JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. **34**, 661–669 (1999)

Comparative Mass Spectrometric Analyses of Photofrin Oligomers by Fast Atom Bombardment Mass Spectrometry, UV and IR Matrix-assisted Laser Desorption/Ionization Mass Spectrometry, Electrospray Ionization Mass Spectrometry and Laser Desorption/Jet-cooling Photoionization Mass Spectrometry

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Photofrin® (porfimer sodium) is a porphyrin derivative used in the treatment of a variety of cancers by photodynamic therapy. This oligomer complex and a variety of porphyrin monomers, dimers and trimers were analyzed with five different mass spectral ionization techniques: fast atom bombardment, UV and IR matrix-assisted laser desorption/ionization, electrospray ionization, and laser desorption/jet-cooling photoionization. All five approaches resulted in very similar oligomer distributions with an average oligomer length of 2.7 ± 0.1 porphyrin units. In addition to the Photofrin analysis, this study provides a side-by-side comparison of the spectra for the five different mass spectrometric techniques.

KEYWORDS: photofrin; photodynamic therapy; oligomer distribution; mass spectrometry; fast atom bombardment; matrix-assisted laser desorption/ionization; electrospray ionization; laser desorption/jet cooling photoionization

INTRODUCTION

Photofrin® (United States Adopted Name (USAN): porfimer sodium) has been developed as a parenteral drug for use in photodynamic therapy (PDT) for the treatment of solid tumors. PDT involves dosage of a patient with a photosensitizing drug, preferential retention of the drug by tumors and light activation of the drug which results in control or palliation (partial remission) of the tumor. Photofrin was approved by the US FDA in 1995 for opening airways obstructed by advanced esophageal cancers that cannot be treated with thermal laser therapy. In 1997, it was approved for early stage lung cancer and in 1998 for advanced stage lung cancer. Photofrin is also available for several other indications. It was approved in Germany for the treatment of early stage lung cancer, in France and The Netherlands for early and advanced cancers of the lung and esophagus, in Canada for bladder and esophageal cancer and in Japan for the early stages of lung, esophageal, gastric and cervical cancers.

Photofrin is a complex mixture of non-metallic oligomeric porphyrins, linked primarily through ether bonds[1,2] (**1**, Fig. 1). A brief review of the literature serves to illustrate the difficulty in characterizing the complex Photofrin mixture. Initial characterization efforts employed the unpurified mixture obtained following a 1 h reaction in base (0.1 M NaOH) of the hematoporphyrin acetates, a product often referred to as hematoporphyrin derivative (HPD) or sometimes as Photofrin I. At first, HPD was considered to be a mixture of hydrolysis products that probably formed aggregates,[3,4] although dimers and higher oligomers were suggested as contaminants.[5] After Dougherty *et al.*[2] had determined that only a portion of HPD localized in tumors, the active components in PDT were proposed to be hematoporphyrin ether-linked dimer isomers, referred to as dihematoporphyrin ether (DHE).[6] Purification of this tumor-localizing fraction, often cited as Photofrin II, led to the development of a diafiltration process for the manufacture of Photofrin as a pharmaceutical. Sommer *et al.*[7] also postulated the presence of

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CCC 1076–5174/99/060661–09 $17.50

Received 31 December 1998
Accepted 5 March 1999

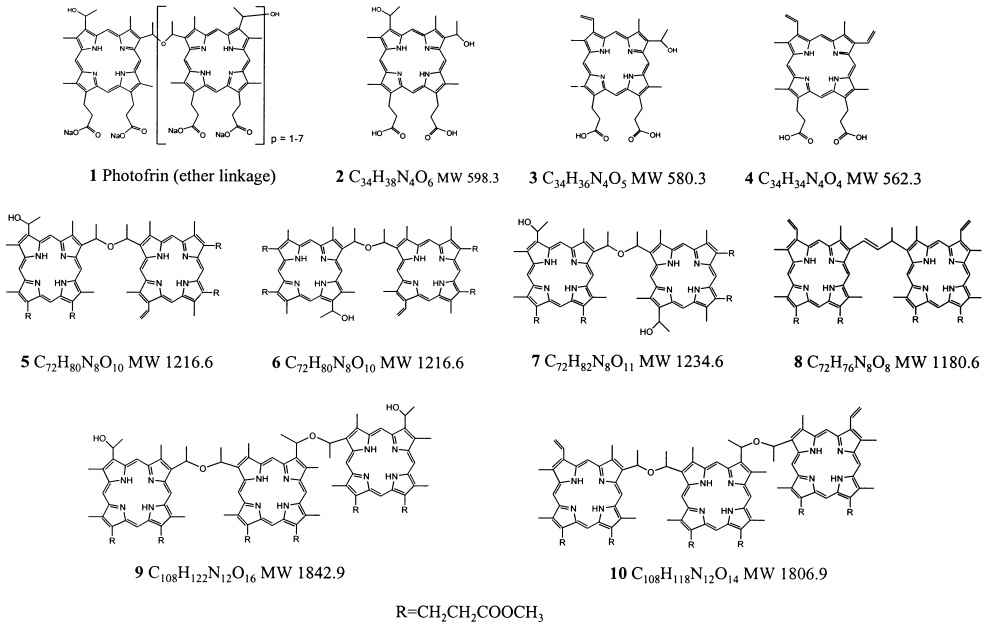

Figure 1. Proposed structure of Photofrin (**1**) (with ether linkages), Photofrin monomer precursors hematoporphyrin (**2**), hydroxyethylvinyldeuteroporphyrin (one of two isomers) (**3**), protoporphyrin (**4**) and Photofrin methyl ester model dimers (**5, 6, 7, 8**) and trimers (**9, 10**).

a few dimers, with the possibility of oligomers. Kessel and Cheng[8] investigated the activity of the higher molecular mass fractions, previously thought to be comprised of aggregates and dimers, and identified the presence of oligomers higher than dimers. Controversy existed for a while over the nature of the porphyrin oligomer linkages. Ester bonds hydrolyze in aqueous base, whereas ether bonds are stable. Using this information, with additional evidence, Kessel *et al.*[9] proved the predominance of ester-linked porphyrins. Byrne *et al.*[1] helped resolve the discrepancy between ether and ester bond linkages in Photofrin by comparing the method of preparation of the hematoporphyrin diacetate. The HPD prepared by Kessel *et al*. from pure hematoporphyrin diacetate instead of mixed acetates was initially much richer in ester-linked material. However, in time the ester linkages in this material were also replaced by the more stable ether linkages.

