Detection of Adducts with Matrix Clusters in the Positive and Negative Ion Mode MALDI-TOF Mass Spectra of Phospholipids

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It is usually accepted that neutral phospholipids (PLs) generate singly positively charged ions, whereas negative PLs are easily detectable in the negative ion mode when analysed by matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS). In this study, we demonstrate that some caution is required in the interpretation of MALDI-TOF mass spectra of PLs, since also neutral PLs have appeared to be detectable in the negative ion mode as well. Neutral and negatively charged phospholipids can generate adducts with the most commonly used matrix – 2,5-dihydroxybenzoic acid – yielding singly negatively charged ions that are detectable in the spectra. This further contributes to the complexity of the spectra and potentially leads to severe misinterpretation, particularly when unknown mixtures of PLs are analysed by MALDI-TOF MS.

Key words: Phospholipids, MALDI-TOF MS, Adducts, Matrix Clusters, Positive Ion Mode, Negative Ion Mode

Introduction

Matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS*) is increasingly applied for the analysis of lipids and phospholipids (PLs) [1]. Results from our laboratory [2, 3] and from the work of others [4] imply that MALDI-TOF MS can even be applied for quantification of lipids because of the high reproducibility. Various matrices have been used for this purpose, but the most established one is 2,5-dihydroxybenzoic acid (DHB) [1]. This matrix does not interfere with peaks arising from analytes, does not lead to marked fragmentation reactions and can be used for the acquisition of positive as well as of negative ion mass spectra [5]. Additionally, in contrast to matrices with olefinic residues, DHB has only a weak tendency to give signals of matrix oligomers [1, 5].

All lipid and PLs classes can be analysed by MALDI-TOF MS, and they are mostly detectable as singly charged ions. These ions are generated by charge compensation and subsequent ionisation. Na^+ and H^+ adducts are the most abundant molecular species since sodium ions are predominant in samples of biological origin. On the other hand, H^+ sources are commonly added to the matrix solution, since this approach results in increased signals arising from lipids [6 – 8]. In addition to their detection in the positive ion MALDI-TOF mass spectra, acidic PLs can be detected as singly negatively charged ions.

We have previously reported that PLs with a choline head group – phosphatidylcholines (PC) and sphingomyelins (SM) – can generate clusters with one negatively charged DHB molecule [9]. Negatively charged clusters generated in that way can then be easily detected and analysed by mass spectrometry. This observation was made with lipoproteins that contain huge amounts of PC and SM.

In the present report, we demonstrate that additional caution is required in the spectra interpretation, because also other PL species – neutral as well as negatively charged ones – can generate clusters with DHB as such or with sodiated DHB (DHB : Na) providing more complex peak patterns than previously assumed. The intensity of these PL : matrix clusters seems to depend on the purity of the applied DHB, and particularly on the content of inorganic salts in the solvents used for matrix and/or lipids.

Results and Discussion

In this report, we demonstrate that neutral and acidic PLs can generate adducts with the DHB matrix, which

*Abbreviations: MALDI-TOF MS – matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry; PL – phospholipid; DHB – 2,5-dihydroxybenzoic acid; DPPA – 1,2-dipalmitoyl-*sn*-glycero-3-phosphate; DPPC – 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE – 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine; DPPS – 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; PI – phosphatidylinositol; SM – sphingomyelin.

