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Characterization of Phospholipids by Electron Impact, Field Desorption and Liquid Secondary Ion Mass Spectrometry

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Dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were characterized by electron impact (EIMS), field desorption (FDMS) and liquid secondary ion (LSIMS) mass spectrometry. The results showed that the fragmentation processes of the three phospholipids are very similar. In the EI mass spectra, no molecule-ion peaks were found, the peaks of the fatty acid chains being dominant. The results of FDMS and LSIMS showed that both techniques worked particularly well with all three kinds of phospholipids. The FD mass spectra of all three phospholipid gave strong MH^+ peaks, and no fragmental peaks were found except that of DMPC. With LSIMS, the fragmentations of all the phospholipids were extensive and clear, and gave dominant peaks of MH^+. The cleavages around phosphate groups were the most abundant. An interesting phenomenon was found in the LSIMS measurements that all three kinds of phospholipids formed intense peaks of molecule-ion clusters, $[2M + H]^+$, $[3M + H]^+$ and $[4M + H]^+$, etc. It was found that the formation of the molecule-ion clusters was related to several factors: concentration, ionic strength, pH, etc., among which concentration is the most important. The degree of cluster formation increased with increase in concentration of phospholipid. The results also showed that the nature of the matrix is critical to a successful LSIMS analysis of phospholipids, and (*S*)-glycerol was confirmed as a very satisfactory matrix for these phospholipids.

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INTRODUCTION

Phospholipids are the main constituent of biological membranes, which play an important role in cell structure and function. As the surface compounds of the cells, lipids are considerably related to cellular recognition, specificity of the species, organism immunification, etc. The analysis of different kinds of phospholipids is thus essential to a full understanding of the contribution of phospholipids to membrane function. Many approaches have been taken for phospholipid analysis.[1–4] One approach involves cleavage of the molecules into lipid and phosphate constituents in order to cope with the detergent properties of these compounds.[1,2] With this approach, however, the structural identity of individual phospholipid molecular species may be lost. A more satisfactory method is removal of the phosphate group by hydrolysis with phospholipase C and analysis of the product 1,2-diacylglycerols by gas chromatography/mass spectrometry (GC/MS) as their trimethylsilyl or *tert*-butyldimethylsilyl derivatives.[5–7] The advantage of this approach is that the structural and positional integrity of the fatty acyl chains is retained. Although high-performance liquid chromatography (HPLC) allows the separation and quantitation of many phospholipid classes, no information is available concerning the number, type or structure of the individual molecular species within each class.[8] The combination of HPLC with UV detection has been used for the analysis of some important classes of lipids, especially lysophosphatidylcholine and diacylglycerol, but it is hampered by poor sensitivity because of their low UV absorbance. The soft ionization technique fast atom bombardment (FAB) has been shown to be an excellent technique for the analysis of phospholipids molecular species.[9–11] Intact phospholipids have been examined and in some cases considerable structural information was derived from a single experiment.[9,10,12–14]

Dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) are three kinds of important synthetic membrane phospholipids for the preparation of different kinds of model membranes in research on lipid–protein interactions. To our knowledge, there have been no report of systematic studies on these phospholipids by mass spectrometric techniques. In this work, we characterized these phospholipids by electron impact mass spectrometry (EIMS), metastable ion mass spectrometry, field desorption mass

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spectrometry (FDMS) and liquid secondary ion mass spectrometry (LSIMS), and studied the factors that induce molecule-ion cluster formation of the phospholipids in the process of LSIMS measurements.

EXPERIMENTAL

Materials

DMPG, DMPC and DMPE were purchased from Sigma, gave a single spot on thin-layer chromatograms using $CHCl_3$–MeOH–H_2O (65:25:4, v/v/v) as the migration solvent and were used without further purification. (*S*)-Glycerol was purchased from Fluka. Other reagents were analytically pure Chinese products. The structures of the phospholipids examined in this work are shown in Fig. 1.

Mass spectrometric measurements

Mass spectrometric analysis was carried out on a Finnigan MAT 90 double-focusing magnetic mass spectrometer equipped with a field desorption ion source and a liquid secondary ion source (with Cs^+ gun). The EIMS experiments were carried out under the following conditions: ionization energy 70 eV, emission current 1 mA, source/probe temperature of 220/200 °C, resolution 1000 (10% valley definition) and mass range 50–1000 u. Field desorption mass spectra were obtained under the following conditions: heating current 0–40 mA, high voltage 8 kV, mass range 50–3500 u and resolution 2000 (10% valley definition). The measurement of liquid secondary ion mass spectrometry was done under the following conditions: thioglycerol as the matrix, bombarding energy of Cs^+ ion beam 20 kV, heating current of emitter 1.4 A, emission current 5 μA, source temperature 25 °C, mass range 50–3500 u, resolution power of 2000 at 10% valley and acceleration potential 5 kV.

Metastable ion analysis. The data for metastable ion analysis were obtained from the first field-free region (*B*/*E* = constant); argon had been admitted to the collision activation chamber and thus multi-step fragmentation processes were detected because of collisional activation of the metastable transitions.

Liquid secondary ion mass spectrometric measurements. The phospholipids were dissolved in 5 μl of $CHCl_3$–MeOH (3:1, v/v), 1 μl of this solution was deposited on the target and dried in vacuum, then 1 μl of (*S*)-glycerol was placed on the target to dissolve the samples. The mass spectra are the results of more than ten accumulated scans.

