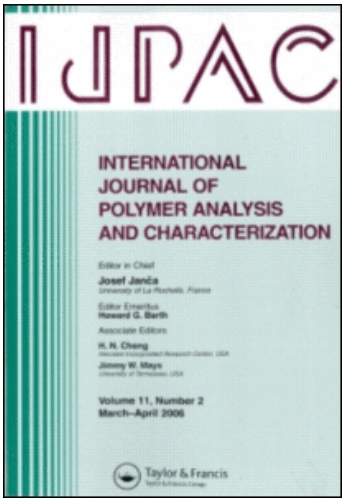

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Characterization of Macromolecules by Electrospray Ionization Mass Spectrometry*

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Application of electrospray ionization (ESI) mass spectrometry (MS) to the analysis of oligomers and polymers is reviewed through several representative examples. The benefits and limitations of the technique are discussed considering the molecular weight and complexity (polydispersity) of the analyte. Recent results demonstrating the advantage of using hyphenated techniques such as size exclusion chromatography (SEC) coupled with ESI-MS are highlighted.

Keywords: Mass spectrometry; Electrospray ionization; Size-exclusion chromatography; 2-Hydroxypropyl-β-cyclodextrin; Octylphenoxypoly(ethoxy)ethanol

INTRODUCTION

The possibility of obtaining gaseous ions *via* the use of charged droplets has captured the attention of mass spectrometrists since the late 1960s. Projects by Dole and co-workers,[1] involving spraying dilute solutions of macromolecules such as polystyrene through a syringe held at high potential into a gas-filled chamber for mass spectrometric analysis, have been the origin of ESI. These early efforts ended up with the developers concluding that fundamental problems,

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apparently due to the formation of multiple-charged species and clusters, (then) prevented the determination of accurate molecular weights of polymers, and work had been abandoned on ESI mass spectrometry until the 1980s. Rediscovered by Fenn and co-workers, [2] it has now been developed into a powerful ionization technique used to desorb molecules at atmospheric pressure from a dilute solution of the sample and found applications in the mass spectrometric analysis of oligomers and polymers. [3]

PRINCIPLES AND APPLICATIONS

Mass spectrometers are available with electrospray interfaces of various designs. They all rely on a strong electric field nebulizing a liquid into an aerosol of charged microdroplets. The solution containing the analyte is supplied at a constant flow rate through a small-diameter metal, fused-silica, or glass capillary or needle (isolated by fused silica tubing from the device such as a syringe pump that maintains the sample flow). The solution leaving the capillary is electrosprayed at atmospheric pressure into a plume of charged microdroplets, as shown schematically in Figure 1. Solvent evaporation upon heat transfer *via* the ambient gas from the heated part of the ion source, such as the metal capillary shown in the scheme leads to the shrinking of the droplets and to the accumulation of excess surface charge resulting in the generation of gas-phase ions.

Although there is still no consensus on the mechanism by which sample ions are obtained by ESI for mass spectrometric analysis, a widely accepted model proposed by Iribarne and Thomson [4] assumes that solute ions from charged droplets are formed by field-assisted desorption (also referred to as "ion evaporation"). According to this model, the surface electric field becomes high enough (up to several V/nm) to desorb analyte ions from the shrinking droplets. The strong electric field evolved on the surface of the droplet assists the solute ion in overcoming the energy barrier that blocks its escape. Routine electrospray interfaces also provide additional ways (pneumatically assisted electrospray or ionspray, sheath liquid, *etc.*) of stabilizing the production of the charged microdroplets and/or to increase liquid