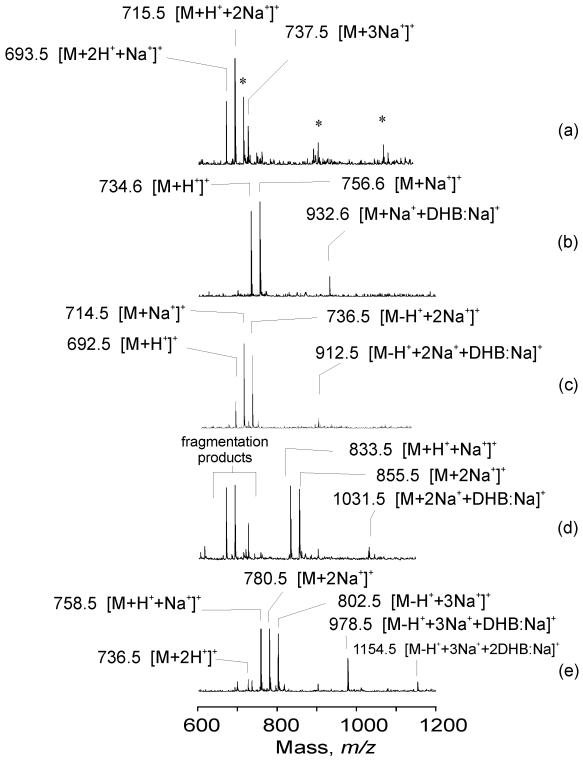

Fig. 1. Positive ion MALDI-TOF mass spectra of DPPA (a), DPPC (b), DPPE (c), DPPI (d) and DPPS (e). All spectra of the PLs were recorded with DHB as matrix. Peaks are labelled according to their *m/z* ratios, and their identity is indicated. "M" indicates the completely dissociated PL molecule, and characteristic matrix peaks are indicated by an asterisk.

contributes to the complexity of the spectra and can lead to severe misinterpretation.

Fig. 1 shows the positive ion MALDI-TOF mass spectra of DPPA (a), DPPC (b), DPPE (c), DPPI (d), and DPPS (e). Peaks are labelled according to their *m/z* ratio, and their identity is indicated in the Figure. In general, neutral PLs require only one cation (for cationisation) to be detectable as positive ions [1, 6 – 8]. On the other hand, acidic PLs first require neutralisation with the adequate number of cations (depending on their charge) and the addition of one more cation in order to be detected in the positive ion mode MALDI-TOF mass spectra. These PLs are more easily detected as negative ions. Peaks that arise from characteristic matrix adducts (labelled with an asterisk in Fig. 1) are described in detail elsewhere [7]. "M" corresponds to the molecular mass of the completely dissociated PL molecule, whereas "DHB : Na" indicates clusters of DHB with one H^+ exchanged by a sodium ion. This molecule thus remains uncharged. The addition of one DHB:Na produces a PL-matrix adduct with mass difference of 176 Da compared to a completely sodiated PL (*cf.* below).

The spectra of each of the above mentioned PLs were discussed in detail earlier [1, 6 – 8], and here the spectra characteristics will only be shortly summarised with the emphasis being on adducts with the matrix, or matrix cluster(s).

DPPA (1a) bears two negative charges, and after charge compensation with two cations and ionisation with a further one this PL is detectable at *m/z* = 693.5, 715.5 and 737.5 (corresponding to the addition of 2 H^+ and Na^+, H^+ and 2 Na^+ as well as 3 Na^+, respectively). In the positive ion MALDI-TOF mass spectra of DPPA, cluster adducts were not observed. Peaks at higher *m/z* are characteristic matrix peaks [7]. The positive ion MALDI-TOF mass spectrum of DPPC (1b) contains two peaks (at *m/z* = 734.6 and 756.6) that are generated by the cationisation of DPPC either with a proton or a sodium ion, respectively. The addition of a cluster DHB : Na to DPPC results in the peak at *m/z* = 932.6 DPPE (1c) yields more complex positive MALDI-TOF mass spectra in comparison to PC because the ethanolamine head group possesses an exchangeable proton. Thus, DPPE yields three peaks (*m/z* = 692.5, 714.5 and 736.5) corresponding to the charge compensation with H^+, Na^+ and the replacement of H^+ by Na^+, respectively. Also in this case, the DPPE-matrix cluster (the peak at *m/z* = 912.5) is generated only from the completely sodiated form of this PL.

