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Complementarity of Solvent-Free MALDI TOF and Solid-State NMR Spectroscopy in Spectral Analysis of Polylactides

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We report systematic studies of solvent-free modification of matrix-assisted laser desorption/ionization time-of-flight (SF MALDI-TOF) mass spectrometry in analysis of synthetic polymers employing solid-state NMR spectroscopy as a supporting technique. In the present work oligomeric ($M_n = 4000$ g mol^{-1}) poly(L-lactide) (PLLA) was employed as a reference sample. The analyte was embedded into four matrixes commonly used in MALDI-TOF analysis of polymers: 1,8-dihydroxy-9-anthracenone (DT), 2,5-dihydroxybenzoic acid (DHB), 2-(4 hydroxyphenylazo)-benzoic acid (HABA), and *trans*-3-indoleacrylic acid (IAA). Solid-state NMR measurements clearly showed that the initial crystallinity of PLLA had no influence on quality of SF MALDI-TOF spectra since the crystalline structure of the analyte was not preserved during analyte/matrix grinding. Interestingly, the matrix remained crystalline during the sample's preparation. It was also found that, on the contrary to the dried droplet (DD) method, the SF approach leads to highly resolved mass spectra for a large variety of matrixes. Finally, problems of polymorphism and mechanochemical processes that can occur during the analyte/matrix grinding are briefly discussed.

Since its discovery in 1988, matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has became one of the most useful analytical techniques for the determination of molar mass, molar mass distribution, and chain microstructure of synthetic polymers.[1−3] The crucial step in successful analysis by MALDI MS is the preparation of the sample, which significantly affects the desorption/ionization process.[4−6] For natural polymers, such as peptides, proteins, oligonucleotides, and oligosaccharides, solvent-based techniques of sample preparation have been elaborated and resulted in the well-resolved spectra. These procedures although successfully applied for the analysis of a number of synthetic polymers have shown also several disadvantages. The best results were obtained when all components of the sample (polymer, matrix, and cationizing agent) were soluble in the same solvent. However, in this approach (known as "dried droplet" MALDI (DD MALDI)) the optimal conditions for solubility are often difficult to fulfill.[7−9] To avoid these difficulties and to extend the applicability of MALDI to insoluble polymers, a solvent-free method (SF MALDI) of sample preparation has been recently introduced.[10−12] Notably, some of the mass spectra obtained using this new sample preparation method had enhanced signal-to-noise ratio (S/N) and improved resolution.[13]

Although only a few methods of solvent-free sample preparations for MALDI analysis are in general use,[14] the quality of the spectra for all of these methods is dependent on the molar ratio of sample components (polymer/matrix/cationization agent). Additionally, the method and time length of sample grinding and milling also affect spectral quality.[15] The refinement of all details regarding sample preparation and optimization of measurement conditions have been the subject of recent investigations.[16] The time needed for sample preparation has also been critically discussed.[17] Hanton et al., while optimizing the vortex method, examined the morphology of solvent-free prepared samples using

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atomic force microscopy (AFM), scanning electron microscopy (SEM), and time-of-flight secondary ion mass spectrometry (TOF-SIMS).[18] Very recently, Weidner and Falkenhagen have employed imaging mass spectrometry to localize the regions of varied polymeric compositions in MALDI samples.[19]

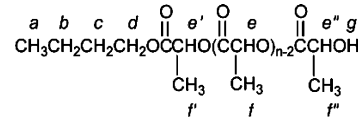
As a part of our ongoing interest related to the application of analytical techniques supporting the optimization of mass spectrometric measurements,[20] we present herein results obtained by means of solid-state NMR (SS NMR) spectroscopy. SS NMR spectra give a "fingerprint" of local structure and represent the local electronic environment for each nucleus under investigation.[21] This technique allowed us to investigate the matrix−analyte interaction and to evaluate the influence of sample grinding, which is required for preparation of the sample for SF MALDI experiments, on the final morphology of the synthetic polymer. The simplest piece of information that can be obtained from high-resolution spectra recorded under magic-angle spinning (MAS) is the isotropic chemical shifts of solids. However, the other NMR parameters, such as the chemical shift anisotropy and dipolar coupling constants, are also accessible. These parameters provide information on electron distribution, intra- and intermolecular interactions (e.g., matrix/analyte), and changes of local conformation.