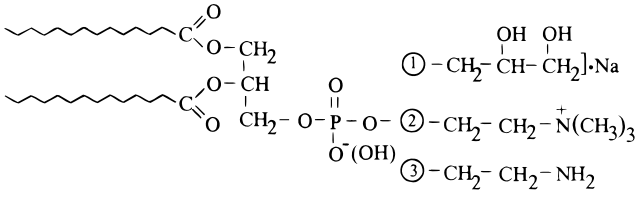


Figure 1. Structures of the phospholipids examined. (1) DMPG (M_r 688.4); (2) DMPC (M_r 677.5); (3) DMPE (M_r 635.5).

Effect of salt on molecule-ion cluster formation. NaCl was dissolved to the matrix (*S*)-glycerol to its saturant concentration at room temperature (about 25 °C). A 1 μl volume of this solution was used to dissolve the phospholipid sample deposited on the target, and then measured by LSIMS.

Effect of pH on molecule-ion clusters formation. The matrix of (*S*)-glycerol was adjusted to acidic pH (about 4.0) with 1 M HCl, or to basic pH (about 10.0) with 1 M NaOH. A 1 μl volume of this solution was used to dissolve the phospholipid samples deposited on the target, and then measured by LSIMS.

Effect of phospholipid concentration on molecule-ion cluster formation. The concentration of phospholipid in the matrix was varied from 10 ng to 15 μg. A 1 μl volume of this matrix was placed on the target and then measured by LSIMS.

Molecule-ion cluster formation from different phospholipid species. DMPG and DMPC [stock solution in $CHCl_3$–MeOH (3:1, v/v)] were mixed in a ratio of 2:1 (w/w). A 1 μl volume of this solution was placed on the target, dried in vacuum, dissolved on the target with 1 μl of (*S*)-glycerol and then measured by LSIMS.

RESULTS AND DISCUSSION

Electron impact and metastable ion mass spectra of phospholipids

The EI mass spectra of DMPG, DMPC and DMPE are shown in Fig. 2. The spectra of these three phospholipids are very similar. No molecule–ion peaks were observed. The base peaks are the same at *m*/*z* 211 which corresponds to the fatty acids chains. The other important peaks appeared at *m*/*z* 285 and 494. The peak at *m*/*z* 494 corresponds to the loss of the polar head group of the molecules. The peak at *m*/*z* 285 corresponds to the loss of a fatty acid chain from the ion of *m*/*z* 494. The other important peaks of each phospholipid species are shown in the spectra. The results showed that the EI mass spectra reflect mainly the structural information of the hydrophobic part of phospholipid molecules. Data analysis of the EIMS and metastable ionization MS results showed that the fragmentation processes of the three phospholipids are very similar. Combining the information from EI and metastable ion (Table 1) mass spectrometry, the main fragmentation processes can be represented as shown in Scheme 1.

Field desorption mass spectra of phospholipids

The FD mass spectra of the three phospholipids are shown in Fig. 3. All the spectra are simple and clear. In the spectra of DMPG and DMPE, the peaks of MNa^+ (*m*/*z* 711.5 for DMPG) and MH^+ (*m*/*z* 636.5 for DMPE)