In (1d) and (1e) the positive ion MALDI-TOF mass spectra of DPPI and DPPS, respectively, are given. These singly charged acidic PLs require charge compensation with one cation and ionisation with a further one to be detectable in positive ion mode. DPPI is detected at *m/z* = 833.5 and at 855.5, corresponding to the addition of H^+, Na^+ and 2 Na^+, respectively, whereas the spectrum of DPPS contains the following peaks at *m/z* = 736.5, 758.5 and 780.5, corresponding to the charge compensation with 2 H^+, H^+ and Na^+, and 2 Na^+, respectively. Moreover, with DPPS an exchange of the proton by a sodium ion on the head group yields one further peak at *m/z* = 802.5. PI as well as PS generate also clusters with DHB : Na, yielding peaks at *m/z* = 1031.5 in the spectrum of DPPI (1d) and at *m/z* = 1154.5 in the positive ion mass spectrum of DPPS (1e). Only for the completely sodiated forms of both of these

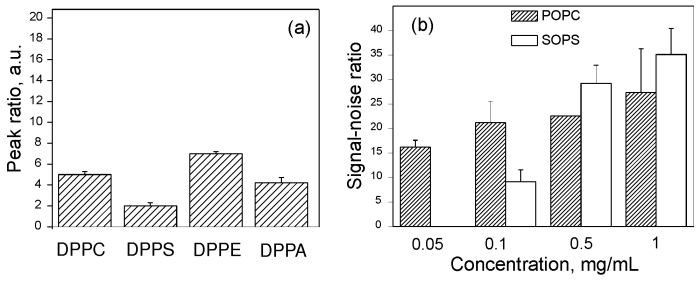

Fig. 2. The ratio between peak intensity of the completely sodiated form of the PL and of the peak intensity of the matrix clusters is given in (a); in (b) the signal-to-noise ratio of the peak arising from the cluster ion $[POPC+Na^+ +DHB{:}Na^+]^+$ and of the ion $[SOPS–H^+ +3Na^+ +DHB{:}Na^+]^+$ in relationship with the concentration of the lipid is shown. All spectra were recorded with DHB matrix, under delayed extraction condition and in the reflector mode. Presented results are the average of four spectra recorded.

Table 1. Identity of peaks detected in the negative ion MALDI-TOF mass spectra of PLs.

Peak position, m/z ratio	Peak identity
647.5	$[DPPA^{2-}+H^+]^-$
734.5	$DPPS^-$
809.5	$DPPI^-$
823.5	$[DPPA^{2-}+H^+ +DHB^- +Na^+]^-$
866.5	$[DPPE^+ –H^+ +DHB^- +Na^+]^-$
910.5	$[DPPS^- +DHB^- +Na^+]^-$
932.5	$[DPPS^- –H^+ +Na^+ +DHB^- +Na^+]^-$
985.5	$[DPPI^- + DHB^- +Na^+]^-$
999.5	$[DPPA^{2-}+H^+ +2(DHB^- +Na^+)]^-$
1042.6	$[DPPE^+ –H^+ +2(DHB^- +Na^+)]^-$

PLs, the addition of DHB : Na is leading to detectable peaks.

MALDI-TOF mass spectra of PLs were also recorded in the negative ion mode, and the detected peaks with their identities are given in Table 1. It is important to note the following: i) acidic PLs, such as DPPA, DPPS and DPPI, which were used in this study, require a lower number of cations in comparison to the positive mode to be detectable as negative ions; ii) neutral PLs are easily detectable as positive ions [6, 7], whereas in the negative ion mode they are detectable exclusively as clusters either with negatively charged DHB or with DHB : Na, in dependence on the nature of the head group. This behaviour, however, has been demonstrated in our previous work for choline-containing PLs [8].

Relative tendencies of various lipid species to produce matrix adducts that were detectable by MALDI-TOF MS in the positive ion mode are presented in Fig. 2(a). The ratio of the peak intensities of the completely sodiated form of DPPC, DPPS, DPPE and DPPA and the matrix cluster is presented. The intensity of the completely sodiated form of acidic PLs is lower compared to the intensity of the peak generated by the addition of a matrix cluster, at least as far as it is detectable in the positive ion mode. The opposite result is obtained for neutral PLs. Therefore, the acidic PLs tested in this study, DPPS and DPPA, show somewhat higher tendency to generate adducts with matrix clusters in comparison to neutral PLs (DPPC and DPPE).