As a reference polymer sample, we employed well-defined (M_n = 4000 g mol^{-1}) poly(L-lactide) (PLLA) allowing rigorous MALDI MS analysis to be carried out. PLLA is among the most prominent biodegradable and biocompatible synthetic polymers used as large-scale, environmentally friendly commodity thermoplastic and fiber-forming materials.[22] Other important, specialty applications of PLLA were developed in the biomedical and pharmaceutical polymers field.[23] Technology of PLLA production is currently based on the annually renewable raw materials of agricultural origin.[24]

EXPERIMENTAL SECTION

Materials. Matrixes 1,8-dihydroxy-9-anthracenone (DT), 2,5-dihydroxybenzoic acid (DHB), 2-(4-hydroxyphenylazo)-benzoic acid (HABA), *trans*-3-indoleacrylic acid (IAA) and the cationization agents sodium iodide (NaI) and sodium chloride (NaCl) were purchased from Sigma-Aldrich and used without further purification.

n-C_4H_9O−Poly(L-lactide)−H (PLLA) has been prepared by the ring-opening polymerization of L,L-lactide (L,L-LA) initiated with tin(II) bis(*n*-butanolate)/*n*-butyl alcohol two-component system according to the procedure described elsewhere.[25] The number-average molar mass (M_n) and dispersity (M_w/M_n, where M_w stands for the weight-average molar mass) of the resulting PLLA were determined by the size exclusion chromatography (SEC) (4100 g mol^{-1} and 1.32, respectively). ^{1}H NMR spectrum analysis (C_6D_6, 20 °C) gave the following results (in brackets are given chemical shifts (δ, in ppm), multiplicities of signals, and their relative intensities, respectively):



a (0.67, t, 3.00), *b* + *c* (not directly seen due to the coincidence with *f* protons), *d* (3.85, t, 2.08), *e* + *e*′ (5.05, t, 53.73), *e*″ (4.13, q, 1.10), *f* + *f*′ (1.35, d, 161.2), *f*″ (1.12, d, 3.11), *g* (2.5, d, 0.95). M_n calculated on the basis of NMR data: $M_n(\mathrm{NMR}) = 0.5M_{LA}[(I_e + I_{e'} + I_{e''} + I_f + I_{f'} + I_{f''})/4)/[(I_a + I_d + I_{e''} + I_g)/7] + M_{BuOH}$ (where M_{LA} and M_{BuOH} are equal to 144.13 and 72.06, respectively) is equal to 3950 g mol^{-1}, that is a value being close to that determined by SEC.

Sample Preparation. *Dried Droplet (DD) Method.* The polymer and the matrixes were dissolved in tetrahydrofuran (THF, POCh, Gliwice Poland, 99%) at a concentration of 10 mg mL^{-1}. Solutions of the cationization agents were prepared at the concentration of 10 mg mL^{-1} in THF. Samples for analysis were prepared by mixing the polymer solutions, matrix solutions, and the cationization agent solutions in the ratio 1:10:1 (v/v/v). An aliquot (1 μL) of this mixture was placed on the sample plate and allowed to dry.

Solvent-Free (SF) Method. Polymer, matrix, and cationization agent in weight ratio 1:10:1 were ground with a mortar and pestle or in a ball mill MM200 (Retsch GmbH & Co., Haan, Germany) for an appropriate time (2 or 30 min). A few grains of the mixture were applied to the target plate and pressed with a spatula.

NMR Measurements. The solid-state cross-polarization magic-angle spinning (CP MAS) ^{13}C NMR experiments were performed at a frequency of 75.47 MHz on a Bruker Avance III 300 spectrometer, which was equipped with a MAS probe head using 4 mm ZrO_2 rotors. A sample of glycine (Gly) was used to set the Hartmann−Hahn condition, and adamantane was used as a secondary chemical shift reference at 38.48 and 29.46 ppm from external tetramethylsilane (TMS).[26] The conventional spectra were recorded with a proton 90° pulse length of 3.5 μs and a contact time of 1 ms. The repetition delay was 10 s, and the spectral width was 25 kHz. Free induction decay (FID) spectra were accumulated with a time domain size of 2K data points. The RAMP shape pulse was used during the cross-polarization (CP)[27] and two-pulse phase modulation (TPPM),[28] with tp = 6.8 ms and a phase angle of 20° during the acquisition. The spectral data were processed using the WIN NMR program.[29]

^{1}H NMR spectra were recorded in C_6D_6 (Dr. Glaser, Basel, Switzerland) on a Bruker AC200 spectrometer.