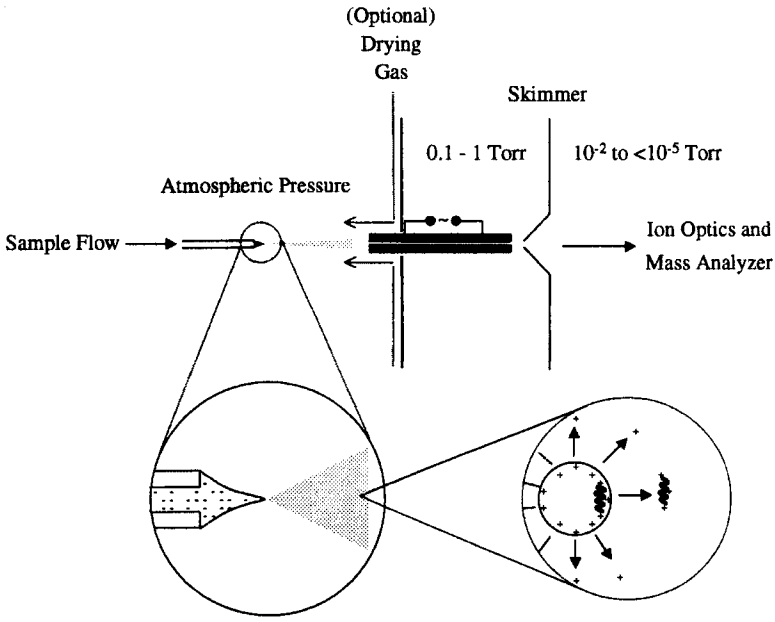

FIGURE 1 Schematic diagram of an ESI mass spectrometer and the process of ion formation.

flow rates at which the instrument can be operated. ESI is conducted at near ambient temperature; therefore, no thermal effects causing the decomposition of the analyte are observed during ionization. The process of ion formation is also extremely soft; usually no fragmentation occurs.

During ESI, charged molecules are produced by ionic species (H^+, Na^+, K^+, *etc.*) being attached to a neutral analyte such as 2-hydroxypropyl-β-cyclodextrin[5] (HPBCD, **1**) used as a pharmaceutical excipient.[6] HPBCD is obtained by the reaction of the β-cyclodextrin, a cyclic oligosaccharide containing seven α-1,4-linked glucose units, with propylene oxide under alkaline conditions. The product is a mixture of oligomers with a molecular weight distribution according to number of the 2-hydroxypropyl substituents attached to the oligosaccharide core. The reaction may target the 2-, 3- and 6-OH groups, and the newly formed 2-hydroxypropyl groups may also propagate the formation of poly(propylene oxide) side chains, as

shown by the structure below:

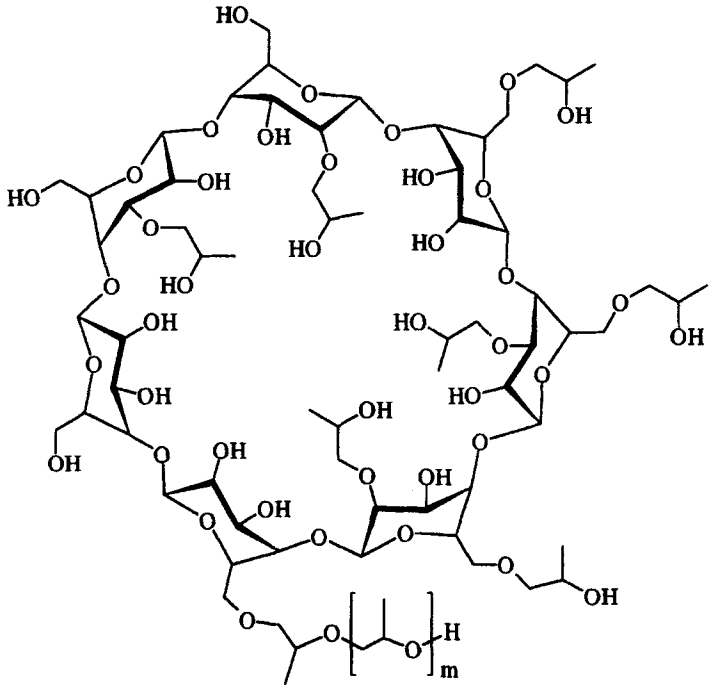

Figure 2 shows ESI mass spectra of HPBCD recorded upon the addition of various ionizing agents to the spraying solution. The positive ions formed are indicated in the spectra. Most polymers do not protonate easily due to the lack of basic functional groups demanding, therefore, the addition of a salt to the solution to be electrosprayed. In negative-ion ESI, removal of protons or attachment of anions yields the ions of the sample molecule, as exemplified in Figure 3.

