Effect of Dihydroxybenzoic Acid Isomers on the Analysis of Polyethylene Glycols in MALDI-MS

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The effects of different dihydroxybenzoic acid (DHB) isomers, when used as matrix materials in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), were investigated in analyses of polyethylene glycol (PEG) polymers. PEG polymers ranging from 400 to 8,000 Da were prepared in different DHB isomer matrices using solvent-based and solvent-free methods. PEG samples were detected only in matrices of 2,3-DHB, 2,5-DHB, and 2,6-DHB while the most intense peaks were observed using 2,6-DHB in both solvent-free and solvent-based preparations.

Key Words: Dihydroxybenzoic acid, Polyethylene glycol, Solvent-free, Solvent-based, MALDI-TOF

Introduction

Conventional organic matrices used in matrix-assisted laser desorption/ionization (MALDI) analyses of biomolecules include α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxybenzoic acid (2,5-DHB). Among the different DHB isomers, 2,5-DHB has been used exclusively due to its strong absorbance at 337 nm, corresponding to the emission from a N_2 laser.[1-4] Recently, the performance of different DHB isomers was investigated for analyses of peptides, proteins, oligonucleotides, and polysaccharides with a 337-nm N_2 laser ionization source.[5] The 2,5-DHB isomer yielded superior results in most analyses while the 2,6-DHB isomer was more effective for oligonucleotides and polysaccharides in positive ion mode and for oligonucleotides in negative ion mode. Other studies have shown that changes in state from liquid to sol-gel[1] or solid[2] induced a redshift in the wavelength of maximum absorbance for the different DHB isomers.

The analysis of synthetic polymers using mass spectrometry is of particular interest,[6,7] as summarized in a recent review article.[8] The advent of solvent-free sample preparation has made MALDI mass spectrometry (MALDI-MS) applicable to polyether chain polymers[9] or polymers that are insoluble in common organic solvents.[10-13] The solvent-free method is known to have several advantages, including investigations of the MALDI mechanism,[9,14] the possibility of multi-sample preparations,[15] analyses of insoluble synthetic polymers,[11] and the removal of potential solvent-induced chemical modifications.[13]

In this study, solvent-free sample preparations employing different DHB isomer matrices for the analysis of various polyethylene glycol (PEG) samples were compared with a more traditional solvent-based sample preparation technique.

Experimental Section

Materials. PEG (average M_n ~400), PEG (average M_n ~ 1,450), PEG (average M_n ~3,350), PEG (average M_n ~8,000), 2,3-DHB, 2,4-DHB, 2,5-DHB, 2,6-DHB, 3,4-DHB, 3,5-DHB, trifluoroacetic acid (TFA), sodium trifluoroacetate (NaTFA), horse heart myoglobin, adrenocorticotropic hormone (ACTH) 18-39, angiotensin I, bradykinin, and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The μFocus MALDI plate was obtained from Hudson Surface Technology, Inc. (Newark, NJ, USA).

Sample preparations. Solvent-free preparations consisted of the DHB isomer matrix, PEG polymer, and a NaTFA cationization agent and were mixed at a molar ratio of 200:1:5, respectively. The mixture was vigorously vortexed with several stainless steel beads as described in the literature,[16,17] which is believed to induce the size reduction of the matrices.[18] The mixture was then deposited on the MALDI plate as a suspension as shown in the literature.[12] Briefly, an approximately 1-μL aliquot (approximately 0.4 mg) of the mixture was removed with a 10-μL pipet tip and mixed with 2-μL of distilled water that had already been deposited on the plate by repeated aspiration and ejection. Approximately 10 nmol of PEG polymer was deposited onto the plate each time.

Solvent-based preparations consisted of a 500 mM DHB isomer matrix in an aqueous solution of 50% acetonitrile/0.1% TFA, 1 mM PEG polymer in 0.5% TFA and 10 mM NaTFA cationization agent in 0.5% TFA. A three-layer preparation method[19] was used to deposit the preparation onto the MALDI plate, where 0.5-μL of the DHB matrix was deposited first, followed by a 1-μL mixture composed of a 0.5-μL PEG polymer and a 0.5-μL matrix, and then 0.5-μL of the cationization agent. The total amount of PEG polymer loaded onto the plate was 500 pmol.

