In order to perform routine analysis precisely and accurately, and to acquire improved mass spectra faster than with solvent-based methods, it seemed reasonable to propose [5] an automated solvent-free sample preparation platform for high-throughput accurate mass determination (automated workstation and automated mass analysis) designed into a commercial MALDI-TOF mass spectrometer. Solvent-free sample preparation directly on the MALDI plate has also been proposed [7]. These first experiments of on-target solvent-free sample preparation for the MALDI analysis of synthetic polymers were obtained as follows [30]: matrix, salt, and polymer (not weighed) were added to the plate, and the direct pressure from the end of the spatula was used to “grind” the solid materials together. The resulting sample was a thin film of powder, similar to the samples prepared with the vortex method and applied to the target with a spatula. While the resulting mass spectra [7] were not of the same quality as the vortex method, they indicated that further simplifications of these solvent-free methods are feasible and show the potential to speed up the process by eliminating the transfer step.

To further simplify homogenization/transfer and to reduce multi-sample preparation time requirements, different methods were developed for solvent-free MALDI analysis of synthetic polymers that are not only simple but practical [4, 6]. First, the sample volume was reduced, thus increasing the number of samples that can be prepared [4] compared to the original one-sample vortex/BB method [7]. For this, a simple 96-sample holder was used with small metal beads and plastic vials identical to the ones used in the MBM method [25]. This setup homogenized up to 96 analyte and matrix samples simultaneously using simply a vortexer for only 1 min; however, 5 min considerably improved the quality of the mass spectra. The analytical results of the multiple-sample vortex solvent-free MALDI analysis were then essentially identical to the results of the single-sample vortex approach but greatly reduced the time necessary to individually homogenize each sample.
With this approach, each sample needed to be transferred to the MALDI target individually.

A second approach integrated sample transfer into the homogenization step of the previously described multi-sample preparation method [4]. The homogenization of analyte and matrix powder and salt is obtained using a TissueLyser® (QIAGEN) which is conventionally used for disruption of biological samples. A few grains of each synthetic polymer are combined in 2-mL plastic vials followed by the addition of either dithranol or 2,5-dihydroxybenzoic acid (DHB) matrices, then NaCl, and finally the metal beads (1.1 mm). Weighing of the powders or counting the beads was not employed in these experiments. The approximate amount of powders added were <1 mg (analyte) to ca. 10 mg (matrix) and the volume of the beads is roughly equal to the volume of the MALDI sample. Eight uncapped vials were placed in each adapter set sample holder and each set was capped by a MALDI plate followed by the adapter container lid. Each set was placed in one of the two TissueLyser arms and shaken for 2 min at which time the sample is homogenized and transferred to the MALDI plate simultaneously. The MALDI plates with transferred analyte/matrix/salt were dusted off in a fume hood with difluoroethane gas to remove excess powder. This approach in which analyte/matrix homogenization and transfer occur simultaneously greatly simplifies preparation of multiple MALDI samples and provides considerable time-saving in addition to reducing the amount of analyte consumed for sample preparation. The disadvantage of this approach is that it relies on a cost-intensive homogenization devise.

A third approach using a common vortex device was developed which essentially combines the concepts of the two previous methods allowing for on-target homogenization/transfer of multiple samples simultaneously. This approach was first established with 36 samples and then adapted and increased to 100 samples per MALDI plate (Figures 7.3 and 7.4) [4]. Thirty-six wells (organized 6 by 6) in a bacti plate (Nunc, Roskilde, Denmark) exactly fit a PerSeptives Biosystems (Framingham, MA) MALDI plate. Each polymer was transferred either as powder or in solution to each of the wells with addition of 3-10 metal balls (1 mm). If organic solvent was used, it was evaporated at room temperature or at 37 °C in the oven, drying the entire set of samples at the same time. Premixed matrix: salt powder was used according to Reference 5 and was added to the dried analyte. The set was capped by a MALDI-plate, which was tightly affixed with tape and vibrated by the vortexer for ca. 5 min, at which time each sample had been automatically transferred to the MALDI plate. The time to load the wells, homogenize the analyte: matrix: salt with simultaneous transfer to the MALDI target is under 1 min per sample [4]. This method was adapted to 100 samples (organized 10 by 10) [6] and more (unpublished results) [6]; the custom-made adapter set (Figure 7.3a) gives spot sizes of about 1 mm diameter (Figure 7.3b). The mass spectra are of good to excellent quality as exemplified for polymethylmethacrylate (PMMA) 5 kDa using either DHB (Figure 7.4a) or dithranol (Figure 7.4c), each of which were premixed with sodium trifluoroacetate. Carryover was not observed as evaluated by the blank mass spectrum of the target spot with only matrix (DHB) and sodium salt (Figure 7.4b) between the spots containing polymer (Figure 7.4a,c).