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MS Measurements. Mass spectra were recorded on a Voyager Elite mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with a nitrogen laser (337 nm). Acceleration voltage of 20 kV, grid voltage of 92.1%, and guide wire voltage of 0.02% were used in all cases. Spectra were obtained in the linear, positive ion mode. The delay time in delayed extraction was optimized and set to 100 ns. The energy of the laser beam was set slightly above threshold level. Spectra were acquired in m/z range from 700 to 15 000 with low mass gate set to $m/z = 500$. External calibration was performed using appropriate samples of poly(ethylene glycol). Spectra were recorded using the automatic mode of operation. For each sample, the summed spectrum consisted of the sum of 250 spectra from 25 different points on the sample spot (25 points × 10 spectra = 250 spectra). At least three summed spectra were recorded for each sample. All spectra, resolutions, and S/N ratios were processed and calculated by Data Explorer ver.4 program (Applied Biosystems Inc., Foster City, CA).

SEC Measurements. SEC traces were recorded using a LKB 2150 HPLC pump and two sets of TSK gel columns (G 2000 HXL and 6400 HXL). A Wyatt Optilab 903 interferometric refractometer was applied as a detector. Methylene chloride was used as an eluent. A flow rate of 0.8 mL min^{-1} was applied. The actual number-average molecular masses (M_n) of PLLA were determined using the poly(L-lactide) standards prepared in our laboratory.

RESULTS AND DISCUSSION

^{13}C CP/MAS Measurement of PLLA: Influence of Grinding on Crystallinity of Samples. The quality of MALDI mass spectra can be significantly affected by sample preparation. Recently Meier and Schubert revealed that in the DD approach, use of the same solvent for preparing solutions of polymer, matrix, and inorganic salt gave the best result, but binary solvent systems can be used as long as both solvents are compatible.[30] Trimpin et al., in examining DD MALDI and SF MALDI methods, concluded that minimum crystallinity and intimate contact between analyte and matrix were beneficial to the MALDI process.[31] Moreover, incorporation of analyte into a matrix crystal is not necessary, and indeed, the crystallinity of the sample can obstruct the laser beam and require higher laser threshold power. Our results show that SS NMR spectroscopy is a technique that provides important information regarding the influence of sample grinding on the crystallinity of pure PLLA and PLLA−matrix−salt samples.

We examined the effect of slow and rapid cooling of the PLLA sample as well as the role of sample grinding on the quality of the ^{13}C CP/MAS spectra (Figure 1). In analysis of spectra, two elements were taken into consideration, change of line shape of PLLA signals and the ratio of individual components for each peak of functional groups which reflects crystallinity of the polymer. As a control sample, we used the spectrum of PLLA crystallized in chloroform (labeled hereafter as **C**). When the sample was rapidly cooled to room temperature (Figure 1b), the resolution was worse than that for **C** and the signals were very broad,

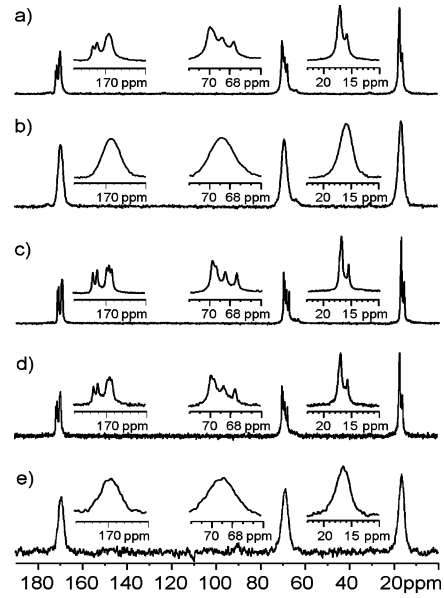

Figure 1. ^{13}C CP/MAS spectra of PLLA prepared under different conditions: (a) PLLA crystallized from the chloroform—sample **C**; (b) melted sample "a" and rapidly cooled to room temperature; (c) melted sample "a" and gently cooled with a gradient 5 K/min to room temperature—highly crystalline (sample **HC**); (d) sample "c" after 30 min of grinding; (e) sample "a" after 30 min of grinding.

indicative of amorphous phases. In contrast, when the sample was gently cooled with a low temperature gradient of 5 K/min, we obtained very sharp signals, typical of a sample with high crystallinity (described hereafter as **HC**) (Figure 1c). The problem of quantitative analysis of morphology of PLLA has been thoroughly discussed by Thakur et al.[32] Employing the previously reported deconvolution procedure, we found that samples **C** and **HC** contained ca. 70% and 95% of crystalline phase, respectively.