ESI mass spectrometry has found applications for the molecular-weight determination and structural analysis of biopolymers, especially proteins.[7] Fewer reports have been published on its application to synthetic polymers, although poly(ethylene glycols) (PEGs) up to 5,000,000 Da have shown to be ionized by this technique.[8] Ions in the ESI mass spectra of macromolecules fall, due to multiple

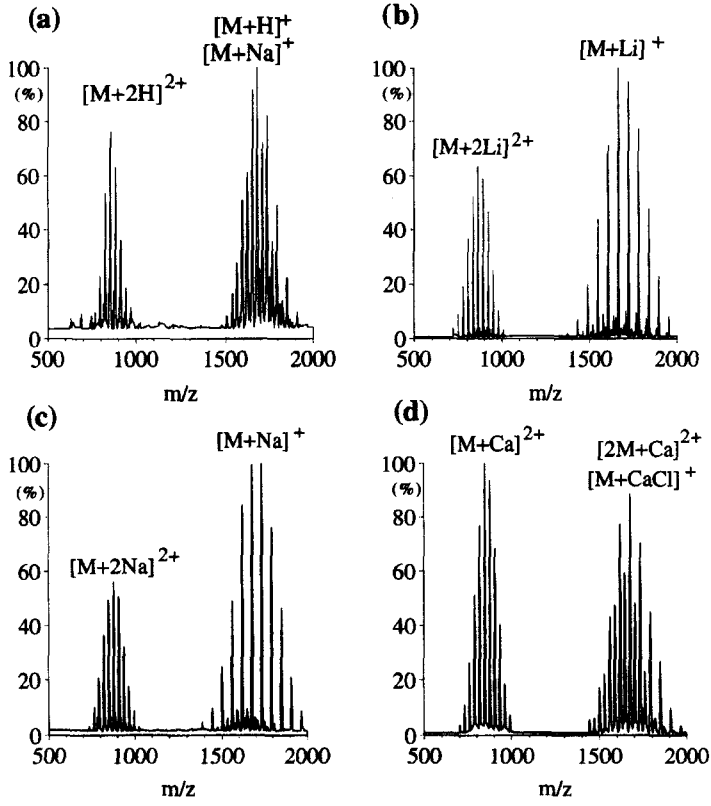

FIGURE 2 Positive ESI mass spectra of 2-hydroxypropyl-β-cyclodextrin (**1**) electrosprayed from water/methanol (50/50, v/v) solution (sample concentration 0.1% w/v) containing (a) 3% (v/v) acetic acid, (b) $5 \cdot 10^{-4}$ M LiI, (c) 10^{-5} M NaI and (d) $5 \cdot 10^{-5}$ M $CaCl_2$ as cationizing agents. The analyses were done by using a low-resolution, single-quadrupole instrument (Vestec/PE Biosystems, Framingham, MA) at a continuous flow rate of 5 μL/min. The type of positive ions of the analyte identified in the ESI mass spectra are indicated above the respective group of ions.

charging, consistently within a certain mass-to-charge (m/z) window (usually m/z 500 to 3000 u), regardless of the actual molecular weight of the samples. It is very crucial to recognize the ability of ESI to form multiple-charged ions, and ESI mass spectra may require interpretation.

Because the multiple charging common to ESI complicates the spectra, ESI has been used mostly for low-molecular-weight polymers.[3] ESI spectra of polydisperse, polymeric samples are complex,