Great strides have been made in the analysis of large molecules by mass spectrometry (MS). Previously this type of analysis suffered from an inability to vaporize and ionize large molecules without substantial fragmentation. While a moderate amount of controlled fragmentation can be useful for structure analysis, extensive fragmentation of larger molecules tends completely to obscure molecular identification. Now a number of new techniques have been developed that make it possible to obtain parent molecular masses for biomolecules and polymers with masses up to several hundred thousand dalton. Great progress has been reported with the following ionization techniques: (1) fast atom bombardment (FAB),[10–14] (2) UV and (3) IR matrix-assisted laser desorption/ionization (MALDI),[15–18] (4) electrospray ionization (ESI)[19] and (5) two-step laser desorption/laser photoionization (LD/PI),[20,21] which can also include jet-cooling (LD/jet-PI).[22–28] As is usually the case with analytical approaches, each of these techniques has its own advantages and disadvantages. It is therefore especially interesting to compare them side-by-side while studying the same challenging problem of structural characterization. Such direct comparisons are also important for other reasons. When studying complex oligomer distributions, the issues of minimizing fragmentation and non-covalent multimer formation become especially relevant, since fragments of larger oligomers could be counted as smaller oligomers and non-covalent multimers can be counted as larger oligomers, thus skewing the observed distribution in either direction. This is problematic especially in the practical situation when the real oligomeric distribution is unknown and needs to be determined in the absence of pure oligomer standards. In such cases, comparisons between a number of techniques are most valuable. If indeed all the techniques result in comparable distributions, the confidence in the final result is significantly enhanced. In the present study, the comparative mass spectral analysis of Photofrin, a complex mixture of porphyrin oligomers was evaluated by five different ionization techniques. One underlying assumption in the study was that the detection efficiency for each of the observed oligomers was mass independent in each of the ionization techniques used. The determination of the composition of Photofrin was needed for the characterization of the material as a drug for regulatory agencies.

In earlier work, FABMS was used to characterize Photofrin, which was found to be a mixture of oligomers containing up to nine porphyrin units.[29–32] A FAB matrix was developed which consisted of a glycerol–thioglycerol–trifluoroacetic acid (TFA) mixture to suppress the effect on the spectra of the high salt content present in Photofrin. In recent work by Zhan *et al.*[21] using an LD/PI-MS instrument without jet cooling, only Photofrin porphyrin monomers were observed which led them to suggest that Photofrin consists of micelles of monomeric porphyrin species. More recently Fenyo *et al.*[33] used UV-MALDI-MS for the analysis of synthetic porphyrin 'arrays' and observed low abundance parent ion masses with significant amounts of fragmentation. Fragmentation was very extensive when a matrix was not used since

the porphyrins are photochromic. Even more recently, Aplin and co-workers[34] reported the use of UV-MALDI-MS for the characterization of synthetic conjugated porphyrin oligomers (dimer through hexamer) and observed that the $M^{+\bullet}$ was the dominant ion in each spectrum. The inconsistencies between these results point to the central difficulty in the mass spectrometric analysis of complex oligomeric compounds and our desire to characterize Photofrin more fully by comparing the mass spectra of the drug obtained using a variety of mass spectral 'soft' ionization techniques.

Owing to the chemical complexity of Photofrin and the limited wavelength range possible for photodynamic therapy with Photofrin, second-generation compounds for photodynamic therapy are being developed which are pure single chemical entities and are applicable over a variety of wavelength ranges.[35]

EXPERIMENTAL

Materials

Photofrin, manufactured as a sodium salt (**1**), was obtained from QLT Photo Therapeutics (Vancouver, BC, Canada) and Lederle Parenterals (Carolina, Puerto Rico). Typically, the sodium content of the salt form of Photofrin was ~6% (w/w),[32] and the total porphyrin monomer (**2, 3, 4**) content was not more than 17% (w/w) as determined by a gradient reversed-phase high-performance liquid chromatographic (HPLC) method with a C_{18} column. The desalted form of Photofrin was prepared by collecting and washing with water the precipitate generated from a solution of Photofrin titrated with 1 M HCl to pH 4. Photofrin was converted to the corresponding permethyl ester by reaction with diazomethane.[36] The monomeric hematoporphyrin reference standards hematoporphyrin, **2** (M_r 598.3), hydroxyethylvinyldeuteroporphyrin (two isomers), **3** (M_r 580.3), and protoporphyrin, **4** (M_r 562.3) were purchased from Porphyrin Products (Logan, Utah, USA) and used as received. Four porphyrin methyl ester dimers, **5** (M_r 1216.6), **6** (M_r 1216.6), **7** (M_r 1234.6) and **8** (M_r 1180.6), and two porphyrin methyl ester trimers, **9** (M_r 1842.9) and **10** (M_r 1806.9), were synthesized as regiochemically pure components. The synthesis of regiochemically pure ether linked dimers (**5, 6, 7**) and trimers (**9, 10**) required the ready availability of large quantities of isomerically pure monoacetylmono(1-hydroxyethyl)deuteroporphyrin-IX dimethyl esters which were prepared in a single step from hematoporphyrin-IX dimethyl ester as described previously.[37] Dimers **5** and **6** were prepared from 4-acetyl-2-(1-hydroxyethyl)- and 2-acetyl-4-(1-hydroxyethyl)- deuteroporphyrin dimethyl ester, respectively. These were separately converted to the corresponding 1-bromoethyl analogs, which on reacting with 2-(1-hydroxyethyl)-4-vinyldeuteroporphyrin-IX dimethyl ester gave the corresponding acetyl dimers.[38] The acetyl groups of the isolated intermediate products were then reduced to the corresponding 1-hydroxyethyl derivatives after reacting with a sodium borohydride–methanol solution. Ether-linked dimer **7** and trimers **9** and **10** were synthesized in a similar manner using appropriate starting materials.[36] Carbon-linked dimer **8** was prepared from 2-acetyl-4-(1-hydroxyethyl)deuteroporphyrin-IX dimethyl ester on treatment with trifluoromethanesulfonic acid (triflic acid) (Aldrich). The isolated intermediate product was then reacted with sodium borohydride followed by dehydration with *p*-toluenesulfonic acid in refluxing *o*-dichlorobenzene to produce the desired product.[39]