All three methods are efficient in that small sample amounts are required; the sample: matrix: salt ratio is not critical, so that there was no need of weighing, and
the time necessary to achieve sufficient homogenization of multi-samples is about 5 min. These methods work well and are reliable for synthetic polymers in a mass range between ~500Da (PEG 600) and ~10,000Da (PS 13,100). The simultaneous multi-sample approach for direct on-target MALDI sample preparation standardizes and automates much of the labor-intensive work to prepare a MALDI plate, suggesting that high-throughput solvent-free MALDI-MS analysis of low-molecular-weight polymers is possible. The method can also be used as a rapid means of optimizing conditions such as matrix choice, which can be especially important for difficult samples.

7.4 THEORETICAL CONCEPTS

7.4.1 Derived from the Pressed Pellet MALDI Method

Investigations of the theoretical aspects of the MALDI processes have also been pursued based on solvent-free MALDI methods. The pressed pellet method usually employs a low and restricted matrix-to-analyte ratio as recognized for both synthetic
Figure 7.4. Solvent-free MALDI mass spectrum of PMMA 5 kDa. Mass spectra were of good quality and reproducibility using the 100-sample on-target homogenization/transfer solvent-free MALDI method (vortex device) using either DHB (a) or dithranol (c). The mass spectra (b) from the spot (only DHB matrix/sodium trifluor acetate salt employed) in between the two samples reveals that even though this setup is not as a tidily closed system as compared to the TissueLyser® approach, there is no cross-contamination observed. The mass measurements were performed on a PerSeptives Biosystems (Framingham, MA, USA) Voyager-DE STR MALDI mass spectrometer.
polymers and biopolymers. For example, qualitatively best results in pressed pellet MALDI analysis of synthetic polymers were obtained for weight ratios of about 5:1 to 1:1 of 3-aminoquinoline: low-molecular-weight polyamide as compared to results obtained for the ratios 10:1 and 0.2:1 [15], which corresponds roughly to an optimal molar analyte: matrix ratio of 1:50; in traditional solvent-based and solvent-free loose powder MALDI analysis, optimal molar ratios are, depending on the matrix, approximately 1:500 (dithranol) and are nonrestrictive (Figure 7.2; PS : dithranol) [19]. It has been shown that more common molar analyte: matrix ratios of 1:500 can be applied using the pressed pellet method accepting reduced qualitative results for simple PS standard, 2 kDa, and dithranol as matrix as evaluated by the achieved resolutions of about 3000 with the pressed pellet, 4000 with the solvent-based, and 5000 with the loose powder MALDI method [19]. As mentioned before, the homogenization time (mortar and pestle, BM, or vortex) is applicable over a broad period.

For peptide and protein analysis utilizing the mortar and pestle/pressed pellet method, molar ratios employed are about 1:100 to 1:500 [21] and revealed a greatly limited accessible molecular weight range exemplified by the restricted analysis of even small proteins. For example, the signal quality obtained from the pellet was evaluated as weak for bovine insulin (5.7 kDa), and no ion signal was observed for cytochrome c (Cyt c, 12kDa) either using 2,4-, 2,5-, or 2,6-DHB [21]. Extending the homogenization period improved the spectra quality to some degree [21]. In other studies, the mass measurement of Cyt c using small amounts of 2,5- and 2,6-DHB assessed to provide good mass spectra via the mortar and pestle/pressed pellet method (no further experimental details provided toward the employed ratio) [31]. In solvent-based MALDI analysis, typically used final molar analyte: matrix ratios are about $10^{-5}$ for these types of compounds [31]. These initial pressed pellet-based studies prompted further investigations toward MALDI mechanistic aspects: One study (mortar and pestle) provided evidence for gas-phase ionization processes of a peptide by employing various different molar matrix: salt: analyte ratios between 500:1000:1 to 10:20:1 as well as using different matrices such as sinapinic acid, DHB, 2,4,6-trihydroxyacetophenone, and dithranol [32], Another study showed that longer BM grinding times (from 5 s to 7 min) extends the accessible mass range up to 12 kDa as evidenced by the mass analysis of Cyt c using either 2,5- or 2,6-DHB as matrix (molar analyte: matrix ratios of 1:250); bovine serum albumin (BSA, 66kDa) was still inaccessible [33].