We next examined the effect of grinding samples **C** and **HC**. After 30 min of grinding, the ^{13}C/CP MAS spectrum of **HC** indicated that crystallinity of sample **HC** is preserved (Figure 1d). In contrast, sample **C**, which originally containing 70% of crystalline phase, became almost fully amorphous after 30 min of grinding (Figure 1e).

We tested the influence of the matrix material on of the crystallinity of PLLA during sample grinding. Figure 2 compares the spectra of samples (PLLA/matrix/salt; 1:10:1) with IAA matrix (Figure 2, parts a and b) and with HABA matrix (Figure 2, parts c and d) after 2 and 30 min of mechanical grinding. In both cases, we used PLLA with high crystallinity (**HC**). From line shape analysis of PLLA resonances (indicated by filled triangles) is it clear that during grinding, both samples lost crystallinity and, after 30 min of milling, became amorphous.

Solid-State NMR Analysis of Matrix and Matrix−Analyte Interaction. Since the analyte is present only in small concentration relative to matrix, the analysis of matrix−analyte interaction is challenging. Previous analyses of such interactions and morphology of samples have mostly used optical techniques. Our data presented above verify that solid-state NMR spectroscopy is a technique which provides straightforward information on the crystallinity of analyte embedded into matrix. In this section, we

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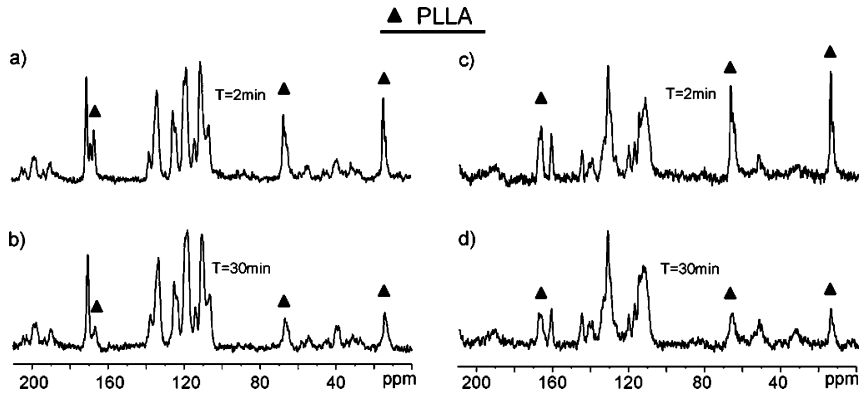

Figure 2. ^{13}C CP/MAS spectra of samples containing **HC** PLLA/matrix/NaI (1:10:1) recorded with 8 kHz spinning rate at room temperature, mixed using a ball mill MM 200 Retsch GmbH & Co.: (a) PLLA/IAA/NaI, mixing time 2 min; (b) PLLA/IAA/NaI, mixing time 30 min; (c) PLLA/HABA/NaI, mixing time 2 min; (d) PLLA/HABA/NaI mixing time 30 min. Signals of PLLA are labeled by filled triangles (▲).