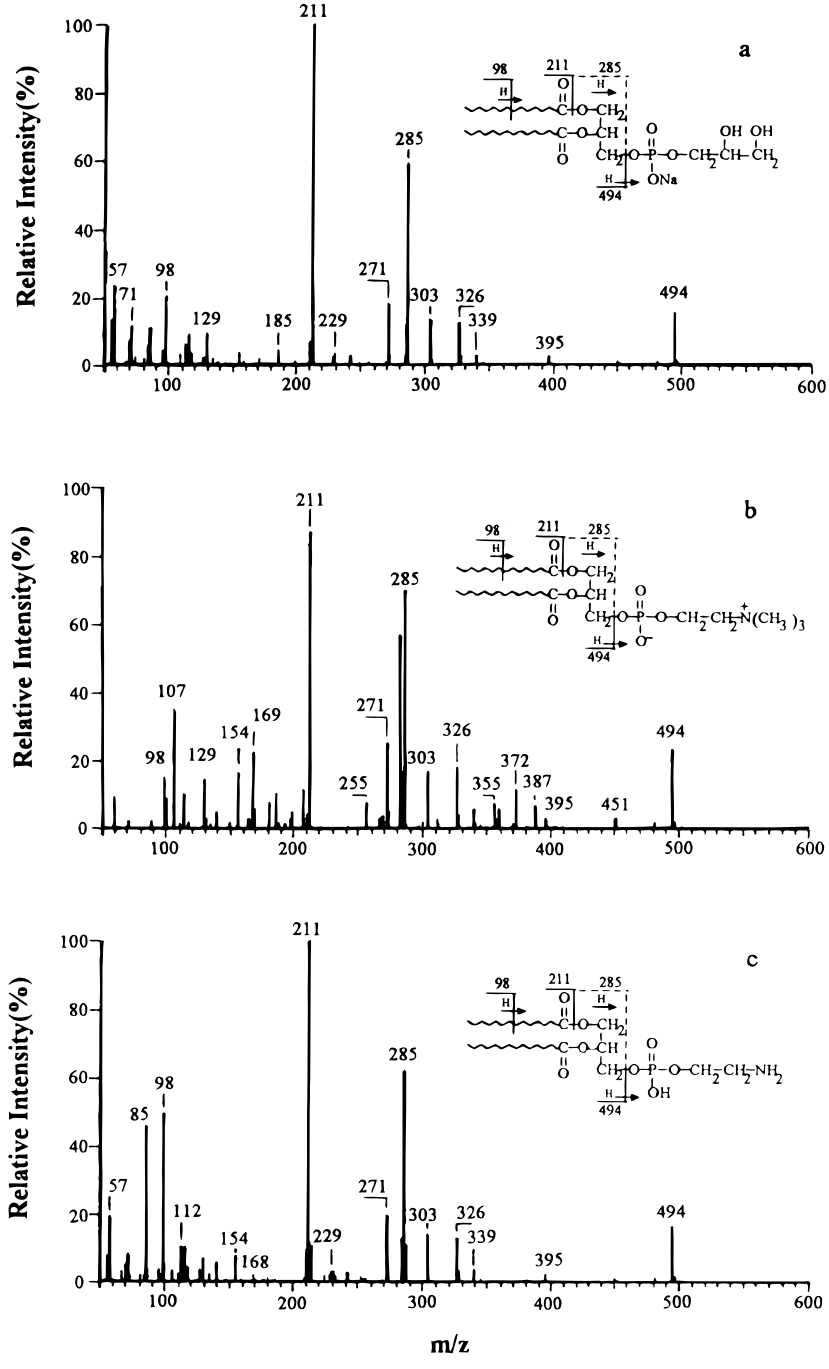


Figure 2. EI mass spectra of (a) DMPG, (b) DMPC and (c) DMPE.

are dominant and no substantial fragmentation can be observed. The MNa^+ peak is much higher than the MH^+ peak in the DMPG spectrum, which is probably caused by the effect of the high Na^+ concentration of the sample. The results indicate the DMPG and DMPE molecules are very stable under the FDMS ionization conditions. However, the spectrum of DMPC [Fig. 3(b)] is different from those of DMPG and DMPE. The intensity of the MH^+ peak (*m/z* 678.5) is relatively low, whereas the fragment peak at *m/z* 496 corresponding to the loss of the polar head group from MH^+ is dominant. This suggests that DMPC is unstable with the increased heating current required for desorption of this compound.

Liquid secondary ion mass spectra of phospholipids

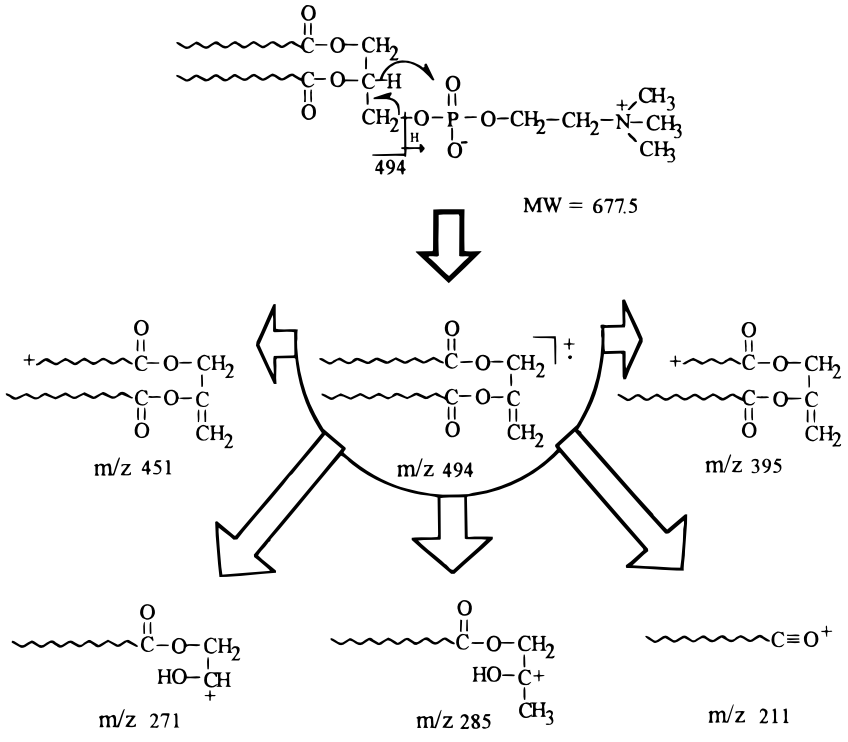
The LSI mass spectra of the all three phospholipids showed a wealth of fragment ion peaks (Fig. 4). In the spectrum of DMPG [Fig. 4(a)], the base peak is MNa^+ at *m/z* 711.6, and the protonated molecule-ion peak of MH^+ at *m/z* 689.5 appears with relatively low intensity,

Table 1. Metastable ion mass spectrometry of DMPG, DMPE and DMPC

Lipid	Parent ions (*m/z*)	Fragment ions (*m/z*)
DMPG	494[a]	451,[a] 437, 409, 395, 372, 319, 304, 285, 271, 211[b]
	395	381, 369, 303,[b] 286,[a] 272, 234
	326	312, 302,[a] 303,[b] 283, 271, 272, 249, 226, 210, 140, 127, 114
	303	261, 231, 226, 207, 171[a]
	285[a]	242, 218, 210,[a] 184, 154, 143, 129, 114, 107
	211[a]	167, 154, 108, 98, 95,[a] 93[a]
	185	170, 163,[a] 153, 139, 126, 111, 98[a]
	129	111, 98,[a] 85, 72
	98	81, 68, 55[a]
DMPC	501	465, 451,[a] 437, 423, 409, 395,[a] 381, 353
	494[a]	466, 451,[a] 437, 436, 409, 395, 333, 314, 285, 271, 211[b]
	451	436,[a] 347
	395	381,[a] 367, 353, 347, 286, 280, 266, 256, 242, 234, 231, 210, 281, 163
	372	353,[a] 339, 325, 311, 283, 269, 211
	326	282, 268, 240, 226,[a] 212, 184
	303	260, 226, 171[a]
	285[a]	268, 252, 242, 226, 210,[a] 198, 184,[b] 170, 154, 143, 129, 107
	211[a]	169, 167, 154, 110, 108, 98, 95, 93
DMPE	494[a]	466, 451, 437, 423, 395, 303, 285, 271, 211[a]
	395	381, 303,[b] 285,[a] 272
	303	285,[b] 252, 171[a]
	285[a]	269, 242, 218, 210,[a] 184, 170, 154, 129, 114, 107
	211[a]	167, 154, 108, 98, 95[a]