Mass spectrometry

Fast atom bombardment mass spectrometry (FABMS). The low-resolution positive ion FABMS experiments were performed on a Micromass ZAB-SE high-performance mass spectrometer equipped with a Micromass SIOS-OPUS data system and a Micromass cesium ion gun which was operated at 35 kV. The accelerating voltage was 8 kV. The FAB matrix used for the Photofrin samples consisted of a glycerol–thioglycerol–TFA mixture prepared as a 50 : 50 : 0.5 (v/v) solution. This matrix mixture previously was found to neutralize the suppression of signal in the FAB mass spectrum owing to the high salt content of Photofrin (~6% (w/w) NaCl).[32] To optimize the sensitivity over the wide mass range needed for analyzing Photofrin, the instrument was scanned over the two ranges, m/z 350–2600 and m/z 2000–5000, with scan rates of 25 and 40 s per decade, respectively. The instrument resolution was set at 700. The Photofrin samples were prepared in deionized water at a concentration of ~10 μg μl^{-1}. About 2 μl of the sample were added to the FAB tip with ~2 μl of the FAB matrix. The spectra were acquired by signal averaging ~10 replicated scans. The two spectra were normalized using the data appearing in the overlapping region from m/z 2000 to 2600. The instrument was calibrated with cesium iodide.

UV and IR matrix-assisted laser desorption/ionization mass spectrometry (UV- and IR-MALDI-MS). The mass spectrometer used for acquiring the UV- and IR-MALDI mass spectral data was a laboratory-built reflector-type time-of-flight mass spectrometer and was operated in the positive ion mode. UV-MALDI studies utilized a nitrogen laser (Laser Science, Newton, MA, USA), wavelength 355 nm, with a pulse width of 5 ns and spot size of 100 μm. IR-MALDI studies utilized an Er : YAG laser (Spektrum, Berlin, Germany), wavelength 2.94 mm (3401 cm^{-1}) with a pulse width of ~90 ns and spot size of ~150 μm. The UV and IR laser irradiances were each about 10^6–10^7 W cm^{-2}. Ions were accelerated through total potential differences of 12.3 kV for static extraction of the ions. The spectra acquired consisted of a sum from 10 to 20 single acquisitions. The matrix used for both UV- and IR-MALDI was 2,5-dihydroxybenzoic acid prepared as a saturated solution in 2 : 1 (v/v) 0.1% aqueous TFA–acetonitrile. The Photofrin samples were prepared for MALDI/MS to a concentration of about 0.10–0.75 mg ml^{-1} in ultrapure water. Aliquots of the samples (0.5–3.0 μl) were mixed with equal volumes of the 2,5-dihydroxybenzoic acid matrix, deposited on stainless-steel targets, air dried and then inserted into the instrument. The final matrix-to-analyte molar ratio was ~10^6 for UV-MALDI and ~10^5 for IR-MALDI, corresponding to the matrix conditions for optimum signal intensity and resolution.

Electrospray ionization mass spectrometry (ESIMS). Electrospray ionization mass spectra were obtained in both

positive and negative ion modes with a Micromass Quattro I triple quadrupole mass spectrometer equipped with a Micromass electrospray source, r.f. hexapole lens and Megaflow gas nebulizer probe. For positive ion studies, the capillary sprayer voltage was set to ~3.0 kV, the high voltage lens set to 250 V, the nozzle–skimmer voltage was varied over the range of 20 to 200 V, the nozzle–skimmer offset was 5 V and lens 3 was set in the range from −60 to −100 V (for the study of negative ions, the polarities were reversed.) The nozzle–skimmer voltage was varied to study the effects of collision energy on ion abundances and fragmentation of the multiply charged Photofrin species. The source temperature was maintained at ~75 °C. The nebulizer and bath gases were nitrogen delivered at flow-rates of 10 and 300 l h^{-1}, respectively. The Photofrin samples were prepared to a concentration of ~10 pmol μl^{-1} in the following solvent systems: for positive ion studies 1 : 1 (v/v) 30% aqueous acetic acid–acetonitrile, and for negative ion studies 2 : 1 (v/v) 15% aqueous ammonia–acetonitrile. About 10 μl of each of the Photofrin samples were infused into the source of the mass spectrometer at a flow-rate of ~10 μl min^{-1} utilizing a carrier solvent of 1 : 1 (v/v) acetonitrile–water with a dual syringe pump (ABI, Model 140B). Data were acquired at 16 data points per *m/z* over the mass range *m/z* ~300–4000 with scan times of 15–30 s. About 10–20 spectra were averaged, smoothed and baseline subtracted. The mass spectrometer was calibrated with sodium iodide or polyethylene glycol mixtures.