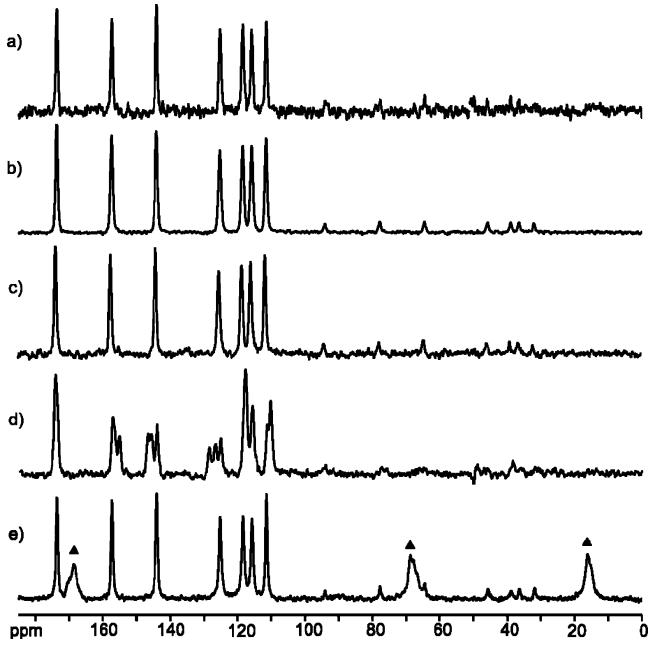

Figure 3. ^{13}C CP/MAS NMR spectra of the DHB matrix recorded with 8 kHz spinning rate at room temperature: (a) DHB crystallized from chloroform−acetone (3:1 v/v); (b) spectrum of DHB ground for 30 min; (c) spectrum of DHB and NaI (10:1) ground for 30 min; (d) spectrum of DHB and NaI (10:1) recrystallized from THF; (e) spectrum of PLLA/DHB/NaI mixture, ground for 30 min. **HC** PLLA was used for all sample preparations. Signals of PLLA are labeled by filled triangles (▲).

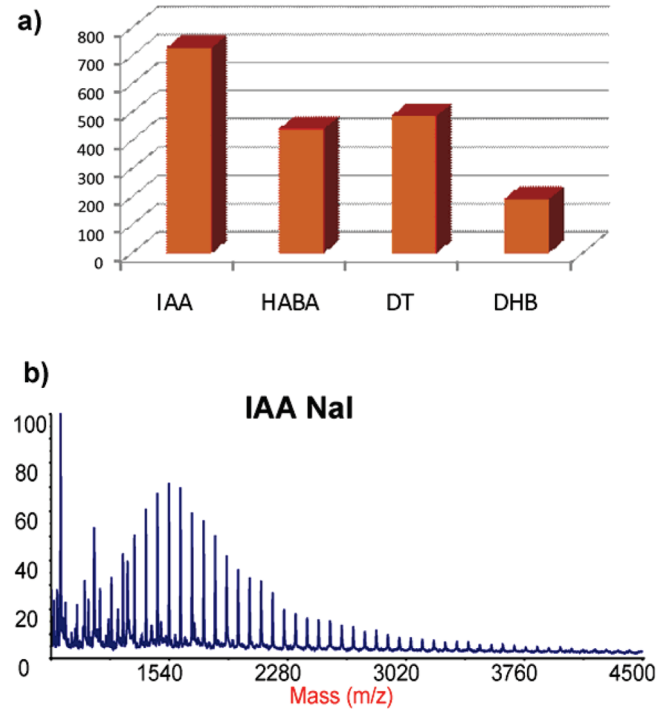

Figure 4. (a) Average S/N values for PLLA in different matrixes in the presence of NaI cationization agent using the dried droplet method. (b) The best quality MALDI-TOF mass spectra of PLLA/IAA/NaI mixture obtained by using the dried droplet method.

present ^{13}C CP/MAS data for matrix, matrix/salt, and matrix/analyte/salt samples and have focused our attention on the morphology of the matrix and on the intermolecular contacts between matrix and PLLA.

NMR measurements of DHB were carried out under MAS conditions with a spinning rate of 8 kHz (Figure 3). A comparison of spectra for DHB recrystallized from chloroform−acetone (3:1 v/v) (Figure 3a), for a DHB ground for 30 min (Figure 3b), and for a mixture of DHB/NaI 10:1 ground for 30 min (Figure 3c) clearly shows that neither the presence of the cationization agent NaI nor sample grinding influenced the morphology of the matrix under investigation. During solid-state grinding, a variety of solid-state transformations, including crystalline to amorphous (and vice versa) as well as polymorphic conversions can be induced. Several studies have been reported in the literature, mostly for pharmaceutical compounds, with examples of barbituric acid and piroxicam being particularly notable.[33] To the best of our knowledge, the problem of the influence of matrix polymorph formation on the quality of mass spectra has not been discussed. In the course of our studies, while examining DT, DHB, HABA, and IAA matrixes, we did not observe any mechanochemical processes and formation of new polymorphs. These results are especially interesting in the light of our DD matrix results. It is clear from the SS NMR study that DHB in the presence of NaI recrystallized from THF forms polymorphs (Figure 3d). NMR results for other matrixes are available as Supporting Information.