[a] Dominant peak in the spectrum.
[b] Second most intense peak in the spectrum.

which is probably caused by the effect of the high concentration of Na^+ in the solution as in the FDMS results above. The peak at *m/z* 195 is dominant, which caused by the cleavage around the phosphate group with the loss of hydrophobic chains and the migration of hydrogen. The other main peaks appear at *m/z* 637, 555, 502, 496, 484, 409, 143, 131 and 125. In the spectrum of DMPC (Fig. 4(b)), the base peak is at *m/z* 184, which is also the result of the cleavage around the phosphate group as the fragmentation of DMPG. It contrast to the FDMS results, DMPC molecules are stable under the LSIMS ionization conditions. The peak of MH^+ at *m/z* 678.6 is intense, and the peak of MNa^+ at *m/z* 700.5 was also observed. The results further con-



Scheme 1. Fragmentation of dimyristoylphosphatidylcholine in EIMS.

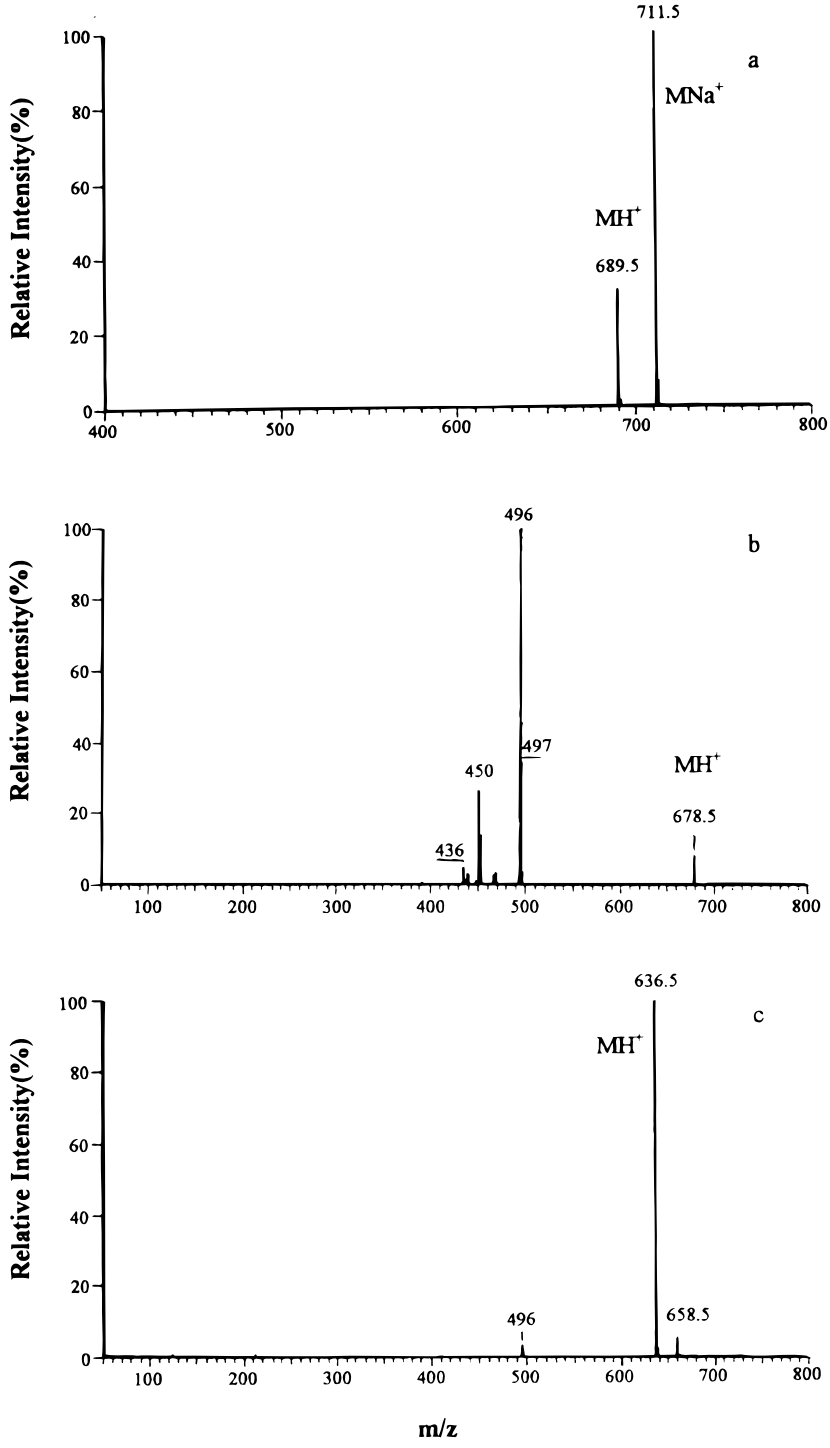


Figure 3. FD mass spectra of (a) DMPG, (b) DMPC and (c) DMPE.