Laser desorption/jet-cooling photoionization mass spectrometry (LD/jet-PI-MS). Details of the set-up for LD/jet-PI-MS have been described.[26] Photofrin samples were prepared by depositing material out of solution on graphite substrates and allowing the solvent to evaporate. Desorption was achieved with pulses from a frequency-doubled Nd : YAG laser, wavelength 532 nm, at fluences of the order of 1 mJ cm^{-2}. Desorbed material was entrained in a supersonic expansion with Xe drive gas, injected by a pulsed solenoid valve. Desorption took place on the vacuum side of the valve, within 2 mm of the 1 mm opening. Downstream the entrained molecules were photoionized at 193 nm (excimer laser) or 125 nm (obtained from sum frequency mixing in Hg vapor). The ions were detected with a reflectron time-of-flight mass spectrometer (R. M. Jordan, Grass Valley, CA, USA). This set-up is optimized for detecting desorbed neutral molecules with photoionization.

RESULTS AND DISCUSSION

The strategy for characterizing Photofrin was as follows. The desalted and permethylated forms of Photofrin were analyzed using the five 'soft' ionization methods of FAB, UV- and IR-MALDI, ESI and LD/jet-PI. The spectra obtained were used to determine and compare the Photofrin oligomer distributions for each of the ionization methods. In addition, various Photofrin monomer precursors and various Photofrin model dimers and trimers were also analyzed using the above five 'soft' ionization methods. The mass spectra of the precursor and model compounds were compared to determine the softness of the ionization methods with emphasis on the degree of fragmentation and the extent of cluster/micelle formation. The results of these observations for the Photofrin precursor and model compounds were then correlated with the mass spectral results obtained for the desalted and permethyl ester forms of Photofrin.

Desalted photofrin

Desalted Photofrin was analyzed by FABMS, UV- and IR-MALDI-MS, ESIMS and LD/jet-PI-MS. The desalted form of Photofrin was preferred over Photofrin. Desalted Photofrin produced abundant proton adduct molecular ions while ionization of Photofrin generally was suppressed owing its high salt content. In the FABMS, UV- and IR-MALDI-MS and ESI-MS (nozzle–skimmer voltage 100 V) positive ionization modes, principally singly charged porphyrin oligomers were observed with masses up to about 4100 Da (Fig. 2(A)–(D)). Each porphyrin oligomer consisted of a variety of components differing by 18 Da, consistent with the elements of H_2O. The distributions of the oligomers were nearly identical for all these mass spectra (Fig. 3). The average oligomer length for Photofrin was 2.7 ± 0.1 porphyrin units. We are only interested in characterizing the oligomeric species present in Photofrin since they constitute the active components for photodynamic therapy, therefore only Photofrin components containing two or more porphyrin units were used in the distribution calculation. The majority of monomeric porphyrin species present in Photofrin are residual starting materials, as demonstrated by HPLC; nevertheless, some of the ion abundances of the monomeric peaks can be induced as fragment ions during the ionization process.

In the ESIMS positive and negative ionization modes, singly and multiply charged porphyrin oligomers were observed with corresponding masses up to 5000 Da. When the ESIMS nozzle–skimmer collision energy in the positive ionization mode is elevated from 20 to 200 eV and in the negative ionization mode from 20 to 100 eV, the abundances of multiply charged ions were significantly reduced and nearly all of the observed ions were singly charged (Figs 4(A), 2(D) and 4(B) for positive ions and Fig. 4(C) and (D) for negative ions). Dehydration of the individual oligomers increased with increasing voltage and the oligomer distribution closely resembled that of the distributions observed in the FAB and UV- and IR-MALDI mass spectra (Fig. 2(A), (B) and (C), respectively). The high-voltage nozzle–skimmer experiments demonstrated that Photofrin is a covalent mixture of porphyrin oligomers since the observed components only partially dehydrated and did not break down into monomeric species which would be expected if Photofrin was a cluster or micelle mixture of porphyrin monomers. This is further supported by the proprietary gradient reversed-phase HPLC method which uses 1 : 1 : 1 water–methanol–tetrahydrofuran as the sample diluent. Micelles are unlikely to survive in this high level of organic solvent.

ESIS positive ion mass spectra of Photofrin samples differing in concentration by over three orders of magnitude (~1.3 ng μl^{-1} to ~1.3 μg μl^{-1}) were obtained and showed no significant change in oligomer distribution as a