All mass spectra were obtained using a Kratos Axima CFR (Shimadzu, Kyoto, Japan) time-of-flight mass spectrometer equipped with a 337-nm N_2 laser in positive linear mode. Each mass spectrum was a result of summing 500 laser shots, with the laser irradiating a solid sample spot on a stainless steel MALDI target. External calibration was performed with ACTH 18-39, angiotensin I, bradykinin, and horse heart myoglobin.

Results and Discussion

Effect of DHB isomers on the detection of PEG samples. The structures of the DHB isomers are provided in Figure 1. Mass spectra of PEG 400, PEG 1,450, PEG 3,350, and PEG 8,000 prepared using solvent-free and solvent-based methods are shown in Figures 2, 3, 4, and 5, respectively, where only $[PEG+Na]^+$ ions were observed. PEG samples were detected with a monomer spacing of 44 Da only with 2,3-DHB, 2,5-DHB, and 2,6-DHB matrices. 2,6-DHB exhibited the most efficient ionization of all of the analyzed samples in both solvent-free and solvent-based preparations. A summary of MALDI intensities obtained for PEG samples using 2,3 DHB, 2,5-DHB, and 2,6-DHB matrices is shown in Table 1.

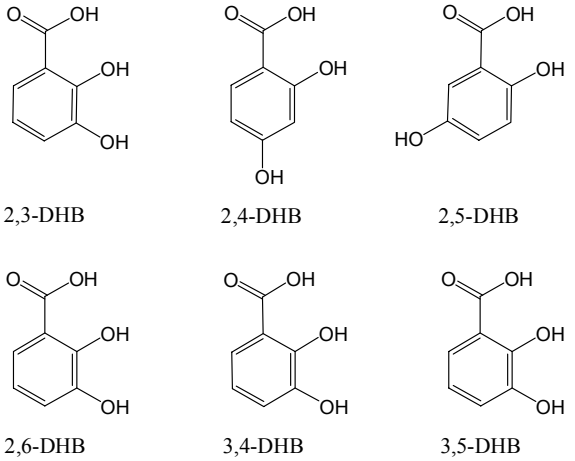

Figure 1. The molecular structures of the different dihydroxybenzoic acid isomers.

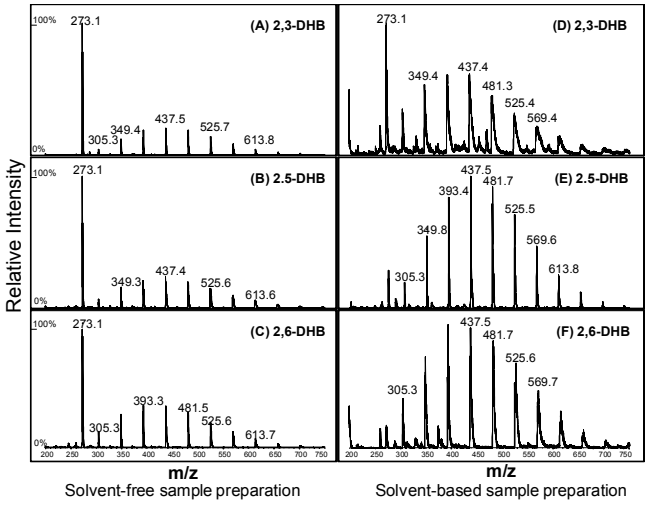

Figure 2. MALDI mass spectra of PEG 400 are shown in the range of m/z 200-750 for solvent-free (A-C) and solvent-based (D-F) sample preparations in DHB isomer matrices. The peak at m/z 273.1 was assigned to $[2DHB\text{-}H_2O\text{-}OH^-]^+$. Only $[PEG+Na]^+$ ions were observed. PEG exists in a form of $HO\text{-}(\text{-}CH_2CH_2O\text{-})_n\text{-}H$, where n is ranging from 6 to 13. For example, the peak with m/z = ~ 437.5 has n = 9.