firmed that the fragmentation of DMPC in FDMS was caused by the thermal induction of the heating current. The other important peaks appear at *m/z* 522, 496, 468, 450, 290, 104, 86 and 58. In the spectrum of DMPE (Fig. 4(c)), the base peak appears at *m/z* 496, which is the product of the cleavage around the phosphate group. However, in contrast to DMPG and DMPC, the charge remained in the hydrophobic part of the molecules after the cleavage (Scheme 2). The protonated molecule-ion peak at *m/z* 636.5 is intense, whereas the peak of MNa^+ at *m/z* 658.5 appears with low intensity. Other intense peaks appear at *m/z* 426, 339, 285, 254, 211, 142 and 124. Obviously, the fragmentations of the three kinds of molecules are different.

Comparing the three spectra, it can be seen that the changes in the polar head group of the phospholipids strongly affect the fragmentation process of these compounds. The cleavages around the phosphate groups are the most important to all the three phospholipids, but the resulting fragment ions are different. For

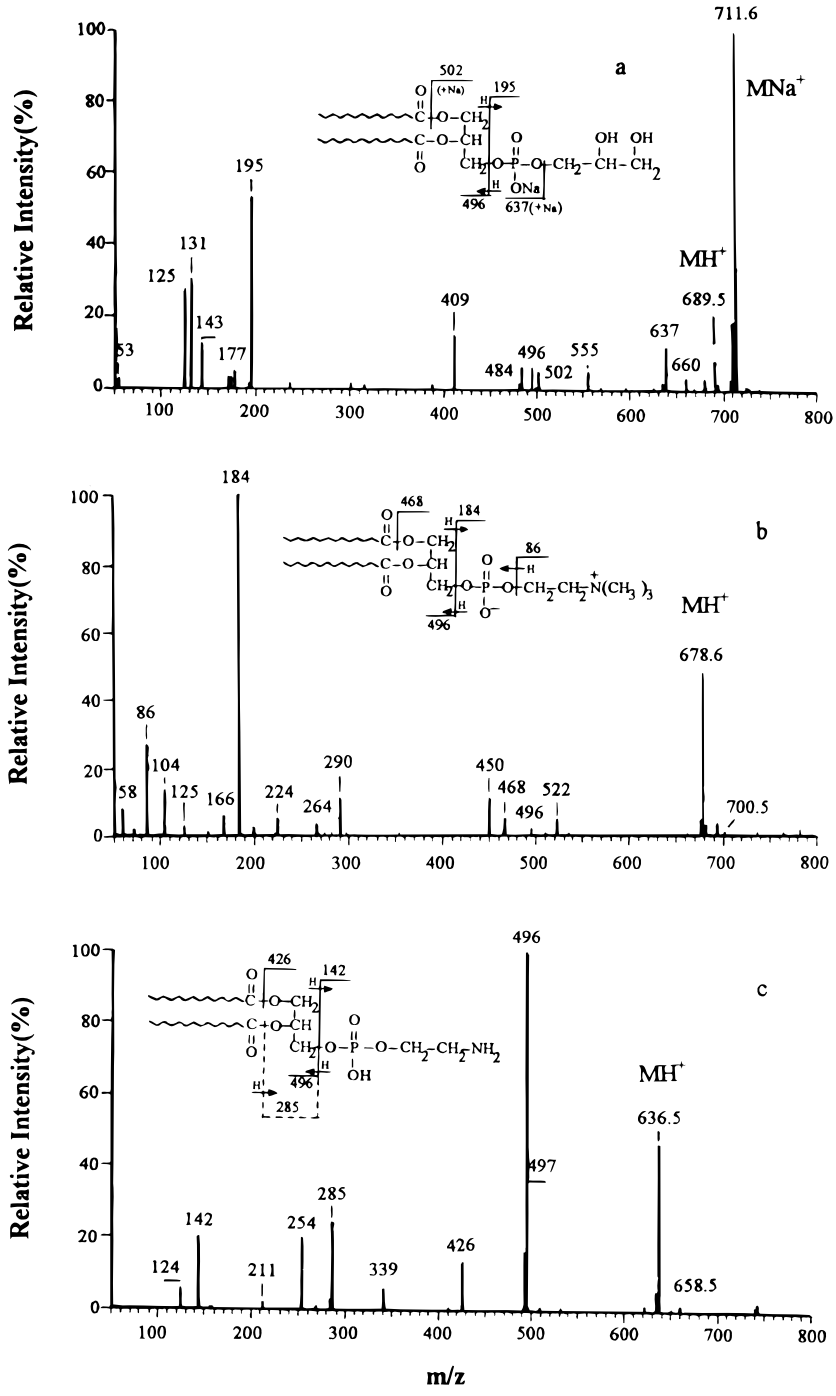
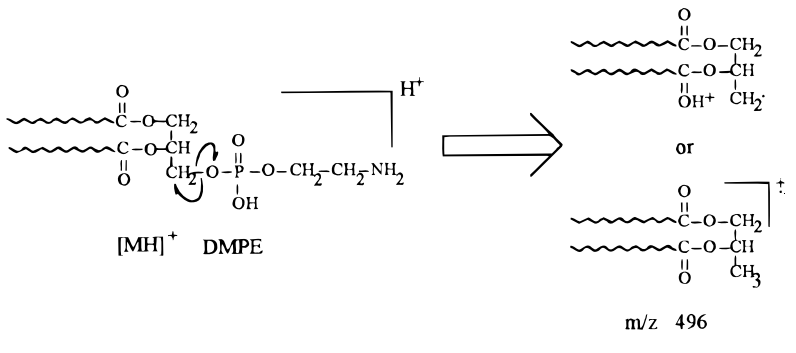


Figure 4. LSI mass spectra of (a) DMPG, (b) DMPC and (c) DMPE. (*S*)-Glycerol was used as the matrix.