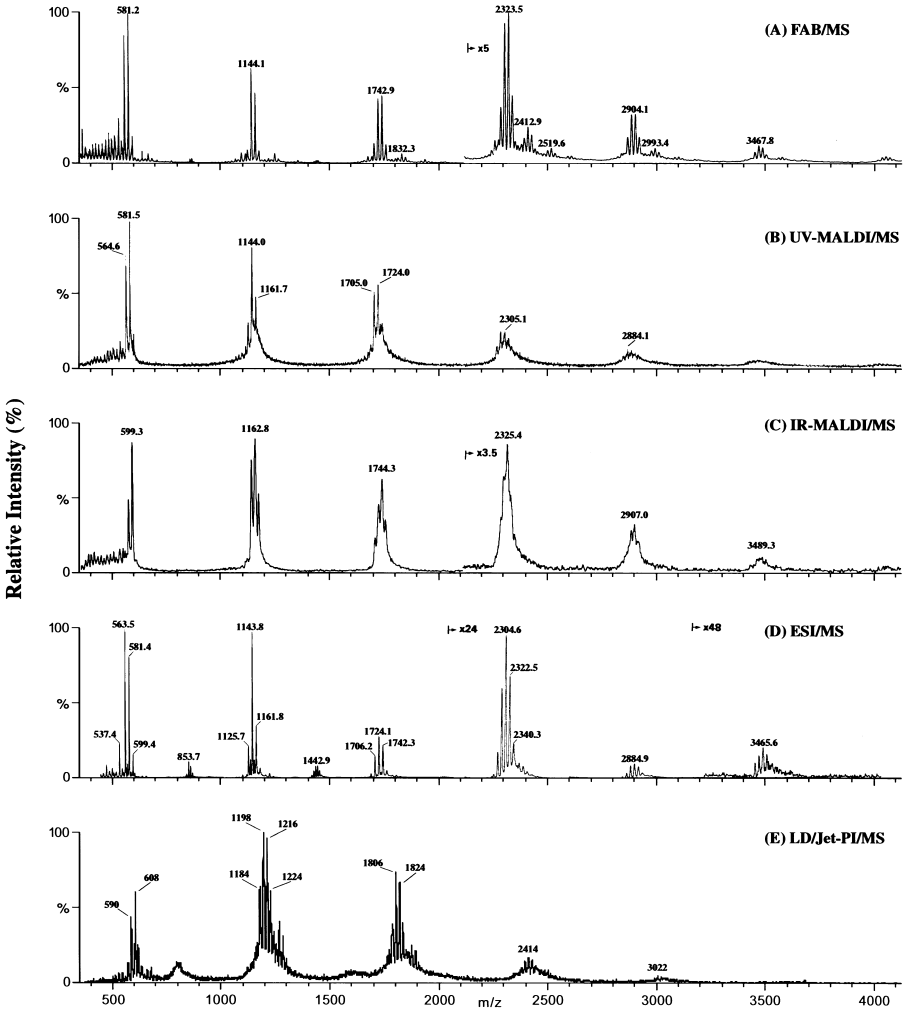

Figure 2. Mass spectra of desalted Photofrin, in the positive ionization modes, obtained by (A) FABMS, (B) UV-MALDI-MS, (C) IR-MALDI-MS and (D) ESIMS (nozzle–skimmer voltage 100 V). (E) Mass spectrum of permethyl ester of Photofrin, in the positive ionization mode, obtained by LD/jet-PI (193 nm)-MS.

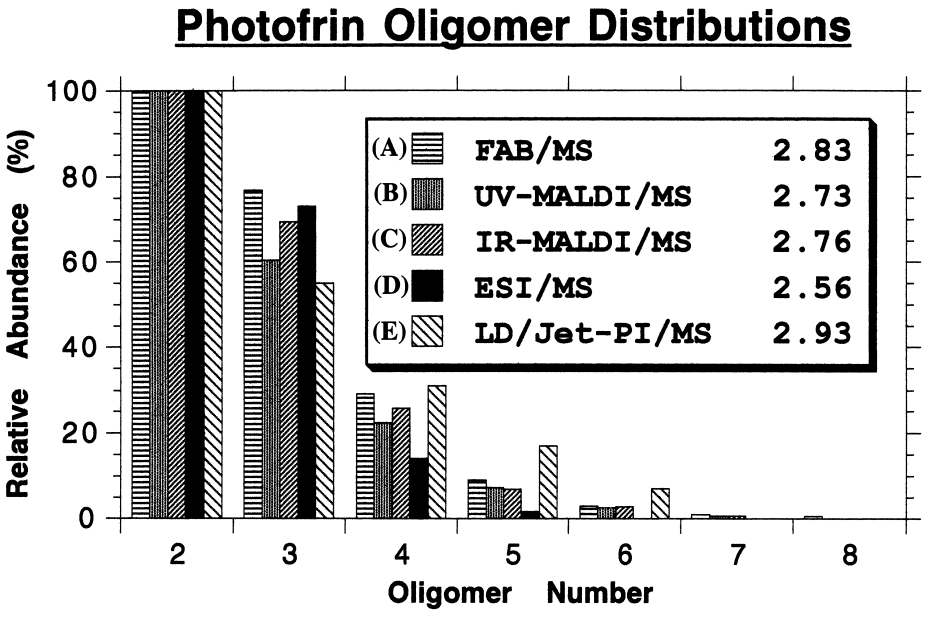

Figure 3. Distributions of the Photofrin oligomers obtained with desalted Photofrin by (A) FABMS, (B) UV-MALDI-MS, (C) IR-MALDI-MS, (D) ESIMS (nozzle–skimmer voltage 100 V) and with permethyl ester of Photofrin by (E) LD/jet-PI-MS. The calculated average oligomer numbers for each of the ionization modes measured for Photofrin are listed in the inset.