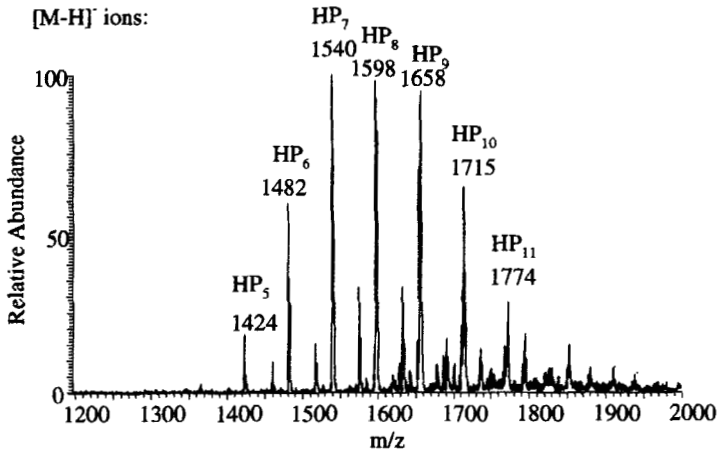

FIGURE 3 Negative ESI mass spectrum of the 2-hydroxypropyl-β-cyclodextrin (**1**) sprayed from a water/methanol (50/50, v/v) solution containing 0.01% (w/v) NH_4OH at 3 μL/min. Recorded on an LCQ, a quadrupole ion-trap instrument (Finnigan MAT, San Jose, CA).

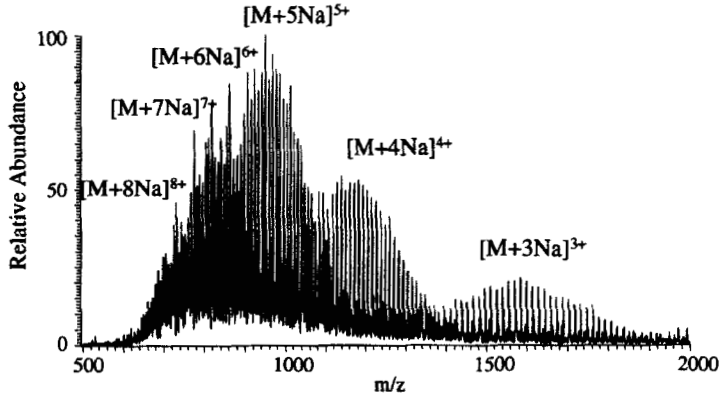

FIGURE 4 (+) ESI mass spectrum of a PEG, $M_n = 4600$. A methanol/water solution (50/50, v/v) solution of the sample (0.1%, w/v) containing 25 μM NaI was electrosprayed at 3 μL/min. Instrument: Quadrupole ion-trap (LCQ).

because the multiply charged ions of the oligomer distribution often heavily overlap, as shown in Figure 4. The resolving power of the mass analyzers also has to be considered. Widely available ESI – quadrupole mass spectrometers with low (≤ 1000) resolving

power are prone to be inadequate for the analysis of higher molecular-weight polymers.[9] Quadrupole ion-traps, state-of-the-art time-of-flight (TOF) analyzers, or magnetic-sector instruments may give medium mass resolution ($\geq$ 5,000), and especially an ESI – Fourier-transform mass spectrometer (FTMS) offers high mass accuracy and resolution that may extend the application of the technique to higher polymers. For example, McLafferty and co-workers used ESI/FTMS to characterize PEG 20,000 on a 6-Tesla FTMS.[10] Over 5000 isotope peaks resulting from 47 oligomers in ten charge states were distinguished with resolving powers in excess of 50,000.

Early analytical ESI mass spectrometry of synthetic polymers concentrated on the direct application of the technique for the determination of the molecular weight distribution. Mass spectra of polymers can be obtained in minutes, and they provide far more detailed mass information than conventional techniques. Polymers may be complex mixtures with heterogeneity not only in size (molecular weight distribution), but also in chemical composition and end-groups. Furthermore, distributions in architecture add another level of complexity. All the above characteristics can often be elucidated by mass spectrometric analyses. When multiple-charging characteristic to higher molecular-weight samples is encountered, data reduction is often needed in order to extract the required information. In addition to "human data reduction", deconvolution techniques may be employed to provide molecular-weight information on many components from an unseparated mixture.[10–14]

In general, there may be no major inaccuracies involved in the calculation of average molecular weights for oligomers and polymers with narrow molecular-weight distributions, as long as the mass resolution of the instrument allows for the separation of the individual n-mers according to their m/z values. The number-average molecular weight (M_n) of HPBCD (1) calculated from the ESI mass spectra (Fig. 2) is given as an example in Table I. Considering the experimental error, they are in agreement with the value determined by ^{1}H-NMR spectrometry, although a moderate influence of the ESI conditions and of the ions on the estimated M_n is apparent. However, obtaining realistic mass distributions and reliable molecular-weight averages from mass spectra can be a major concern with polydisperse samples. To reflect accurate molecular weight distributions,