Scheme 2. Proposed pathway for the formation of the fragment ion of *m/z* 496.

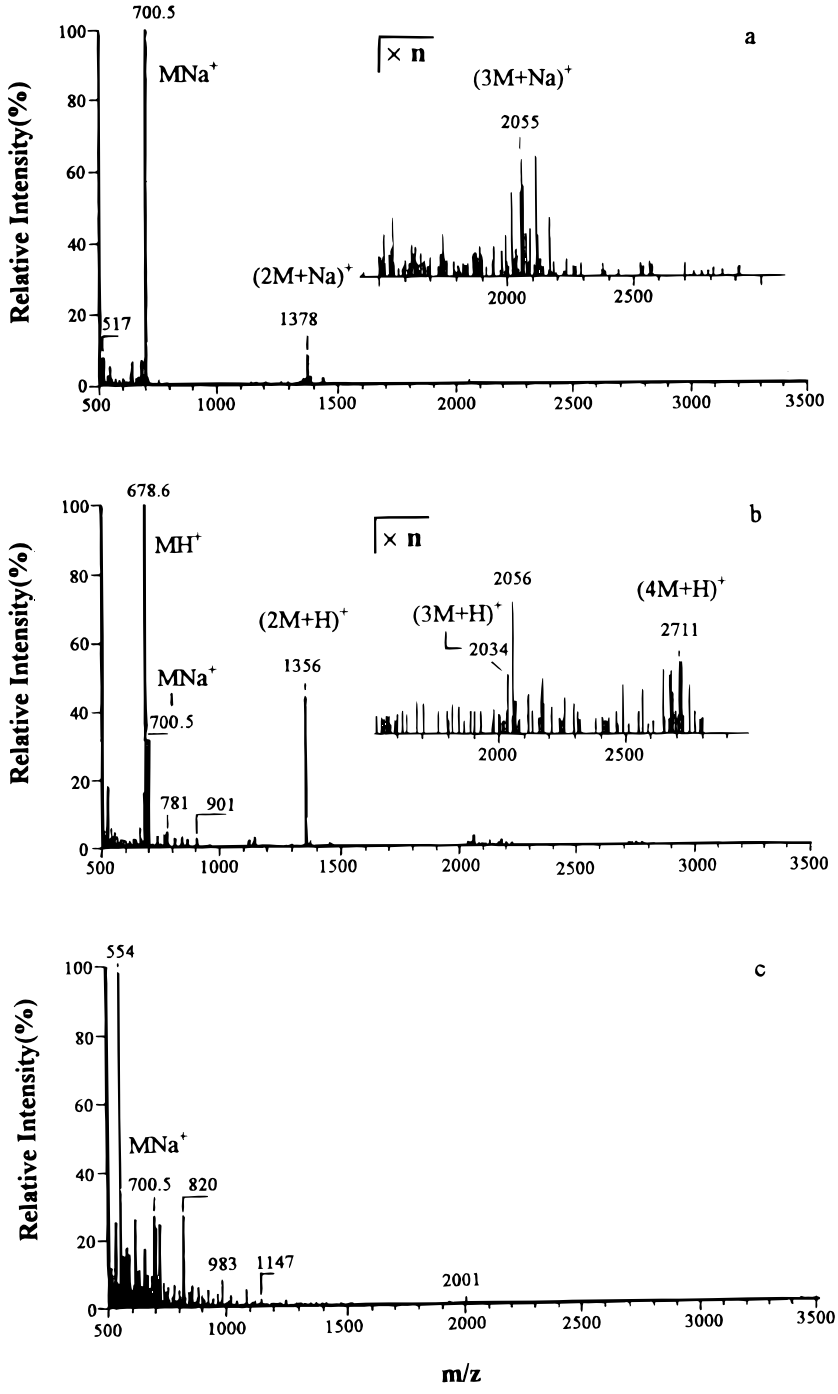


Figure 5. Effects of Na^+, H^+ and OH^- on the molecule-ion cluster formation by DMPC in LSIMS analysis. (a) Addition of NaCl (<0.1 M); (b) addition of HCl (to pH 4.0); (c) addition of NaOH (to pH 10.0). (*S*)-Glycerol was used as the matrix.

DMPG and DMPC the resulting ion is part of the polar head group (*m/z* 195 and 184), whereas for DMPE the resulting ion is the hydrophobic part of the phospholipid molecules (*m/z* 496). This is probably because the protonated position on the three kinds of phospholipid molecules were different in the process of LSIMS ionization. For DMPG and DMPC, the protonation occurred at the polar head group of the molecules, but for DMPE, the protonation occurred at the oxygen of the carbonyl of the hydrophobic part. Therefore different ions were produced by the cleavages around the phosphate group.

Of further interest is the presence of the high-mass peaks at *m/z* 1300–1500, 1900–2200, 2600–2900 and 3300–3500 [see Fig. 6(d)]. These signals are related to the dimer, trimer, tetramer and pentamer, respectively, formed by the phospholipid molecules. The intensities of these peaks decreased with increase in the size of clusters. This implies that the stability of molecule-ion clusters decreases with increase in the size of clusters. Peaks of $[2M + H]^+$ have been observed in the FAB spectra of individual phospholipid molecular species.[15–17] However, polymer formation from the same or different kinds of phospholipid monomers

under LSIMS conditions has not been reported previously.