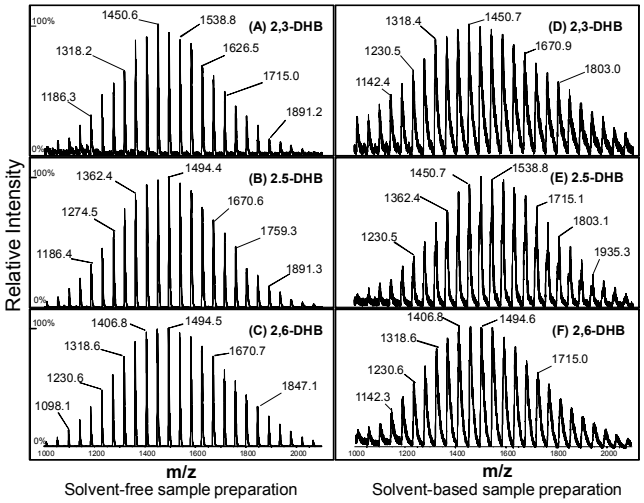

Figure 3. MALDI mass spectra of PEG 1,450 are shown in the range of m/z 1,000-2,100 in (A-C) solvent-free and (D-F) solvent-based sample preparations in DHB isomer matrices. Only $[PEG+Na]^+$ ions were observed. PEG exists in a form of $HO\text{-}(\text{-}CH_2CH_2O\text{-})_n\text{-}H$, where n is ranging from 22 to 46. For example, the peak with m/z = ~1494.4 has n = 33.

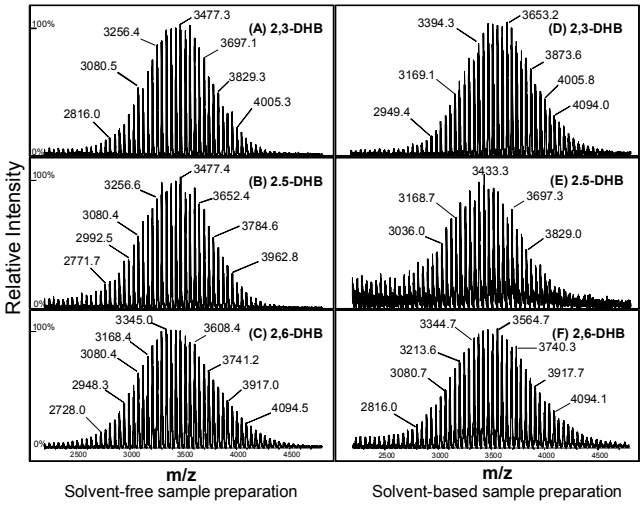

Figure 4. MALDI mass spectra of PEG 3,350 are shown in the range of m/z 2,200-4,800 in (A-C) solvent-free and (D-F) solvent-based sample preparations in DHB isomer matrices. Only $[PEG+Na]^+$ ions were observed. PEG exists in a form of $HO\text{-}(\text{-}CH_2CH_2O\text{-})_n\text{-}H$, where n is ranging from 50 to 108. For example, the peak with m/z = ~3477.8 has n = 78.

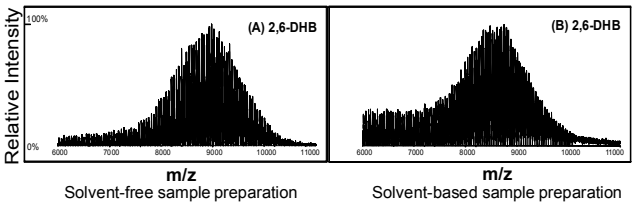

Figure 5. MALDI mass spectra of PEG 8,000 are shown in the range of m/z 6,000-11,000 in (A) solvent-free and (B) solvent-based sample preparations in DHB isomer matrices. Only $[PEG+Na]^+$ ions were observed. PEG exists in a form of $HO\text{-}(\text{-}CH_2CH_2O\text{-})_n\text{-}H$, where n is ranging from 170 to 240.