To check whether the concentration of an analyte affects the intensity of peaks arising from adducts of PLs with the matrix, positive ion MALDI-TOF mass spectra were recorded at various concentrations of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (SOPC). Results of these experiments are shown in Fig. 2(b), and it can be observed that the signal intensities increase with increasing PL concentration. A signal arising from the matrix adduct of SOPC was not detectable at the lowest concentration of this PL, most probably due to already observed higher detection limits for acidic PLs, *i. e.* higher concentrations of them are required for their detection in the spectra in comparison to the neutral ones [10]. On the other hand, the intensity of the matrix adduct of POPC was rather high even at the lowest concentration. At highest concentrations tested, the intensity of signals arising from acidic PLs was comparable, even somewhat higher than the intensity of signals arising from POPC. Finally, by this experiment we have tested whether the generation of adducts with matrix clusters is related exclusively to PLs with saturated fatty acid residues. It has been shown that adducts with matrix cluster(s) are observable also in the case of PLs with unsaturated fatty acid residue(s).

In summary, we have demonstrated that different PLs can generate complex adducts with the DHB matrix and/or matrix clusters, thus leading to complex positive and negative ion MALDI-TOF mass spectra of PLs. The addition of one or, in some cases, two DHB : Na clusters was observed in the positive ion MALDI-TOF mass spectra of PLs – except in the case of highly acidic PA – as well as in the negative ion mode mass spectra of acidic PLs. Peaks generated by

the addition of one negatively charged DHB molecule were observed in the negative ion MALDI-TOF mass spectra of neutral PLs – DPPC and DPPE. The appearance and intensity of the peaks corresponding to these cluster ions cannot be completely explained at this stage. However, it might be that these additional peaks decrease the intensity of the PL peaks of interest, thus enhancing the risk of their misinterpretation. This risk is particularly high when unknown lipid/PL mixtures of biological origin are analysed by MALDI-TOF MS. To our knowledge, generation of PL-DHB clusters is favoured by (i) a high ion concentration and (ii) enhanced laser intensities. The tendency of various lipids to generate adducts with the matrix will be further investigated, and this report is a preliminary warning to those who use DHB/trifluoroacetic acid for the lipid analysis by MALDI-TOF MS.

Experimental Section

Chemicals

Matrix for MALDI-TOF mass spectrometry – 2,5-dihydroxybenzoic acid, DHB – as well as solvents – chloroform and methanol (with less than 0.00005 % of sodium and potassium) were purchased from Fluka (Neu Ulm, Germany). Phospholipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS) were obtained from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA) Phosphatidylinositol (1,2-dipalmitoyl-*sn*-glycero-phosphoinositol, DPPI) was purchased from Echelon Research Laboratories (Salt Lake City, Utah, USA). These chemicals were of highest commercially available purity and were used without further purification.

Preparation of samples for MALDI-TOF MS

PLs used in this study were applied as chloroform solutions at various concentrations. A small volume (1.2 μL) of a PL solution was applied onto the sample plate and immediately dried under a moderately warm stream of air. Afterwards, the same volume of a matrix solution (0.5 M DHB in methanol) was added over a lipid film and also immediately dried. This approach was shown to improve the homogeneity of the sample/analyte mixture and the reproducibility of the spectra. In some experiments, DHB was dissolved in methanol saturated with NaCl or KCl.

MALDI-TOF MS

MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry workstation (Perseptive Biosystems, Flamingham, MA, USA). The system utilises a pulsed nitrogen laser emitting at 337 nm. The pressure in the ion chamber was maintained between 1×10^{-7} and 1×10^{-4} Torr. Other conditions were: delayed extraction (DE), accelerated voltage of 20 kV and reflector mode, so that the total field-free time-of-flight distance was 2 m. These conditions significantly improved mass resolution and mass accuracy. An internal calibration was performed by setting the peak of the protonated DHB to its appropriate value (155.034 Da). Laser intensity was kept about 10 % over the threshold to obtain the best signal-to-noise ratio. All lipid spectra were acquired using a low-mass gate at 400 Da to prevent the detector from saturation by ions arising from matrix degradation or photochemical reactions.

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