The presence of singly charged cluster ions or multiply charged single-molecular ions often makes mass spectra too complicated to interpret, especially in the analysis of mixtures. On the other hand, it is useful for the investigation of the interactions among phospholipid molecules. Therefore, it is important to recognize them for both the interpretation of mass spectra and the investigation of the interactions among phospholipid molecules. In the following section we systematically consider the dependence of molecular-ion cluster formation on pH, ionic strength, concentration, etc.

Effect of salt on molecule-ion cluster formation

The LSI mass spectrum of DMPC with Na^+ is shown in Fig. 5(a). On comparison with the spectrum obtained without the addition of salt [Fig. 6(c)], an interesting change was found that the protonated molecule-ion peak MH^+ (*m/z* 678.6) decreased sharply, whereas the

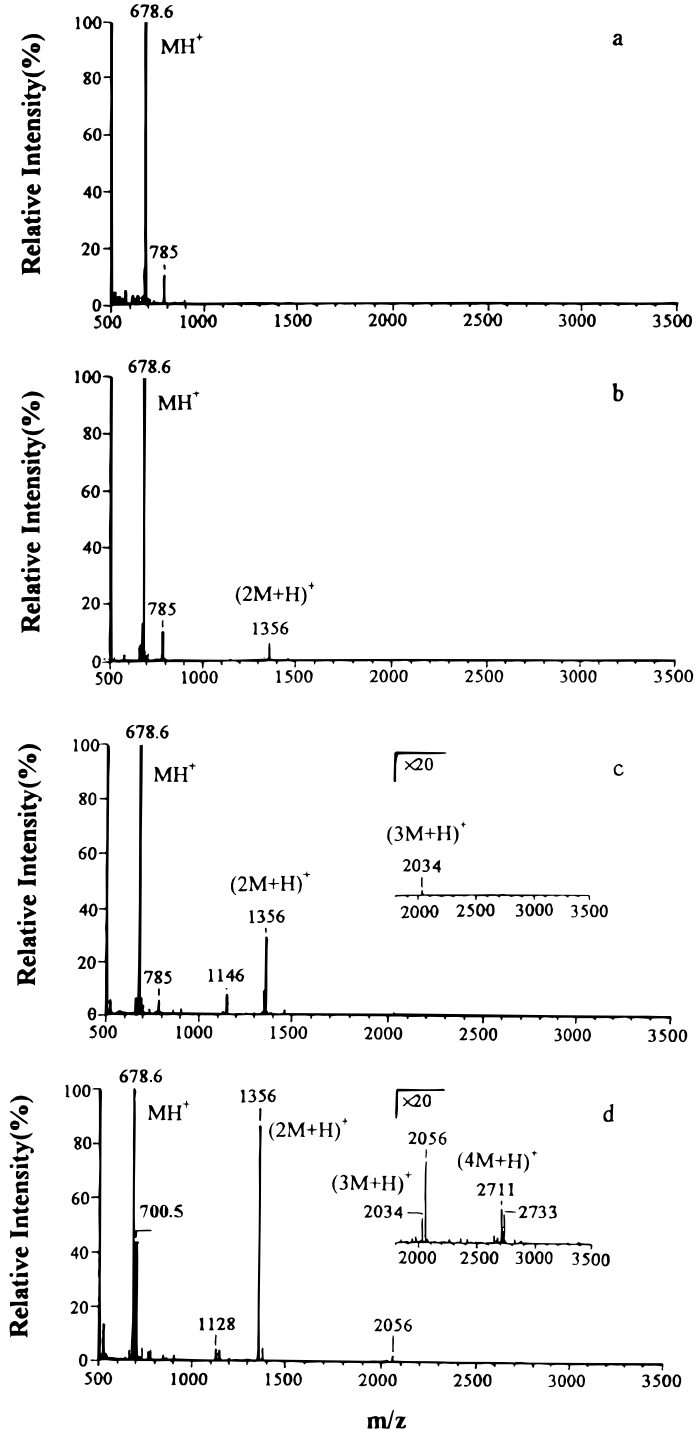


Figure 6. Change in LSI mass spectra with phospholipid concentration. Concentration of DMPC in the matrix: (a) 10 ng μl^{-1}; (b) 100 ng μl^{-1}; (c) 1 μg μl^{-1}; (d) 15 μg μl^{-1}.

MNa^+ (*m/z* 700.5) peak became the base peak. The other change was that the peaks of molecule-ion clusters such as $[2M + H]^+$, $[2M + Na]^+$ and $[3M + H]^+$ decreased sharply and the spectrum became clearer. This result indicates that for normal LSIMS measurements of phospholipids, the addition of salt into the matrix is a very effective method for simplifying the spectra.

Effect of pH on molecule-ion cluster formation

The LSI mass spectra of DMPC at different pH are shown in Fig. 5(b) (pH 4.0) and 5(c) (pH 10.0). In comparison with the spectrum in Fig. 6(d) (obtained with the same phospholipid concentration), it can be seen that the addition of H^+ did not cause obvious changes in the spectra. The MH^+ peak is the base peak, and the cluster-ion peaks of $[2M + H]^+$, $[3M + H]^+$ and $[4M + H]^+$ are also observed, the only difference being that the $[M + H]^+/[nM + H]^+$ ratio increased slightly. In contrast, the addition of OH^- made the intensity of the molecule-ion peak decrease sharply, and resulted in a very poor spectrum.