The intensity values were taken from the most intense peak in each mass spectrum. Figure 6 shows a comparative display of intensities obtained from PEG samples in both the solvent-free and solvent-based preparations.

Solvent-free sample preparation. To effectively deposit the solvent-free samples on the MALDI plate, a pre-deposited, 2-μL distilled water droplet was used as discussed in the experimental section. In this manner, the synthetic polymer was applied to the MALDI plate as a colloid, demonstrating the applicability of this method to insoluble polymers. The on-target homogenization/transfer method[15,20] was also evaluated, in which the solvent-free sample was deposited by the impaction of stainless steel beads, but the technique did not result in secure attachment of the polymer to the MALDI plate. The major problem with the current solvent-free preparation, relative to the solvent-based method, was poor reproducibility due to imprecise measurements of the amount of sample loaded onto the plate and mixture heterogeneity. As shown in Figure 6, the standard deviations obtained from solvent-free preparations (Figure 6A) were much larger than those from solvent-based preparations (Figure 6B). The solvent-free technique also required a longer time than solvent-based preparations due to the more complex loading step when transferring the sample onto the MALDI plate, which involved repeated pipet aspiration and ejection. The absolute sample amount loaded onto the MALDI plate was also much larger in the solvent-free system, which required much more sample to obtain homogenization by vortex mixing. The amount deposited onto the MALDI plate with the solvent-free method was approximately 10 nmol, while the solvent-based required only 500 pmol. Despite the much lower amount of material, the solvent-based method provided similar peak intensities compared to those of the solvent-free method (Table 1 and Figure 6).

Requirement of a good matrix among DHB isomers. The most important requirement of a good matrix is generally a strong absorption at the irradiated laser wavelength. At 337 nm, the relative molar absorptivity of the isomers decreases in the order: 2,5-DHB, 2,3-DHB, and 2,6-DHB in methanol[21] or in aqueous solution (3,426 $cm^{-1}M^{-1}$, 613 $cm^{-1}M^{-1}$, and 151 $cm^{-1}M^{-1}$, respectively.)[1], while the other isomers have negligible absorption. The crystalline structure of the DHB matrix prior to laser irradiation in MALDI-MS experiments induces a redshift and band broadening in both solid[2] or sol-gel phases,[1] which implies that a DHB matrix with a small absorbance at 337 nm in the liquid phase can absorb a great amount of the laser irradiation in the crystalline phase. The acidity of the matrix is another important requirement. pK_a values for 2,6-DHB, 2,3-DHB, 2,5-DHB, 2,4-DHB, 3,5-DHB, and 3,4-DHB in water were reported as 1.30, 2.94, 2.97, 3.29, 4.04, and 4.48, respectively.[21] These data indicate that 2,6-DHB, 2,3-DHB, and 2,5-DHB are the three most acidic DHB isomers. A phenolic hydroxyl group positioned *ortho* to a carboxylic group in an aromatic compound has been suggested to be a prerequisite of matrix compounds to form intramolecular hydrogen bonds in the excited state following irradiation, which would then lead to ionization of the analyte.[3] This assumption is applicable in the current analysis with the exception of 2,4-DHB, which was inactive for PEG samples regardless of fulfilling the aforementioned prerequisite. The 2,4-DHB isomer was presumably not suitable as