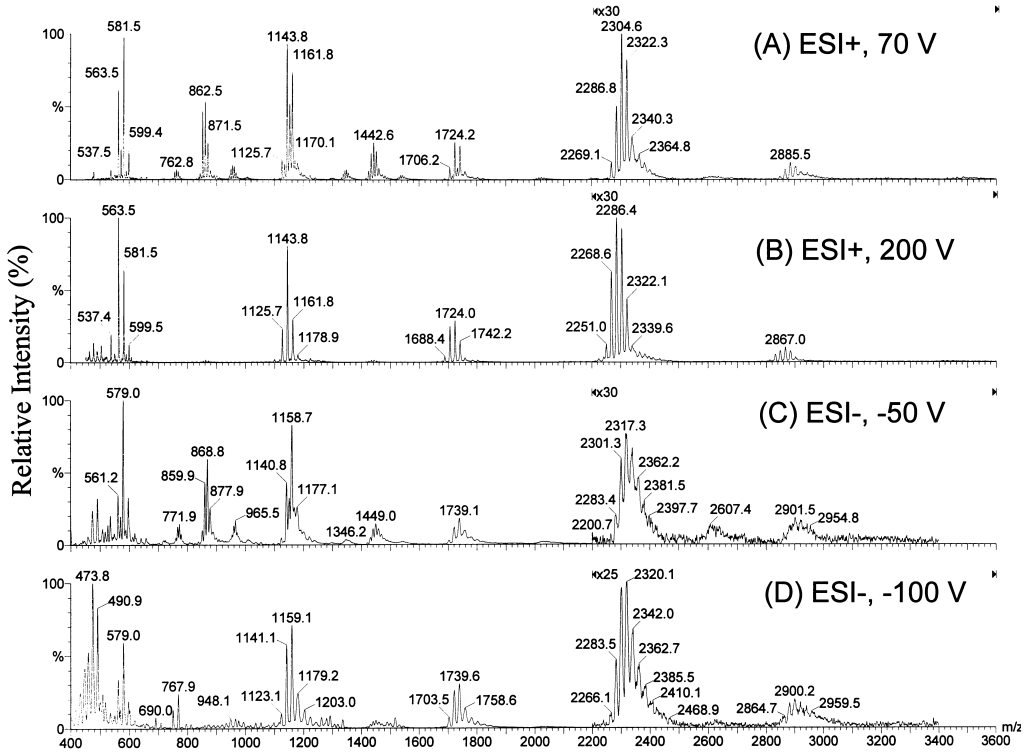

Figure 4. ESIMS nozzle–skimmer study of desalted Photofrin. ESI mass spectra in the positive ionization mode with nozzle–skimmer voltages of (A) 70 V (Fig. 2(D), 100 V), (B) 200 V, and in the negative ion mode with nozzle–skimmer voltages of (C) −50 V and (D) −100 V.

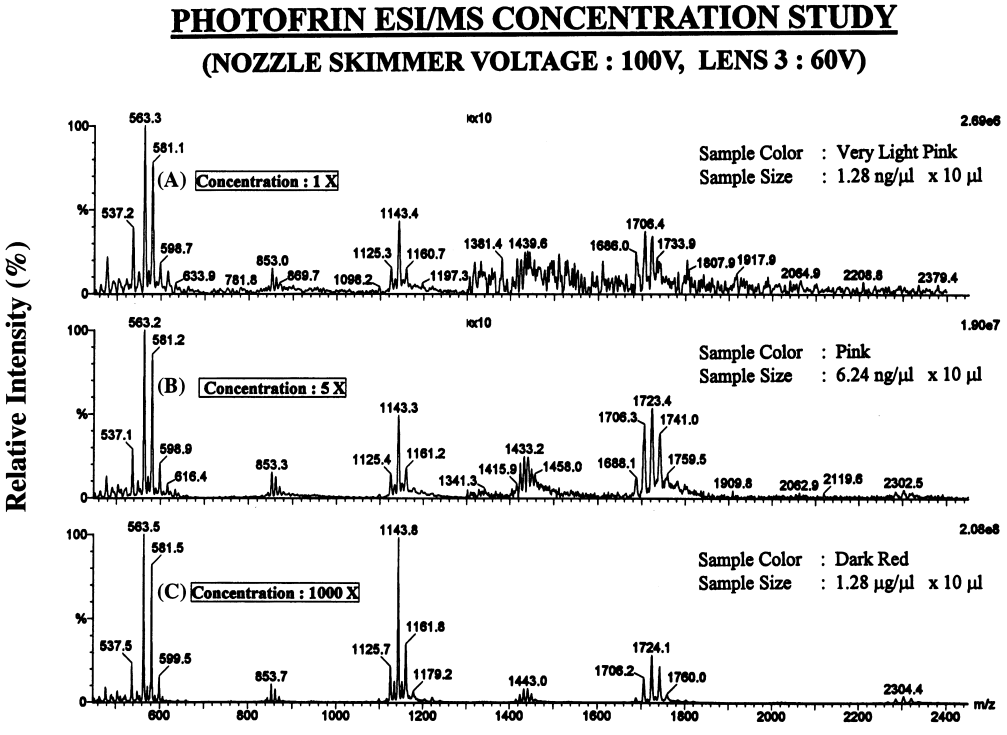

Figure 5. ESIMS concentration study of desalted Photofrin in the positive ionization mode. ESI mass spectra obtained with 10 µl of desalted Photofrin at concentrations of (A) 1.28 ng μl^{-1}, (B) 6.24 ng μl^{-1} and (C) 1.28 µg μl^{-1}.

function of concentration (Fig. 5). These observations are consistent with the behavior of a mixture of covalently bound oligomers rather than micelles of non-covalently bound monomers.

The LD/jet-PI mass spectrum of desalted Photofrin exhibited mostly porphyrin monomers with a small relative abundance percentage (∼20%) of porphyrin dimers. This observation is inconsistent with the greater abundances of higher oligomers observed by FABMS, UV- and IR-MALDI-MS and ESIMS and is due to the extensive degradation/fragmentation of Photofrin as demonstrated below in the discussion of LD/jet-PI-MS of

the permethyl ester form of Photofrin. Nevertheless, the use of jet cooling in LD/jet-PI-MS with desalted Photofrin resulted in a significant amount of dimer, which contrasts with the complete absence of oligomers and only the observation of monomers when jet cooling is absent, as reported by Zhan *et al*.[21]

Permethyl ester of Photofrin

The permethyl ester of Photofrin was analyzed by FABMS, UV- and IR-MALDI-MS, ESIMS and LD/jet-PI-MS. The FABMS, UV- and IR-MALDI-MS and ESIMS distribution results were essentially identical with the respective results for the desalted Photofrin. The LD/jet-PI mass spectrum of permethyl ester of Photofrin is illustrated in Fig. 2(E) and is consistent with the FABMS, UV- and IR-MALDI-MS and ESIMS results for both the permethyl ester of Photofrin and desalted Photofrin. The spectrum, obtained at 193 nm (6.3 eV) and at unit mass resolution, exhibits the presence of up to six porphyrin methyl ester units. The average oligomer length, calculated from the LD/jet-PI distribution, was found to be 2.9 porphyrin units. By comparing the higher mass peaks of the individual porphyrin oligomers of the permethyl ester of Photofrin with the corresponding higher mass peaks of the oligomers of desalted Photofrin, for the five ionization modes, the average numbers of methyl ester (ME) groups per Photofrin oligomer were determined as follows: 2 ME/monomer, 5 ME/dimer, 7 ME/trimer, 8 ME/tetramer, 10 ME/pentamer. These results are closer to the predicted values for ether-linked porphyrin oligomers (2 ME/monomer, 4 ME/dimer, 6 ME/trimer, 8 ME/tetramer, 10 ME/pentamer) rather than ester linked porphyrin oligomers (2 ME/monomer, 3 ME/dimer, 4 ME/trimer, 5 ME/tetramer, 6 ME/pentamer). NMR studies confirmed the presence of ethers but did not exclude esters as potential products.[40]