TABLE I Number-average molecular weight (M_n) and average number of 2-hydroxypropyl groups per β-CD (HP#) for the HPBCD (1) sample analyzed (Standard deviations, when measured, are given in parentheses)

Method	M_n	*HP#*
^{1}H-NMR	1655 ($\pm$ 39)	8.96 ($\pm$ 0.67)
ESI-MS, $[M+Li]^+$ ions	1690	9.56
ESI-MS, $[M+2Li]^{2+}$ ions	1721	10.10
ESI-MS, $[M+Na]^+$ ions	1682 ($\pm$ 16)	9.43 ($\pm$ 0.28)
ESI-MS, $[M+2Na]^{2+}$ ions	1707	9.86
ESI-MS, $[M+Ca]^{2+}$ ions	1679	9.38
ESI-MS, $[M-H]^-$ ions	1605	8.10

the mass spectrometric analysis must not significantly discriminate over a wide mass and concentration range. Possible areas of discrimination are the ionization process, ion transfer/separation, and detection. Polydispersities generated by direct mass spectrometry of broad distribution polymers are often much lower than either predicted (from the synthesis) or determined by other methods (*i.e.*, SEC, light scattering, or viscosimetry). There have been a number of reports on the origins of this discrepancy and on possible solutions.[15, 16] SEC fractionation prior to mass spectrometric analysis is one reported method.[17, 18] The reduction of components entering the ESI source of the mass spectrometer *via* chromatographic separation may be a straightforward solution to the problem of resolving overlapping charged envelopes of polydisperse samples. Size-exclusion chromatography (SEC), where the mode of separation is well understood, predictable (practically no method development for individual samples is required), and performed routinely for oligomers and polymers, is well suited for coupling with mass spectrometry. While off-line fractionation can yield good results,[17, 18] it is labor intensive and time consuming.

The ESI interface has been attractive for the on-line coupling of SEC with mass spectrometry.[19–25] It can directly handle the SEC effluent from routine analytical columns with very little ($< 1\%$) effluent of the total liquid flow required (~ 10 ng of sample per spectra). Therefore, ESI mass spectrometry uses only a very small fraction of the effluent; thus, conventional SEC detectors, such as a differential refractive index or spectrophotometric detector, can be operated parallel with mass spectrometry. The application of the

hyphenated technique, SEC/ESI-MS, is demonstrated in Figures 5 and 6 by the analysis of octylphenoxypoly(ethoxy)ethanol (**2**), an oligomeric surfactant (Igepal®):

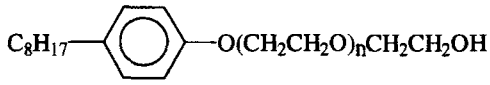

Figure 5a shows the ESI mass spectrum of **2**, when the sample solution (0.1%, w/v) was continuously delivered at a flow rate of 3 μL/min. The prevalent ions detected under these conditions are the monosodiated molecules. The "envelope" of the doubly-charged, disodiated molecules partially overlaps with that of the singly-charged ions, but triply-charged could be detected between m/z 750 to 1000 at very low intensity. From the intensity of the singly- and doubly-charged ions, M_n is calculated to be 1736 Da, the weight-average molecular weight (M_w) is 1771 Da; thereby a polydispersity (PD) of 1.02 is obtained. However, M_n, M_w and PD determined independently by analytical SEC using narrow-molecular-weight polystyrene standards as calibrants are 2001 Da, 2162 Da, and 1.15, respectively. These values suggest an underestimation of the sample's polydispersity by direct ESI-MS, compared to SEC.[19] However, molecular weight information obtained from conventional SEC is also highly dependent upon the accuracy of the calibration procedure. Well-characterized, narrow molecular-weight distribution oligomer and polymer calibrants of similar chemical composition provide the most accurate results. Such calibrants are usually unavailable, and the polystyrene standards are commonly used.[26]

Although a well-defined relationship only exists between the hydrodynamic volume (not molecular weight) of the solute and its retention time (RT) or volume of retention (V_r), the logarithm of relative molecular weight ($\log M_r$) is correlated to V_r or RT in common practice. However, the accuracy of SEC analyses of oligomeric mixtures may suffer the most from this approximation. Oligomers of dissimilar chemical composition can assume significantly different hydrodynamic volumes depending on their conformation in solution, even though their M_r is identical. This effect leads to systematic errors in the estimation of the molecular weight upon