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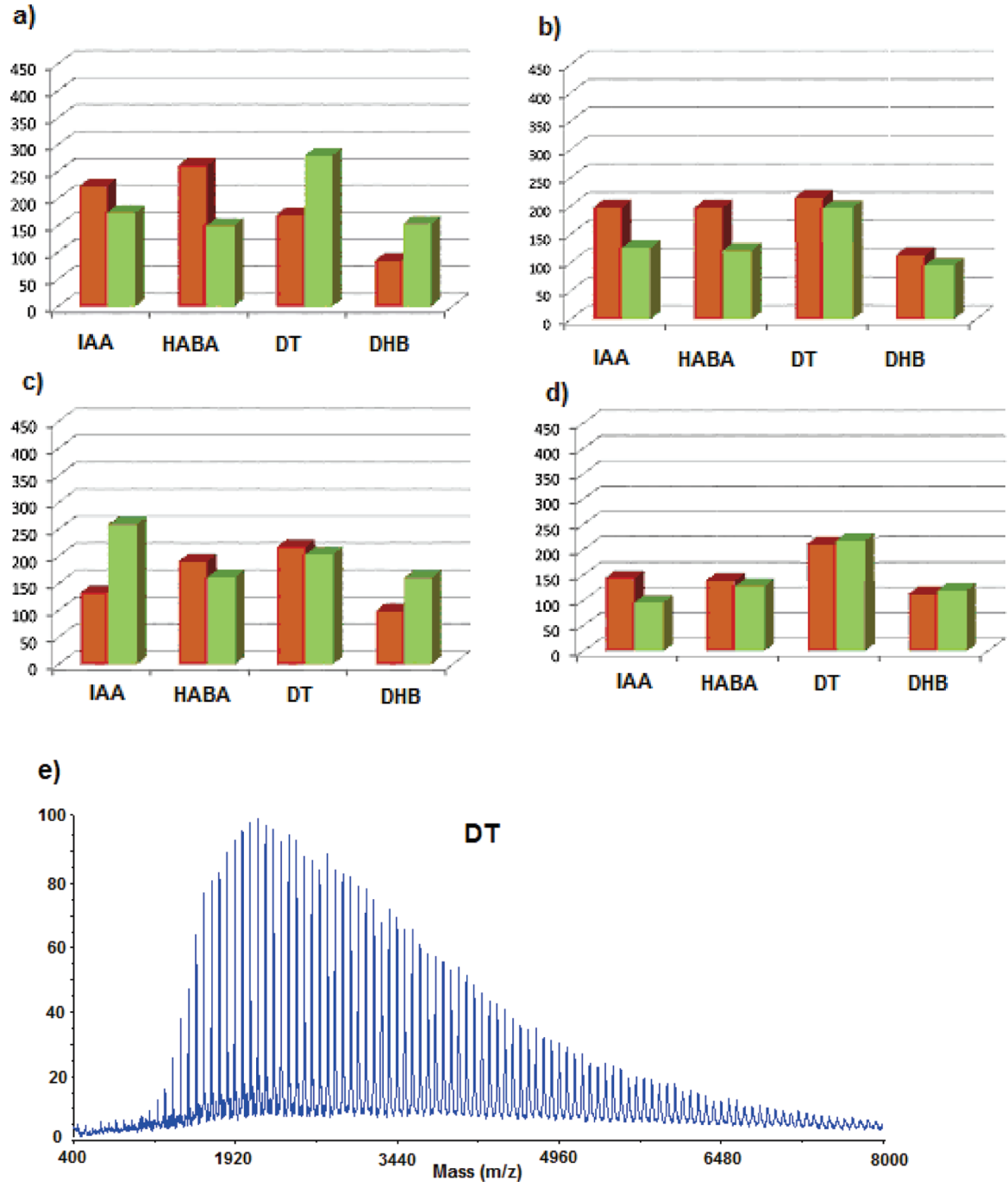

Figure 5. Average S/N values for PLLA in different matrixes (as indicated) in the presence of NaI cationization agent measured by employing a solvent-free MALDI method. Panels a and b represent data for crystalline analyte (**C**, PLLA) ground using a ball steel mill (a) or a mortar and pestle (b). Panels c and d represent data for highly crystalline analyte (**HC** PLLA) ground using a ball steel mill (c) or a mortar and pestle (d). For panels a−d, the brown bars represent S/N data for samples with mixing time of 2 min, whereas the green bars are for samples with mixing time of 30 min. (e) The best quality MALDI-TOF mass spectrum of PLLA/DT/NaI mixture obtained by using a solvent-free method.