Effect of phospholipid concentration on molecule-ion cluster formation

The results in Fig. 6 show that the intensity of the peaks of molecule-ion clusters increased with increase in phospholipid concentration in the matrix, whereas the intensity of the molecule-ion peak decreased. The results indicate that the high concentration of phospholipid contributes to the formation of molecule-ion clusters in the process of LSIMS analysis.

Molecule-ion cluster formation from different phospholipid species

The results are shown in Fig. 7. In the spectrum, the peaks of the protonated molecule-ion MH^+ of the two phospholipids at *m/z* 678.6 (DMPC + H) and *m/z* 689.5 (DMPG + H) appear with low intensities, whereas the peaks of MNa^+ at *m/z* 700.5 (DMPC + Na) and *m/z* 711.5 (DMPG + Na) became the base and dominant peaks, respectively, under the effect of the Na^+ of the DMPG sample. In addition to these peaks, the cluster-ion peaks were also observed. The important cluster-ion peaks were as follows: dimer, *m/z* 1356 $[2DMPC + H]^+$, *m/z* 1378 $[2DMPC + Na]^+$, *m/z* 1389 $[DMPG + DMPC + Na]^+$ and *m/z* 1400 $[2DMPG + Na]^+$; trimer, *m/z* 2056 $[3DMPC + Na]^+$, *m/z* 2066 $[2DMPC + DMPG + Na]^+$, *m/z* 2077 $[2DMPG + DMPC + Na]^+$ and *m/z* 2088 $[3DMPG + Na]^+$; tetramer, *m/z* 2744 $[3DMPC + DMPG + Na]^+$, *m/z* 2755 $[2DMPC + 2DMPG + Na]^+$ and *m/z* 2766 $[DMPC + 3DMPG + Na]^+$; and pentamer, *m/z* 3411 $[5DMPC + Na]^+$, *m/z* 3421 $[DMPG + 4DMPC + Na]^+$, *m/z* 3443 $[3DMPG + 2DMPC + Na]^+$, *m/z* 3454 $[4DMPG + DMPC + Na]^+$ and *m/z* 3465 $[5DMPG + Na]^+$. The results indicate that the clustering ability among different phospholipid molecules is also strong, and cluster formation is probably a stochastic process.

CONCLUSION

The results of this study indicate that both FDMS and LSIMS worked particularly well with the phospholipids, no matter whether the molecular were charged or not. LSIMS provided a wealth of fragment ions for the three phospholipids. These ions provided important structural information such as the length of fatty acid chains and the species of polar head of phospholipid. FDMS of phospholipids provided less fragmentation, thus giving little information about the structure. On the other hand, FDMS was less destructive to the phospholipid molecules than LSIMS/FABMS and EIMS, etc., and it is a better method for obtaining general information about the distribution of the molecular weight of a phospholipid mixture. The traditional techniques of electron impact ionization and metastable

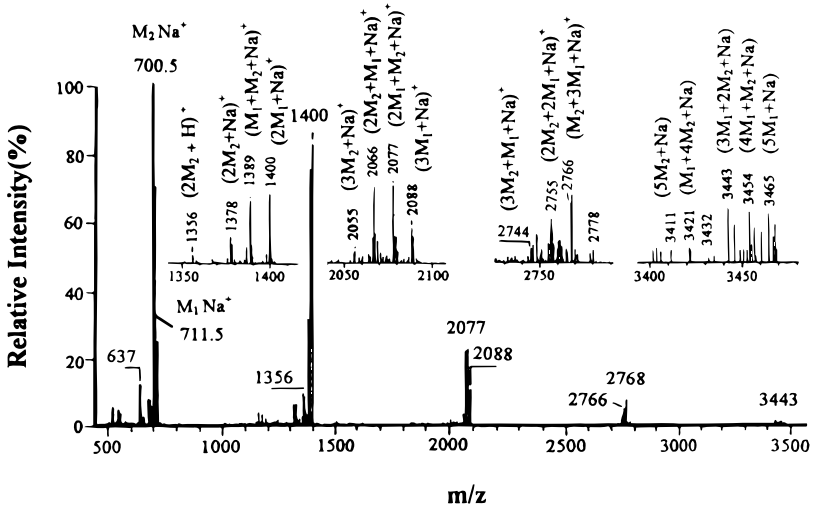


Figure 7. LSI mass spectrum of a mixture of DMPC and DMPG (2:1, w/w) M_1 = DMPG; M_2 = DMPC.

ions can also provide extensive and reasonable structural information, especially for identifying the fatty acid chains of phospholipids, which is mutually complementary with the LSIMS technique to a certain extent for the analysis of phospholipids.

It is evident from these studies that the nature of the matrix is critical to a successful LSIMS analysis. (*S*)-Glycerol was a satisfactory matrix for the LSIMS analysis of all three kinds of phospholipids. With this matrix, the phospholipids all gave dominant peaks of MH^+ (or MNa^+). The fragmentation of all the three phospholipids was extensive and clear. The peaks caused by the cleavages around the phosphate groups were dominant.

The phenomenon of molecule-ion cluster formation is an important characteristic of LSIMS spectra of phospholipids. All three phospholipids formed considerably intense peaks of molecule-ion clusters in LSIMS analysis. The formation of the clusters is related to several factors, among which the concentration of sample in the matrix is the most important. The degree of cluster formation increased with increase in the concentration of phospholipids in the matrix.

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