Monomer precursors of Photofrin and dimer and trimer model compounds of photofrin

The low-mass region of all Photofrin spectra exhibited ions originating from the monomeric species hematoporphyrin **2** (M_r 598.3), hydroxyethylvinyldeuteroporphyrin **3** (M_r 580.3) and protoporphyrin **4** (M_r 562.3). These ions principally originate as unreacted byproducts in the synthesis of Photofrin and have been quantitated by HPLC. During the product development phase, the specification was not more than 17% (w/w) for the total monomer content in Photofrin. The pure monomers, when analyzed by FABMS, UV- and IR-MALDI-MS and ESIMS in the positive ionization modes, produced principally proton adduct molecular ions with no fragmentation or higher mass oligomeric species. When mixtures of these pure monomers were analyzed by FABMS, UV- and IR-MALDI-MS and ESIMS at the analytical concentrations normally used for analysis, no new higher mass oligomeric species related to Photofrin were generated. However, it is instructive to point out that when higher concentrations than recommended were used only the UV- and IR-MALDI methods readily produced abundant mixed higher mass oligomers of the monomeric species. These results demonstrate that the oligomeric species observed above for the desalted and permethyl ester forms

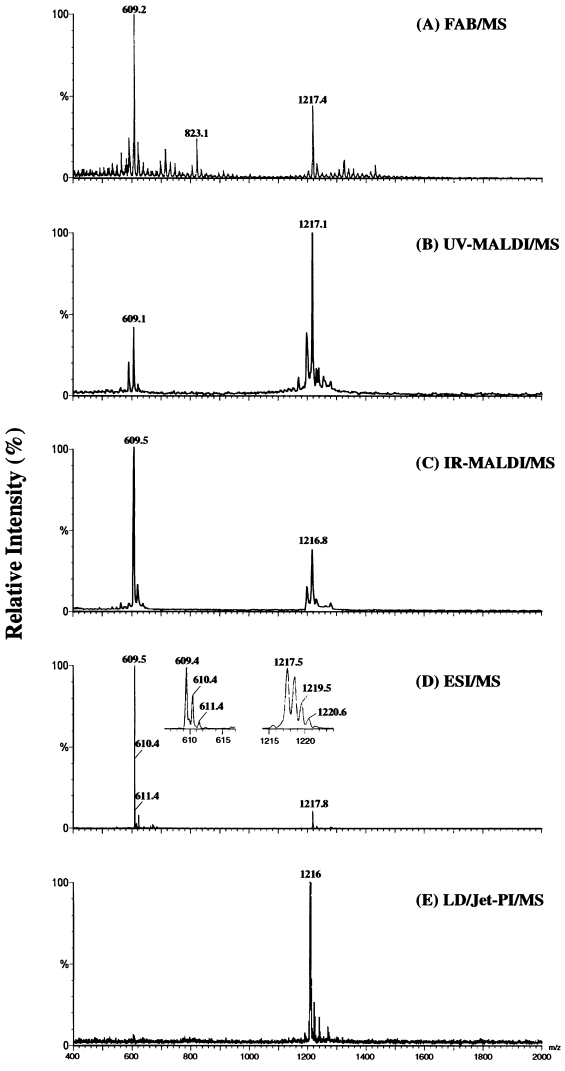

Figure 6. Mass spectra of synthetic Photofrin methyl ester dimer model compound **5** (M_r 1216.6) in the positive ionization mode, obtained by (A) FABMS, (B) UV-MALDI-MS, (C) IR-MALDI-MS, (D) ESIMS (nozzle–skimmer voltage 50 V) and (E) LD/jet-PI (193 nm)-MS.

of Photofrin could not be produced from mixtures of the porphyrin monomers, demonstrating that Photofrin is not micelles of monomeric porphyrin species. Likewise, the FAB, UV- and IR-MALDI, ESI and LD/jet-PI mass spectra of synthetic porphyrin methyl ester dimers (**5–8**) and trimers (**9, 10**), components believed to be present in Photofrin as carboxylates,[41,42] produced molecular ions and some fragment ions of lower mass oligomers. Figure 6 illustrates the mass spectra for the five positive ionization methods for the Photofrin methyl ester dimer model compound **5** (M_r 1216.6). The LD/jet-PI mass spectrum shows no fragment ions while lower mass singly charged ions are observed at m/z 609 in the FAB, UV- and IR-MALDI and ESI mass spectra. Similar results were obtained with the Photofrin methyl ester dimer model compounds **6, 7** and **8**. Figure 7 illustrates the mass spectra for the five positive ionization methods for the Photofrin methyl ester trimer model compound **9** (M_r 1842.9). The IR-MALDI and ESI mass spectra show nearly no fragment ions while some lower mass singly charged ions are observed principally at m/z 609 in