Mechanically grinding a sample of PLLA/DHB/NaI: 1:10:1 for 30 min (Figure 3e) had very little effect on the values of the chemical shifts in the ^{13}C CP/MAS spectra for either the matrix or the polymer, as compared to the spectra in Figures 3a and 1e, respectively. Thus, the intermolecular interactions between the components of the sample are rather weak, and we can rule out the formation of covalent bonds and/or strong hydrogen bonds. According to the results presented in the previous section, PLLA (**HC** sample was used) is amorphous after grinding.

Comparison of the Quality of MALDI TOF Measurements in Different Matrixes: Dried Droplet versus the Solvent-Free Approach. Figure 4 shows in graphical form results of MALDI-TOF measurements for PLLA samples prepared for analysis by means of the DD method. We compared two parameters to assess the quality of the mass spectra: resolution and S/N. Both parameters were calculated with respect to the highest peak in the spectrum, which corresponds to the cationized oligomer of highest concentration in the polymer. It is apparent

from our studies that the quality of the data depends on the matrix used. For the IAA matrix, the results are considerably better as compared to the DHB matrix.

We also investigated PLLA samples in the same set of matrixes prepared for analysis employing a solvent-free method. As in the previous case, we compared resolution and the S/N.

From inspection of data shown in Figure 5 it is clear that for ball milled samples (a and b), the scatter of experimental results is not as significant as in the case of the DD method. A comparative analysis of results showed slightly worse S/N for crystalline PLLA that was manually milled (Figure 5d). This outcome may be attributed to the transformation of crystalline PLLA to an amorphous phase by use of a mortar and pestle, which is not as efficient as in the case of the vortex method. Finally, we carried out SF MALDI measurements for polymorphic samples of DHB containing PLLA recrystallized from THF. Our measurements proved that both polymorphic forms gave very similar results.

Although MALDI MS is not the best method for precise measurement of molar mass (M_n and/or M_w) of synthetic polymers, that typically have molar mass distribution, comparison of M_n and M_w/M_n obtained with DD and SF methods is quite instructive. Namely, from spectra displayed in Figures 4b and 5e the following values have been determined: $M_n = 2100$ and 3400 g mol^{-1}, $M_w/M_n = 1.47$ and 1.21, respectively. Thus, depending on the method of sample preparation mode M_n can be more (DD) or less underestimated (SF), when comparing with the SEC reading (M_n = 4100, see the Experimental Section). Figure 5e shows that in the DD method the low molar mass tail is adventitiously formed, which leads to an artificial decrease of M_n accompanied by M_w/M_n increase (i.e., molar mass distribution broadening). In the case of SF method only one population of signals corresponding to BuO−[(C=O)-CH(CH$_3$)O]$_n$−H macromolecules is exclusively seen (please note that in spite of the dimeric structure of the lactide monomer, macromolecules containing even and odd number of lactoyl units are seen in the spectrum since they are separated by $m/z = 72.065$, whereas L,L-LA molar mass is equal to 144.13 g mol^{-1}; this phenomenon is explained, for example, in ref 25.

CONCLUSIONS

The present study revealed that SS NMR spectroscopy is an excellent tool allowing the investigation of the matrix−analyte interaction at the molecular level. Very recently, Pizzala et al. have proved that this technique can be also used to explore structural characteristics of matrix−cationization agent interactions.[34] Providing a specific set of analytical data (chemical shifts, line shape), SS NMR can be an approach to complement commonly used optical methods which are dedicated to the study of macroscopic effects. Moreover, SS NMR is the technique of choice for the study of polymorphism and mechanochemical processes which can occur during the grinding of samples. We can extract information regarding the degree of crystallinity from line shape analysis of the analyte and/or matrix signals. Our study clearly shows that initial crystallinity of PLLA sample has no influence on the quality of MALDI-TOF spectra. During grinding, the crystalline structure is not preserved and the sample becomes amorphous. Hanton and co-workers have recently proved that very short grinding time is needed for sample preparation for SF MALDI. Our results suggest that, for those cases when analyte is highly crystalline, shorter grinding time in the range of minutes is preferable.

From MALDI-TOF measurements, it is clear that a solvent-free approach gives very good results in analysis of polylactides. The choice of matrix is not as critical as in the case of the dried droplet method. Although we can find an optimal system for the DD method by taking into consideration the matrix, the solvent used for crystallization, cationization agent, crystallization conditions, etc., the solvent-free approach offers reasonable results for a wide range of matrixes.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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