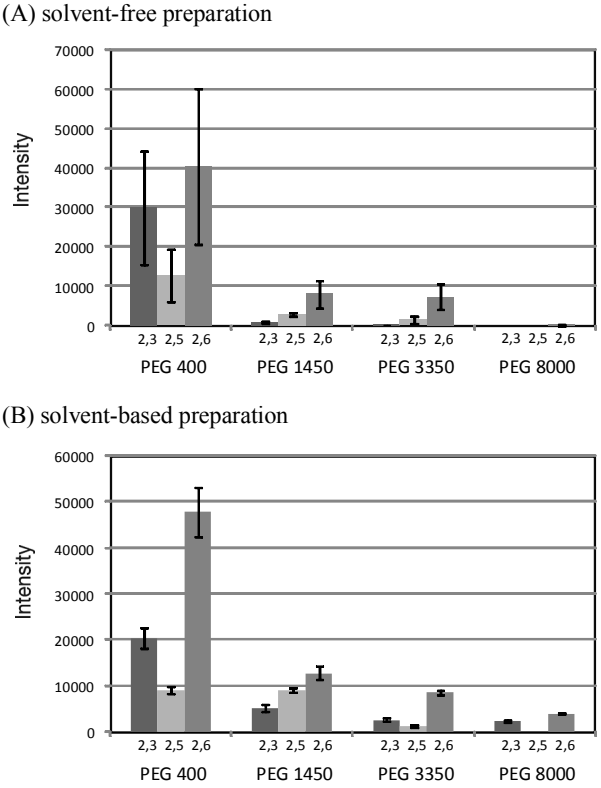

Figure 6. A comparison of MALDI intensities obtained from PEG samples is given for (A) solvent-free or (B) solvent-based preparations. The notations '2,3,' '2,5,' and '2,6,' on the *x*-axis refer to 2,3-DHB, 2,5-DHB, and 2,6-DHB, respectively.

Table 1. Signal intensities of PEG samples in positive ion using different DHB isomer matrices (average of four measurements)

	Solvent-free sample preparation			Solvent-based sample preparation		
	2,3-DHB	2,5-DHB	2,6-DHB	2,3-DHB	2,5-DHB	2,6-DHB
PEG 400	29993.3 ± 14461.7[a]	12705.4 ± 6623.5	40483 ± 19724.2	20430.2 ± 2307.5	9031.6 ± 830.5	47898.4 ± 5389.8
PEG 1450	850.5 ± 249.9	2817 ± 581.3	7990.2 ± 3597.4	5110 ± 848.4	9069.1 ± 506.3	12852.3 ± 1538.4
PEG 3350	145.8 ± 85.9	1540.8 ± 900.5	7338 ± 3307.5	2637.6 ± 466.4	1257.9 ± 237.6	8531.9 ± 435.0
PEG 8000	N.D.[b]	N.D.	98.4 ± 114.2	2256.9 ± 232.1	N.D.	3873.1 ± 142.9

[a]Signal intensity ± standard deviation. [b]Not detected.

a matrix material due to its low absorption at the irradiated wavelength and its high pK_a value.

Best performance of 2,6-DHB isomer. The current observation that 2,6-DHB showed the best performance in the analysis of PEG using MALDI-MS in both solvent-free and solvent-based preparations can be explained by the greater acidity of 2,6-DHB and the gas-phase cationization mechanism. The greater acidity of 2,6-DHB was believed to be one of the determining factors for matrix performance since PEG has anionic characteristics. The anionic sample required a more acidic matrix to achieve the charge compensation necessary to be detected in positive ion mode. Enhanced performance of 2,6-DHB matrix over 2,5-DHB matrix has been observed in analyses of oligonucleotides,[5] polysaccharides,[5] and lipids[21] using positive ion mode by MALDI-TOF MS, where all of these materials exhibited anionic characteristics. Gas-phase cationization is also assumed to contribute in the current observation. Gas-phase cationization was suggested as one of the mechanisms in the MALDI experiment in the analysis of PEG 1500 with 2,5-DHB matrix, where PEG 1500 was detected to be cationized in the gas-phase with Li^+.[22] Conclusively, it is believed that the greater acidity of 2,6-DHB assists the desorption of PEG samples from the plate upon laser irradiation, then sodiation of the PEG takes place in the gas-phase.

Conclusions

PEG samples ranging from 400 to 8,000 Da were prepared using both solvent-free and solvent-based preparation methods and were analyzed by MALDI-MS in matrices composed of different DHB isomers. PEG samples were detected only in matrices of 2,3-DHB, 2,5-DHB, and 2,6-DHB, the latter of which exhibited the best performance in both solvent-free and solvent-based preparations. The solvent-based preparation yielded more sensitive and reproducible results.

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