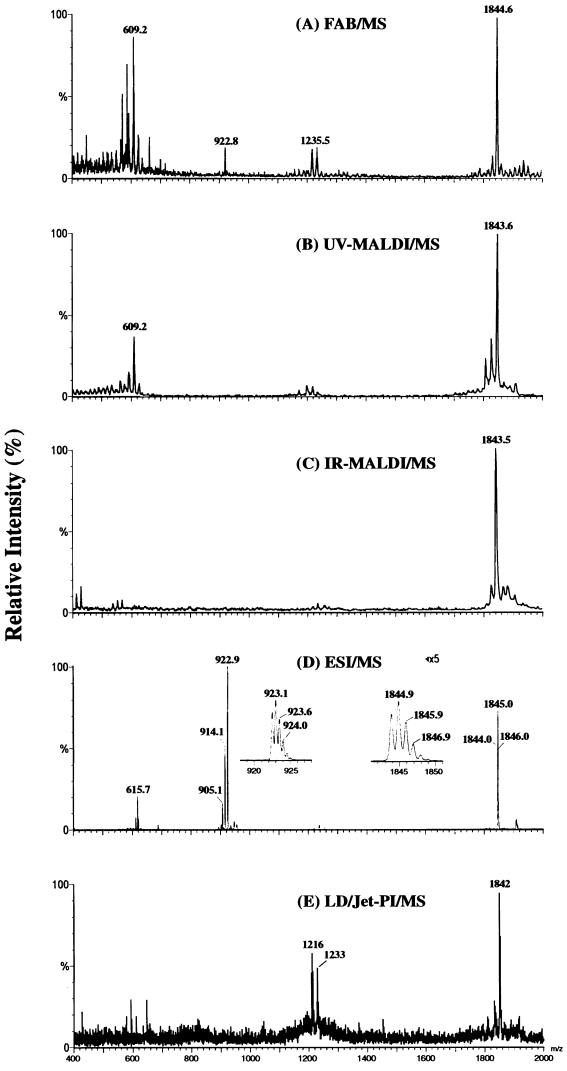

Figure 7. Mass spectra of synthetic Photofrin methyl ester trimer model compound **9** (M_r 1842.9) in the positive ionization mode, obtained by (A) FABMS, (B) UV-MALDI-MS, (C) IR-MALDI-MS, (D) ESIMS (nozzle–skimmer voltage 65 V) and, (E) LD/jet-PI (193 nm)-MS.

the FAB, UV-MALDI and LD/jet-PI mass spectra. Similar results were obtained with the Photofrin methyl ester trimer model compound **10**. The results with the Photofrin monomer precursors and the synthetic Photofrin methyl ester dimer and trimer model compounds, in addition to the dilution and high-energy nozzle–skimmer ESIMS experiments with Photofrin (described above), demonstrate that Photofrin is a mixture of porphyrin oligomers which are relatively stable under the experimental conditions used.

Comparison of LD/jet-PI-MS and LD/PI-MS data for Photofrin and related compounds

Of the five mass spectral techniques described above, the LD/jet-PI-MS is the most relevant for comparison with the LD/PI-MS results of Zhan *et al.*,[21] who reported observing only monomers when analyzing Photofrin and significant fragmentation when analyzing synthetic porphyrin methyl ester dimers and a trimer. The main differences between these two studies are (1) the jet cooling which in our case occurs between desorption and ionization, (2) the use of methyl ester and desalted forms of Photorin vs (undesalted) Photofrin and (3) the use of different wavelengths for ionization.

We have found repeatedly that jet cooling is essential in order to prevent fragmentation upon ionization.[26,43–45] For example, we have shown that even when saturated alkanes are photoionized with only a few tenths of an eV of excess energy, the ions still fragment owing to the initial high internal energy. If, on the other hand, the internal energy is first reduced by cooling the neutral molecules in a supersonic jet, then upon ionization the ion is stable and the parent molecular ion mass can be obtained.[46,47] For molecules the size of photofrin, with their many degrees of freedom, the internal energy is considerable, certainly at the elevated temperatures involved with laser desorption. With jet cooling, as reported above, we observed abundances of about 20% Photofrin dimer with desalted Photofrin and broad oligomer distributions with the permethyl ester of Photofrin. This is further confirmed in the analysis of synthetic porphyrin methyl ester dimers (**5, 8**) and a trimer (**9**) by LD/jet-PI at 193 nm without the presence of significant fragmentation, as described above. On the other hand, Zhan *et al.*[21] observed without jet cooling only monomers for (undesalted) Photofrin and extensive fragmentation for synthetic methyl ester porphyrin dimers and a trimer.

The other issue to be considered in LD/PI-MS two-laser approaches is the wavelength of ionization. We have found, for example, that ionization at 125 nm causes extensive fragmentation, whereas ionization at 193 nm does not. The results of Zhan *et al.*[21] were obtained at 243.5 nm, which we did not try but which possibly may have been a less appropriate wavelength for fragment free ionization.

Given all these considerations, the absence of oligomers in the data of Zhan *et al.*[21] can be explained as fragmentation due to the absence of jet cooling. Additionally, fragmentation may have been intensified to some degree also by the use of (undesalted) Photofrin in part of the work and possibly by the use of a less effective wavelength for photoionization. Although, in our work, we cannot exclude the possibility of fragmentation of larger oligomers, the results with Photofrin dimer and trimer models lend support to the notion that with the help of jet cooling it is indeed possible to photoionize the permethyl ester of Photofrin without significant fragmentation to the lower oligomers. Conversely, we do not observe clusters or micelles of the synthetic oligomers, making cluster formation a very unlikely explanation for the larger oligomer mass ions observed in the LD/jet-PI spectra of the permethyl ester of Photofrin.

CONCLUSIONS

To our knowledge this is the first side-by-side comparison of five mass spectrometric techniques, FAB, UV- and IR-MALDI, ESI and LD/jet-PI, all developed for the study of complex, low vapor pressure compounds. The fact that each of these approaches yields a similar oligomeric

distribution gives confidence in the analyses. At the same time, this result provides increased confidence in the validity and usefulness of these recent mass spectrometric techniques. The results show that Photofrin is a mixture of porphyrin oligomers with an average size of 2.7 ± 0.1 porphyrin units.

Acknowledgements

The authors greatly appreciate the initial exploratory Photofrin MALDI-MS work of Arnd Ingendoh and the graphics arts service provided by Dawn De Thomas and John Vallencourt. One of the authors (R. K. P.) thanks the National Institutes of Health (CA55791) for financial support.

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