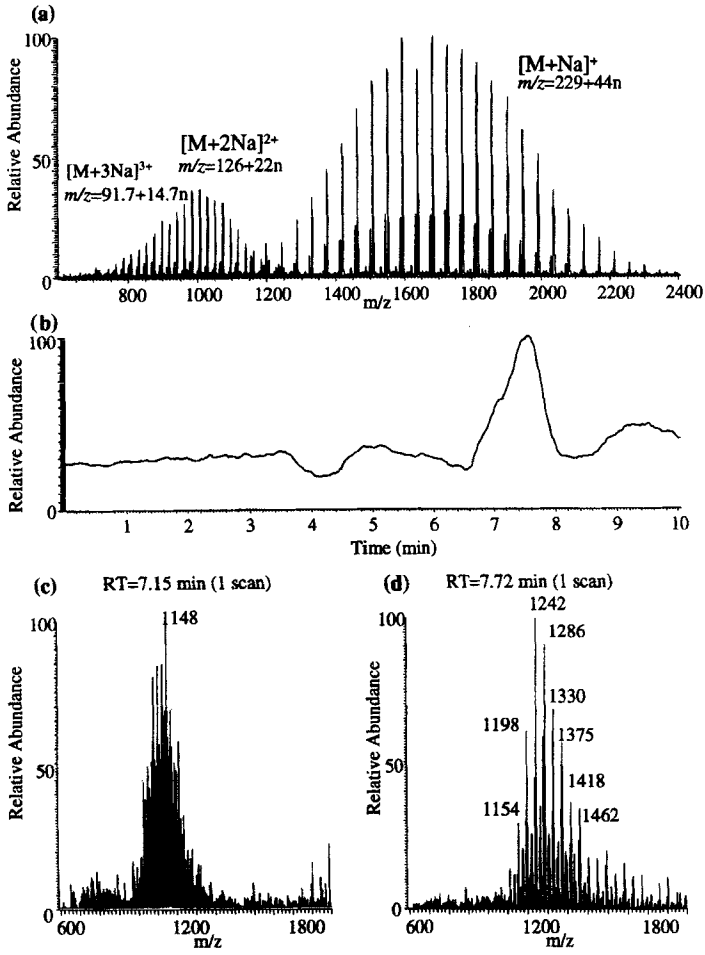

FIGURE 5 (a) (+) ESI mass spectrum of an octylphenoxypoly(ethoxy)ethanol (**2**) recorded upon electrospraying a methanol/water (50/50, v/v) solution of the sample (0.1%, w/v) 3 μL/min; (b) base-peak chromatogram, SEC separation (300 × 7.8 mm i.d. PL-Gel 3-μm Mixed-E column, Polymer Laboratories, Church Stretton, UK) using THF (1.0 mL/min) as a mobile phase (10 μM NaI added) and a 200 : 1 effluent splitting; (c) and (d) ESI mass spectra (single scans) at retention times 7.15 and 7.72 min, respectively. Instrument: quadrupole ion-trap (LCQ).

using calibration relying on polystyrene standards when measuring polymers other than polystyrene. Adsorption on and/or partition of the analyte into the column packing also very often can influence significantly V_r in SEC of oligomers.

SEC combined with ESI mass spectrometric detection offers a solution to this problem by creating a calibration curve from the sample ions detected.[19] Figure 5b displays the base-peak chromatogram, which considers the highest intensity measured in the respective scan for post-run normalization, recorded during the SEC/ESI-MS analysis of **2**. The chromatographic trace indicates the elution of the sample molecules between 6.7 and 8 min. The mass spectra recorded