### Derived from the Loose Powder MALDI Method

Theoretical implications using the solvent-free loose powder transfer method, either in combination with mortar and pestle, BM, MBM, or vortex homogenization, have been obtained on peptides [28, 34], small (bovine insulin, Cyt c) [19, 26, 27, 28] and large proteins (BSA,66 kDa) [28, 34, 35], and small [16, 36] and large molecular weight synthetic polymers (PS 100kDa, PMMA, 100kDa) [19] under various different aspects. Studies revealed that the quality of the analytical result strongly depends on the employed grinding times (MBM) as evidenced by mass spectra with increasing grinding times (0.5-10 min) of a peptide employing α-cyano-4-hydroxycinnamic acid (CHCA) [28], A grinding time of less than 1 min already
gives sufficient quality, yet periods between 1 min and 5 min give the best results as shown by the most intense overall signal intensity. Longer grinding times (10 min) seem to assist a higher degree of \([\text{M} + \text{Na}]^+\) formation as well as a considerably overall signal intensity decrease. A dependence on employed material was also determined, as for example, the least degree of sodiation was observed in the acquired mass spectra using metal beads for homogenization instead of silica or glass beads [25, 28]. In any case, the applicable molar analyte/matrix ratio was not restrictive for biopolymer analysis, specially evidenced by the sensitivity studies [28]; for example, femtomole peptide amounts were accessible [25, 34]. Overall, the general theme is that this nontraditional solvent-free MALDI method allows to employ less laser power and therefore softer MALDI conditions, improving the analytical result, for example, for peptides [18], synthetic polymers [3], and other synthetic macromolecules [5], which triggered more in-depth investigations.

One study employing the loose powder sample transfer method investigated crystallinity dependencies of MALDI samples, and included the analysis of a single crystal of 2,5-DHB incorporating Cyt c, and the powder from ball-milling of the crystal [27]. In addition, comparisons of traditional and nontraditional MALDI sample preparation methods employing other matrices (2,4-DHB, 2,6-DHB, dithranol, and anthracene) were described, all of which gave mass spectra but with different quality [27]. The results of these experiments concluded [26, 27, 34] that the incorporation of analyte in matrix crystals is not helpful for MALDI analysis but obstructive, since it is the crystallinity of the matrix that makes the underlying process energetically less favorable. It was shown that the model of the analyte being embedded in the matrix crystal is not necessary and, in fact, is disadvantageous due to the requirement of increased laser power. The MALDI process is more effective, or “softer,” with improved contact between the analyte and the matrix that results from decreased crystallinity. This also gives a theoretical explanation to the poor analytical results obtained when using the solvent-free pellet method [15, 21, 33].

Other studies were directed at investigating the surface morphology of synthetic polymer MALDI samples produced by the solvent-free vortex homogenization/spatula transfer method using microscopy techniques [36]. Low-molecular-weight (~2000 Da) polymers (PS, PMMA, and polyethynyl formamide (PEF)) were prepared with common MALDI matrices (DHB, CHCA, 3-indolyacrylic acid (IAA), and dithranol) and with metal cationization salts (silver or sodium) using molar ratios of about 1/2000/10. The samples were vortexed for 60 s [7] and analyzed using backscattered electrons from scanning electron microscopy (SEM) in a variable pressure instrument and tapping mode atomic force microscopy (AFM). The results of the AFM and SEM images from MALDI samples are shown in Figure 7.5: PS :CHCA:Ag salt and PEF :IAA:Na salt, respectively (Figure 7.5.I. A, B). The SEM images at 5000× magnification (Figure 7.5.I, II.B) show the samples to be surprisingly homogeneous, considering that only the least powerful of any of the previous electrical homogenization devices was employed and for only 60s. The samples are composed primarily of particles much smaller than 1 μm, closely packed into the film, with a few much larger (few micrometer in diameter) particles on the surface of the film (data not shown), as seen in SEM images of lower magnification.