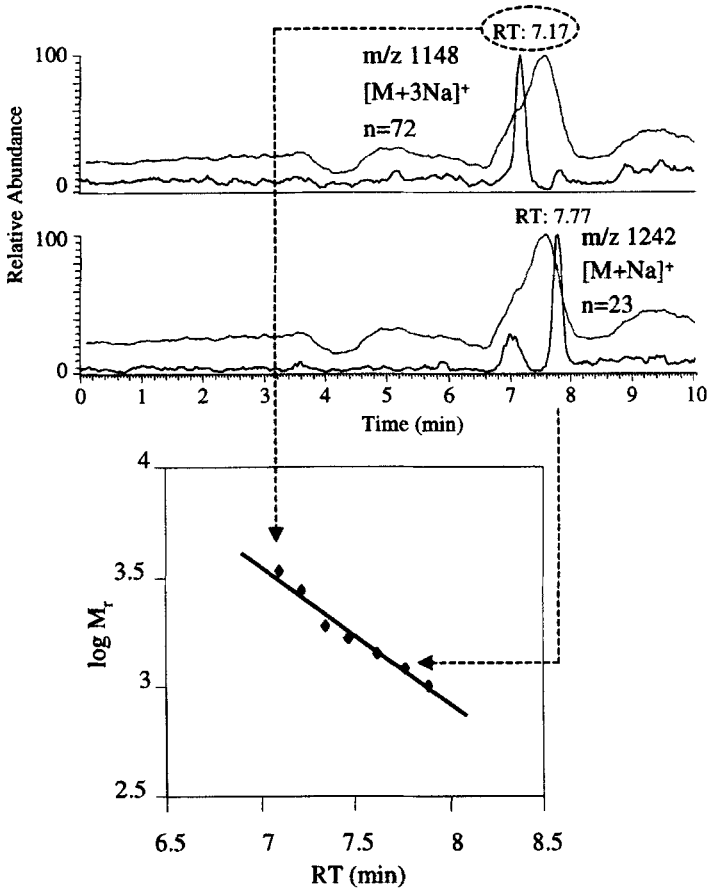

FIGURE 6 Procedure to obtain $\log M_r$ *versus* retention time (*RT*) calibration for octylphenoxypoly(ethoxy)ethanol (**2**) by chromatograms reconstructed from SEC/ESI-MS analysis (for experimental conditions, refer to Fig. 5). The reconstructed chromatographic tracings for the given ionic species (*m/z* 1148 and 1242) were displayed as solid curves with the base-peak chromatogram given as a dotted tracings as a reference.

at 7.15 and 7.72 min, respectively, clearly show that SEC separation results in 6–10 oligomers are detected in single scans (Figs. 5c and 5d). The median of the scan at 7.15 min represents the triply-charged ion of the oligomer of **2** with $n = 72$, while the most abundant ion at 7.72 min retention time is the monosodiated ion, $n = 23$.

Figure 6 illustrates the approach by which ion chromatograms reconstructed ("extracted") from the successively acquired and electronically stored mass spectra can be used for creating the calibration curve. Similar reconstructed chromatograms or selected oligomer profiles (SOPs, obtained by the summation of ion intensities for all charge states detected for the given oligomer) may be created and used as calibration points for every component of a polydisperse mixture.[19] The calibration curve is then applied to the data provided by the traditional SEC detector, and the usual procedure is used to furnish molecular weight values that do not rely upon a polystyrene calibration.[19–21] After an accurate SEC/ESI-MS calibration, the SEC analysis of the oligomeric surfactant **2** yields M_n, M_w and PD of 1971 Da, 2016 Da and 1.08, respectively. This translates to $\geq 10\%$ error in the determination of the average molecular weights done by direct ESI-MS or by SEC relying on mass calibrants unrelated to the sample.

CONCLUSION

In this brief overview, the application of ESI mass spectrometry to macromolecules has been discussed through selected representative examples. Direct application of the technique is appropriate for the qualitative analysis of samples with moderate complexity and molecular weight due to the phenomenon of multiple charging characteristic to ESI, and it is also well suited for sophisticated structural studies involving ultra-high resolution[18] or tandem mass spectrometry.[27] ESI mass spectrometry of mixtures with broad molecular weight distribution should be benefitted by a prior separation that would reduce the polydispersity of the analyte. The utility of the hyphenated techniques, such as SEC/ESI-MS, for obtaining information about chemical composition, resolution of overlapping charged envelopes in the ESI mass spectra of

polymers, SEC calibration, and complex mixture analysis has been demonstrated.

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